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Advances in the evolutionary ecology of termites

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Alberto Arab, Solange Del Carmen Issa Ponce and Daniel Aguilera-Olivares

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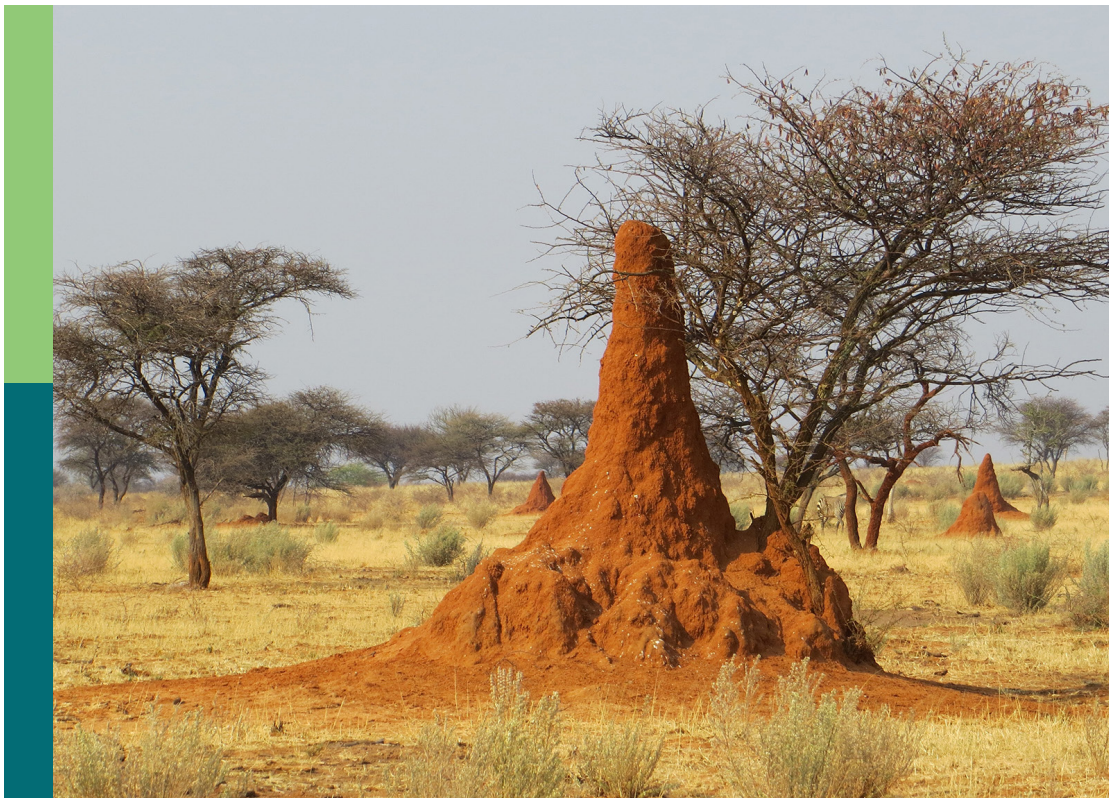

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Advances in the evolutionary ecology of termites

Topic editors

Alberto Arab — Federal University of ABC, Brazil

Solange Del Carmen Issa Ponce — Simón Bolívar University, Venezuela

Daniel Aguilera-Olivares — Universidad de Concepción, Chile

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EDITORIAL
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Editorial: Advances in the Evolutionary Ecology of Termites

Alberto Arab[1], *Solange Issa*[2] *and Daniel Aguilera-Olivares*[3]*

[1] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC (UFABC), Santo André, Brazil, [2] Laboratorio de Comportamiento y Manejo de Plagas, Departamento de Biología de Organismos, Universidad Simón Bolívar, Caracas, Venezuela, [3] Laboratorio de Entomología Forestal, Departamento de Silvicultura, Facultad de Ciencias Forestales, Universidad de Concepción, Concepción, Chile

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Editorial on the Research Topic

Advances in the Evolutionary Ecology of Termites

Termitology has seen great advances in research during the last decades accompanied by an increasing number of scientists and research centers, which is reflected in the growing number of scientists attending meetings (Chouvenc et al., 2018) and published articles. Although ants and bees have been the most prominently researched social insects in general over the last 45 years (**Figure 1A**), termites have been the most researched social insects regarding the topics of ecology and evolution (**Figure 1B**). Thus, in this Research Topic the growing interest in evolutionary ecology of termite research is summarized in six original research articles and two reviews, providing updated information on diverse aspects of the biogeography, evolutionary biology, genomics, systematics, microbiology and chemical ecology of these fascinating insects.

Termites (Blattodea: Isoptera) are eusocial insects that live in colonies containing hundreds to millions of individuals organized into reproductive and non-reproductive castes with specific tasks such as nest construction, foraging, reproduction, brood care, and colony defense (Korb and Hartfelder, 2008). They comprise over 3,000 species distributed in nine families sharing a common ancestor with wood feeding cockroaches of the genus *Cryptocercus* (Krishna et al., 2013; Bourguignon et al., 2015), and are thus considered eusocial cockroaches. Termites have gained the ability to digest lignocellulose through obligate symbiosis, leading to other major adaptations: alloparental care, trophallaxis, and the emergence of sterile castes; all together allowed the evolution of eusociality, the most complex level of social organization, for the first time in the history of the Earth about 150 Mya (Korb et al., 2012; Bourguignon et al., 2015; Chouvenc et al., 2021).

Traditionally, termites have been classified into "lower" and "higher" termites. "Lower" termites (all termite families except Termitidae) consist typically of wood-feeding species that depend mainly on flagellate protists for lignocellulose digestion (Engel et al., 2009). On the other hand, "higher" termites (Termitidae) are the most numerous, diverse and successful group of termites. Their evolutionary success is attributed to their symbiotic association with gut microorganisms (Ohkuma et al., 2009; Dietrich et al., 2014), mainly to the loss of flagellates and the acquisition of specialized bacteria with a high repertoire of lignocellulases, accompanied by a dietary diversification (Bignell and Eggleton, 2000; Brune, 2014). The food sources of "higher" termite lineages include wood, grasses, litter, micro-epiphytes, the mycelia of symbiotic fungi, organic soil and even inorganic soil (Donovan et al., 2001; Eggleton and Tayasu, 2001).

A major topic in termite ecology is the effect of habitat degradation on the diversity of these insects, especially in highly threatened biomes (Bullock et al., 2020). The impact of soil degradation on termite assemblages is investigated in this Research Topic by Duran-Bautista et al. at different land use systems in the Amazon. Local environmental factors such as vegetation type, rainfall,

Edited and reviewed by:
Elise Huchard, UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France

***Correspondence:**
Daniel Aguilera-Olivares
daguilera@udec.cl

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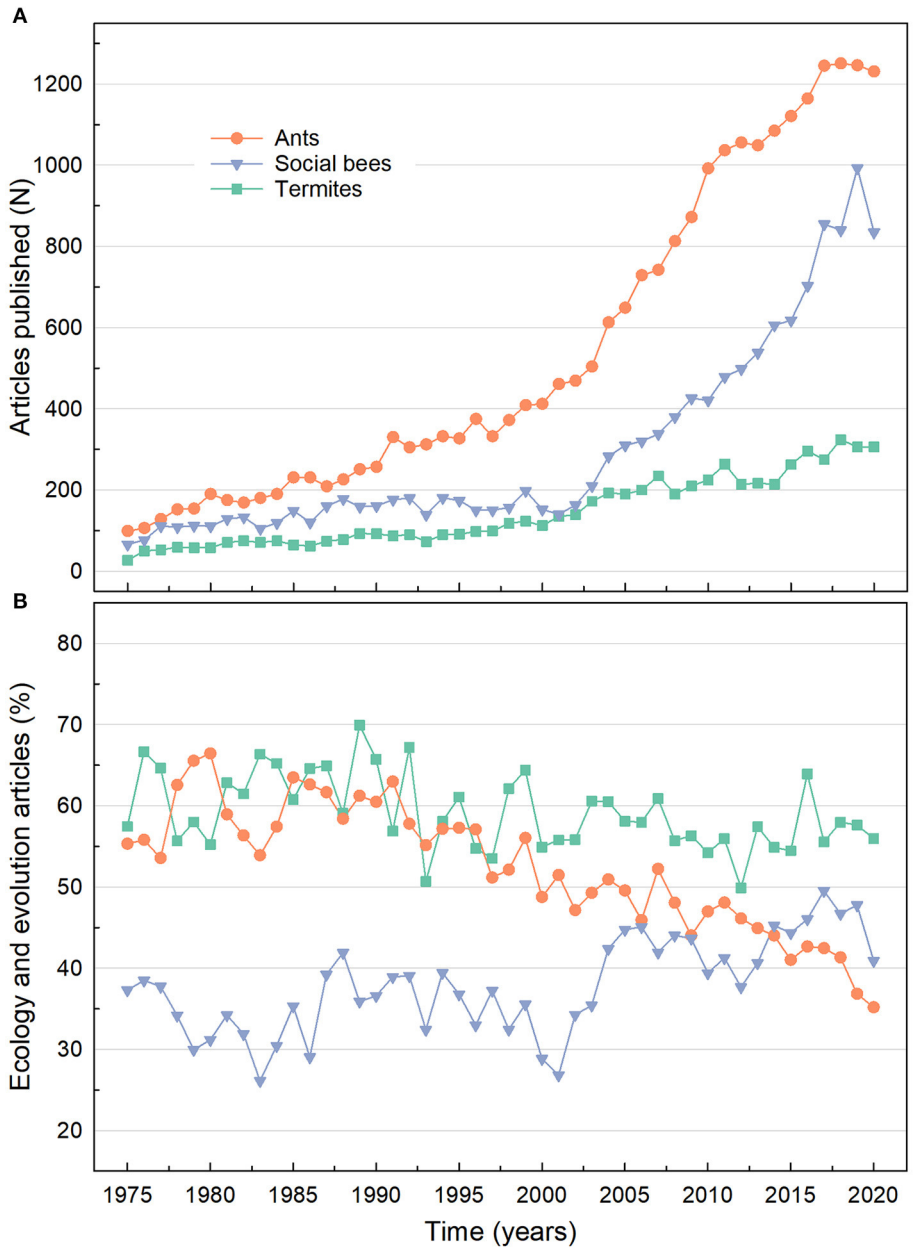

FIGURE 1 | Articles published for ants, social bees, and termites from 1975 to 2020 in all databases available on ISI Web of Knowledge. **(A)** Number of articles published by year for ants (keyword search: "ants"); social bees (keyword search: "orchid bees OR bumble bees OR stingless bees OR honey bees"), and termites (keyword search: "termites"). **(B)** Percentage of articles published by year on ecology or evolution topic for ants [keyword search: "ants AND (ecology OR evolution)"], social bees [keyword search: "(orchid bees OR bumble bees OR stingless bees OR honey bees) AND (ecology OR evolution)"], and termites [keyword search: "termites AND (ecology OR evolution)"].

elevation and latitude, are also known to differentially affect termite diversity. In their Original Research Article, Clement et al. address the question of how vegetation type and rainfall affect termite assemblages by evaluating guilds, activity and diversity of these insects in several Australian biomes.

Molecular analyses and biochemical activity are useful tools to uncover the complexity of the ecological and evolutionary dynamics of termite-symbiont association. "Lower" termites, depend on several lineages of flagellate protists for food digestion (Engel et al., 2009), and because protists are inherited vertically from the parents their distribution seems to be determined by host phylogeny (Tai et al., 2015). In their Original Research, De Martini et al. discuss the drivers of protist diversification in the context of host genetic relationship. Furthermore, the digestion of plant tissues is driven by mutualistic ectosymbiosis in fungus-growing termites (Macrotermitinae), which is considered

an evolutionary novelty in "higher" termites originated about 30 Mya after the acquisition of gut symbionts by the ancestor of Termitidae (Aanen et al., 2002; Bourguignon et al., 2015; Bucek et al., 2019; Chouvenc et al., 2021). In this context, Korb et al. provide new evidence about the transmission of *Termitomyces* to their siblings in fungus-growing termites by evaluating whether the mode of transmission is related to the ecological success of these termites in West Africa. Finally, Moreira et al. investigated the role of nest bacteria and fungi in a food storing Syntermitinae through a combination of microbiome, genomics and transcriptomics. Mounds of these termites are made of soil and feces, and could function as a pre-fermentation chamber for the plant biomass that is stored in fecal made nodules (Menezes et al., 2018). This study showed the role of nest microbiota as a complementary system for lignocellulose digestion in the context of higher termite evolution.

Caste differentiation pathways in termites are regulated by complex postembryonic mechanisms. In this Research Topic, Oguchi et al., reviewed the differentiation pathways underlying the developmental mechanisms and evolutionary origin of the caste of neotenics, which act as secondary or replacement reproductives and constitute a termite synapomorphy. Lastly, Lee et al. investigated how hybridization between phylogenetically closed pest termite species affect the activity of sterile castes. The role of pheromones in communication and physiological regulation in termites from an evolutionary point of view is reviewed in this Research Topic by Mitaka and Akino.

In conclusion, our Research Topic highlight the latest advances in termite evolutionary ecology and cover a wide range of research areas: evolutionary biology, genomics, systematics, microbiology, biogeography, and ethology in 90 termite species, showing the increasing interest of researchers from around the world to study these amazing insects. This knowledge is expected to continue growing during the next years and have an impact into the development of new biotechnological applications such as: biodegradation, development of antibiotics and antifungal, biofuels and pest control industry.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Jordi Figuerola,
Spanish National Research Council (CSIC), Spain

*CORRESPONDENCE
Alberto Arab
albertoarab@gmail.com

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Editorial: Advances in the evolutionary ecology of termites, volume II

Daniel Aguilera-Olivares[1], Solange Issa[2] and Alberto Arab[3]*

[1]Departamento de Silvicultura, Facultad de Ciencias Forestales, Universidad de Concepción, Concepción, Chile, [2]Departamento de Biología de Organismos, Universidad Simón Bolívar, Sartenejas, Miranda, Venezuela, [3]TermitoLab, Centro de Ciências Naturais e Humanas, Universidade Federal do ABC (UFABC), Santo André, Brazil

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Editorial on the Research Topic
Advances in the evolutionary ecology of termites, volume II

The first volume of our Research Topic was published in 2021 and included nine articles (Arab et al., 2021). At June 2023, it has been cited 56 times and it has received more than 5,000 downloads and 35,000 views; additionally, according to the Web of Science (www.webofscience.com), nearly 11,500 termite articles on various topics were published from 1975 to 2021, showing the increasing interest of scientist around the world in these amazing but unpopular eusocial cockroaches. Two years later, another 900 articles were added to this list, and we are publishing the second volume of our Research Topic. Therefore, this Research Topic summarizes the growing interest in the evolutionary ecology of termite research in nine original research articles which expand our knowledge on various aspects of these fascinating insects.

About 3,100 termite species have been described to date, but only 28 species are invasive (~0.9%) and 55 are considered pest (~1.77%) that damage structural wood and other lignocellulosic materials in natural and peri-urban forest habitats (Evans et al., 2013; Evans, 2021; Coêlho et al., 2023). However, due to global warming, the number of both invasive and pest species, including termites, is expected to increase in the coming years (Zanne et al., 2022). For this reason, it is important to understand how colonies organize and what factors influence their distribution. In recent decades, research has increasingly focused on these topics (Figure 1). This Research Topic examines the importance of termite pests in five studies. In their Original Research, Pailler et al. and Lee and Su addressed the foraging ability of four species of termites. Their results suggest that some species have enhanced foraging abilities, which could explain their invasion success. Foraging behavior in termites is primarily executed by workers, although some studies have shown that soldiers can also act as scouts and actively participate in the foraging process (Casarin et al., 2008). In this sense, the organization of social insect colonies requires sophisticated molecular mechanisms to regulate caste composition according to colony requirements, as suggested in their Original Research by Matsunami et al.

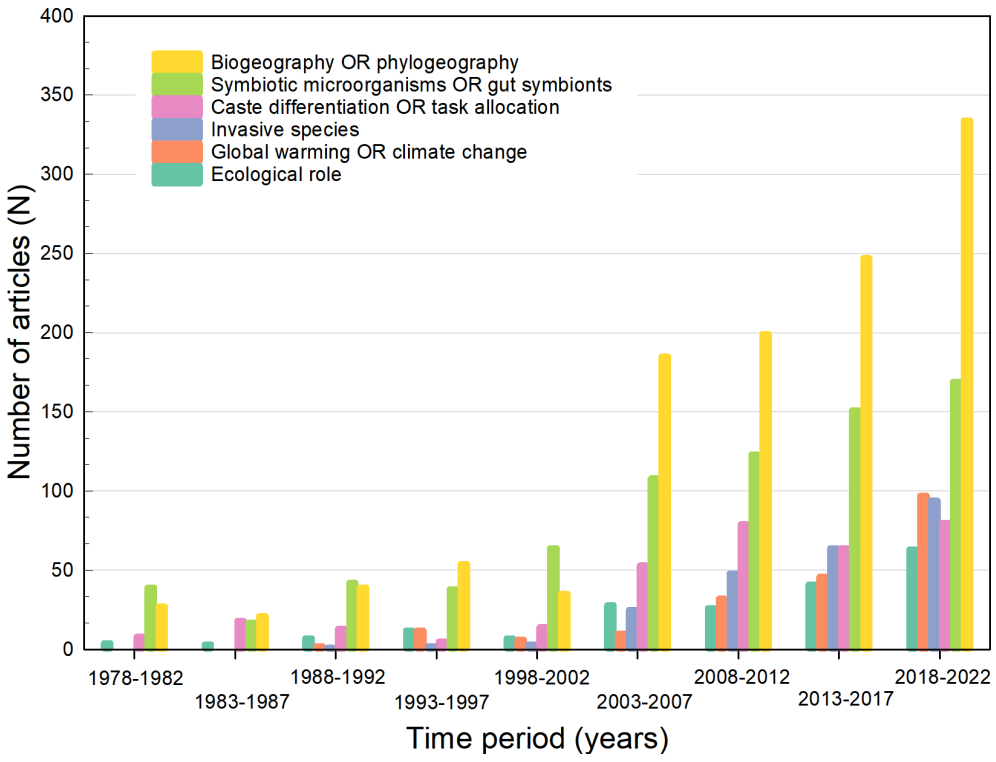

FIGURE 1
Number of articles published on termites from 1978 to 2022 in all databases available on Web of Science related with representative keywords from articles published in the present Research Topic.

The colonizing efficiency of some termites is maintained by the seasonal production of reproductive individuals, as reported by Chouvenc et al. when they dealt with the demographic development of laboratory colonies. However, social environments also put these insects at risk of contact with pathogens and create the potential for infectious events in their colonies (Rosengaus and Traniello, 2001). Protective mechanisms in termites include collective behaviors as self-isolation of infected individuals, allogrooming, cannibalism, and vibratory alarm responses (Rosengaus et al., 1999) as well as individual responses such as the production of antimicrobial compounds (Mitaka et al., 2017). Nevertheless, Moran et al. provide new evidence that infected termites did not communicate their infection status through shaking behaviors, suggesting the occurrence of other mechanisms used in communicating infection. In addition, infected workers travel to the densest part of the colony, where they can potentially benefit from grooming by healthy nestmates. It is also possible for infected individuals to visit nest areas with the strongest antimicrobial activity and disinfect themselves in the process. On the other hand, intraspecific competition could be another source of risk for a termite colony, especially for termites sharing the nesting substrate (Thorne et al., 2003; Aguilera-Olivares et al., 2017). In this Research Topic, Aguilera-Olivares et al. show the effect of intraspecific competition at individual level on morphological traits of soldiers of a one-piece nesting termite. They found that colonies that share a piece of wood produce bigger and more asymmetric soldiers compared to soldiers that develop in colonies that do not share a piece of wood. Thus, large body size in soldiers could be related with the chance of winning a battle.

Molecular and morphological analyses are useful tools to unveil the complexity of the coevolutionary dynamics between termites and their symbionts association. Non-termitid species rely on multiple lineages of flagellate protists for food digestion (Engel et al., 2009), and since protists are inherited vertically from the parents, their distribution among termite groups seems to be mediated by horizontal transfer (Tai et al., 2015). In their Original Research, Radek et al. discuss the drivers of protist diversification among non-termitid species and propose a new genus and family for a flagellate symbiont of the Serritermitidae family.

The diversification of the Termitidae is related to the expansion of the Miocene savannas. These ecosystems provided a new habitat for forest termites and a new food source (C4 grasses) that could withstand high temperatures and water scarcity (Solofondranohatra et al., 2018; Carrijo et al., 2020). In the savannas of southeastern Brazil, Alves et al. found that termites remove and consume dung from herbivore mammals more efficiently that dung beetles, thereby modulating the balance of carbon in this ecosystem.

In conclusion, the volume 2 of our Research Topic, *Advances in the Evolutionary Ecology of Termites*, addresses topics related to caste determination and task allocation, invasive biology, ecology, biogeography and phylogeography of termite species and their relationship with symbionts; which interest has increased in the last decades (Figure 1). A better understanding of termite biology at different organizational levels, *i.e.* from individual to ecosystem

level, will help improve our understanding of the issues related to the impact of global warming and climate change on these insects. Moreover, it will provide valuable insights for directing conservation efforts aimed at preserving this important insect group.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AA and DA-O. The first draft of the manuscript was written by DA-O and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Altered Mobility and Accumulation of Inefficient Workers in Juvenile Hybrid Termite Colonies

Sang-Bin Lee, Thomas Chouvenc, Jayshree Patel and Nan-Yao Su*

Department of Entomology and Nematology, Fort Lauderdale Research and Education Center, University of Florida, Fort Lauderdale, FL, United States

Hybridization of two different species is an important mechanism to have gene flows between species. Recently, mating of two economically important invasive species of subterranean termites (*Coptotermes formosanus* and *Coptotermes gestroi*) have been observed in the field and hybrids colonies have been established in the laboratory. It was previously reported that incipient colonies (∼1 year old) of hybrid *Coptotermes* species contained more termites than colonies of parental species, showing hybrid vigor. In this study, colony vigor and individual termite vigor were investigated in juvenile colonies (∼2 year old), using colony growth parameters and the movement activity of individual termites as proxies for the evaluation of hybrid vigor beyond the initial colony foundation. After 2 years from colony foundations, hybrid colonies showed no more hybrid vigor. In addition, movement activity of termites in hybrid colonies was significantly slower than in termites from conspecific colonies. It is suggested that a reduction in the molting rates of individuals in hybrid colonies may have a negative impact on their physiology and their movement activity. These possible changes in physiology may affect the movement of individuals, and accumulation of these inefficient termites in hybrid colonies may contribute to the loss of hybrid vigor at 2 years of age in hybrid colonies.

Keywords: *C. formosanus*, hybrid vigor, subterranean termite, invasive species, *C. gestroi*, hybridization

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****Correspondence:***
Sang-Bin Lee
lsb5162@ufl.edu

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INTRODUCTION

Sexual reproduction between two different species or relatively distant lineages can potentially allow for hybridization events, where it was estimated that up to 25% of plant species and 10% of animal species may have the ability to hybridize (Mallet, 2005, 2007). Hybridization events may result in various outcomes, such as gene flow between species, individuals with increase genetic heterogeneity, which sometimes results in evolutionary novelties and hybrid vigor (Rhymer and Simberloff, 1996; Seehausen, 2004; Mallet, 2005, 2007; Seehausen et al., 2008; Abbott et al., 2013). However, hybridization often results in low hybrid fitness, which primarily result from low fertility, low vigor, non-adapted traits or genetic incompatibilities. Although hybridization events have been reported in various insect groups, hybridization in social insects such as ants, bees, wasps and termites can provide unique opportunities to study the phenotypic consequences of novel genetic combinations from the individual to the colony level (Feldhaar et al., 2008).

Hybridization in social insects has primarily been documented in haplodiploid hymenopterans, such as ants and bees. Consequences of hybridization in these cases have often included negative

effects such as lack of viable F_1 (Seifert, 1999), infertile individuals (Pearson, 1983; Douwes and Stille, 1991; Umphrey and Danzmann, 1998; Umphrey, 2006) and reduced fertilities in F_1 (Plateaux, 1979; Julian et al., 2002; Schwander et al., 2007). In other cases, hybridization resulted in enhanced traits, such as the hybrids of two fire ant species (*Solenopsis*) that outcompeted native species in disturbed areas (James et al., 2002; Gibbons and Simberloff, 2005). It was also reported that hybridization of honey bees (*Apis*), Africanized honey bees (hybrids between European and African honey bees) not only displayed intermediate traits of both, but also showed high levels of defensive behavior (Guzmán-Novoa et al., 2002; Alaux et al., 2009), causing significant problems to human society, beekeeping and agriculture (Winston, 1992; Scott Schneider et al., 2004).

In termites, hybridization events have been reported in only a few species, in the genera *Coptotermes* (Chouvenc et al., 2015; Su et al., 2017), *Reticulitermes* (Lefebvre et al., 2008), *Zootermopsis* (Aldrich and Kambhampati, 2009), *Pseudacanthotermes* (Connétable et al., 2012), and *Nasutitermes* (Hartke and Rosengaus, 2011). Although termites and social Hymenoptera showed evolutionary convergence, they differ in key biological characteristics as termites are social roaches with a hemimetabolous development, where most individuals retain juvenile form and keep molting to the next larval instar until they differentiate into reproductives or soldiers, excluding workers in derived family Termitidae (Nalepa, 2010, 2011, 2015; Chouvenc and Su, 2014; Roisin, 2016). Colony members that have to periodically engage in molting rely on their nesmates to molt successfully (Xing et al., 2013), and for the re-acquisition of their obligatory gut mutualist (Nalepa, 2015, 2017). Because of the fundamental differences in developmental pathways and the recurrent molting requirement in termites, consequences of hybridization events may differ from those of holometabolous social hymenopterans, where all functional individuals are adults that undergo complete metamorphosis.

Of the 3,000 described termite species, *Coptotermes formosanus* Shiraki [the Formosan subterranean termite (FST)], and the *C. gestroi* (Wasmann) [Asian subterranean termite (AST)], are the most destructive structural pests, and both species are successful invaders (Rust and Su, 2012; Evans et al., 2013; Chouvenc et al., 2016). *Coptotermes formosanus* distributed from subtropics to warm temperate regions, is endemic to southern China and Taiwan, and have invaded Japan and parts of the Southeastern United States (Su, 2003; Chouvenc et al., 2016). However, *C. gestroi* primarily found in the tropics, is native to southeast Asia and has been introduced to many parts of the neotropics (Evans et al., 2013; Su et al., 2017). These two species are allopatric in their native ranges, and it was estimated that both species have evolved separately 15–20 million years ago (Bourguignon et al., 2016).

As a result of human activities and climate change, FST and AST are now sympatric in three locations: Taiwan, Hawaii and southeastern Florida (Chouvenc et al., 2015; Su et al., 2017). Since 2013, simultaneous dispersal flight events were observed in Florida (Chouvenc et al., 2015, 2017b) and heterospecific mating behavior were observed, as AST males readily initiated tandem with females of FST (Chouvenc et al., 2015). From such pairings, hybrid colonies from the two possible mating combinations (♀AST × ♂FST and ♀FST × ♂AST) were successfully established in the laboratory, showing that there were no pre-zygotic barriers for the production of a viable F_1 hybrid brood (Chouvenc et al., 2015, 2017a). Hybrid colonies after 1 year of foundation showed relatively high hybrid vigor by displaying faster colony growth compared to colonies founded with conspecific pairs of FST and AST (Chouvenc et al., 2015). In addition, individuals from hybrid colonies displayed a wider temperature range survivorship than parental species, suggesting that hybrid colonies have the potential to survive in a geographic range combining the tropical, subtropical to temperate region distribution of the two parental species (Patel et al., 2018).

Although heterospecific pairings were observed in the field, it is unknown if such matings can result in successfully hybrid colony establishment in the field, and if such colonies could produce fertile F_1 alates. Laboratory colonies therefore allows for the biological characterization of such hybrids. In this study, we investigated the consequences of hybridization compared to parental species after 2 years of colony foundation from the individual to colony level, to determine if the hybrid colony vigor previously observed in 1 year-old colony (Chouvenc et al., 2015) is maintained as colonies grow. To test this hypothesis, we used 2 year-old colonies to measure their biomass (overall colony growth), the movement behavior of individual termites, and the molting rate of workers in the two heterospecific mating combinations (hybrids) and of the two conspecific colonies (parents).

MATERIALS AND METHODS

Colony Foundations and Rearing

Alates (winged imagoes) of *C. formosanus* and *C. gestroi* were collected in Broward County (Florida, USA) during dispersal flight events using a light trap (Chouvenc et al., 2015). Rearing units were comprised of 6 g of organic soil moistened with distilled water at the bottom of a plastic cylindrical vials (8 cm height × 2.5 cm diameter). Four spruce pieces (*Picea* sp.) were placed on top of the soil and a 3% agar solution (5 cm high) was poured in. A small vertical hole was made through the agar using a piece (8 × 0.5 × 0.5 cm) of wood to serve as royal chamber for the mated pair. After the agar solidified, a male and a female of each species were introduced into each rearing unit, then covered with its lid, which pierced with a safety pin to have four tiny holes to allow air exchange. Four mating combinations were prepared: hybrid_F (HF; ♀*C. formosanus* × ♂*C. gestroi*), hybrid_G (HG; ♀*C. gestroi* × ♂*C. formosanus*), *C. formosanus* (FST; ♀*C. formosanus* × ♂*C. formosanus*), and *C. gestroi* (AST; ♀*C. gestroi* × ♂*C. gestroi*). After 1 year, colonies were transferred to ∼1.5 L plastic boxes (17 × 12 × 7 cm) containing moist organic soil and wood pieces. Distilled water and new wood pieces were provisioned periodically. All rearing

Abbreviations: AST, Asian subterranean termite; FST, Formosan subterranean termite; HF, Hybrid F (♀*C. formosanus* × ♂*C. gestroi*); HG, Hybrid G (♀*C. gestroi* × ♂*C. formosanus*).

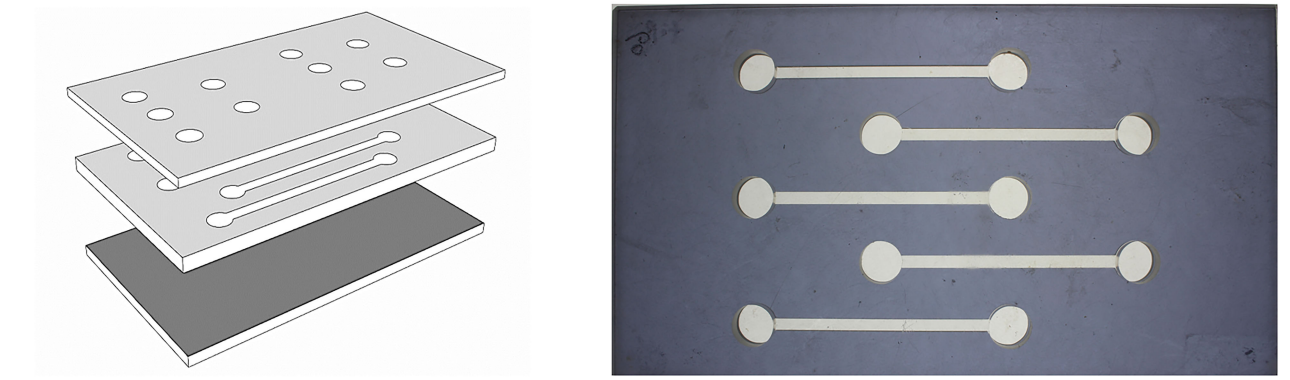

FIGURE 1 | Experimental arena used in behavioral assay. **(A)** The planar arena composed of three layers (top, middle and bottom layer). The top layer contain introduction hole, middle layer produce tunnel and bottom layer. **(B)** Top view of the planar arena.

units and experiments were carried out at 28 ± 1°C and 80 ± 3% RH.

Experiment 1: Colony Census

Two year-old colonies of the four different mating types (i.e., HF, HG, FST, and AST) were processed to measure total biomass of the colony and each castes as a proxy variable for overall colony growth. All colonies had well-developed carton material and had the presence of larvae and younger worker instars, confirming reproductive activity. To collect all termites from the container, the wood, the carton and debris were progressively removed. In the process, termites were collected using aspirators, however, when termites were mixed with nest material, the sorting process of Tamashiro et al. (1973) was used. This procedure was repeated multiple times until all termites were collected in the colonies and separated from nest materials.

Total biomass of the colony and body mass of queen, king, 100 workers, and 20 soldiers were measured from three colonies of each mating combination. For each colony, 100 workers and 20 soldiers were randomly collected three times for measurements. Mass of 100 workers and 20 soldiers were then divided by their numbers to measure individuals mass. Statistical differences of the variables were tested with a permutation analysis of variance (ANOVA) at $\alpha = 0.05$ with mating combinations as a factor, and pairwise comparisons were applied for *post-hoc* analysis. In test, number of permutation was 9,999.

Experiment 2: Individual Behavior Assay

Artificial tunnels within a planar arena were used to observe termite movement behavior (**Figure 1**). The bottom and top layers were made of transparent plexiglass (9 × 16 cm, 5 mm in height), separated by gray middle layer made of plexiglass (9 × 16 cm, 2 mm in height). The surface of the bottom layer was sanded to provide traction for termite movement (Roughness 60, CAMI grit designation) and tunnels were cut out of the middle layer. Releasing chambers were provided at both ends of the arena on the top layer. The tunnel widths were 3 mm, and the distance between releasing chambers was 5 cm.

Workers (undifferentiated larval of the 4th instar) of similar body size were randomly selected from each mating combination (HF, HG, FST, and AST). For each assay, a termite was introduced into each tunnel, such that five workers were tested simultaneously (**Figure 1**). Termites were introduced into the releasing chamber on the left side for consistency across the experiment, covered with a transparent coverslip, and were left for 10 min for acclimatization before recording. A digital camcorder (SONY CX-700, Tokyo, Japan) was mounted on top of the arena for 10 min video recording. At the end of each experiment, Arenas were dismantled and washed with detergents, 95% ethanol and distilled water three times to prevent confounding effects of potential trail-following pheromones. Fifteen workers from each colony and three different colonies of origin were tested for each mating combination, for a total of 45 workers in each of HF, HG, FST, and AST mating combinations.

Videos were analyzed using a processing software, virtualdub (http://www.virtualdub.org/) and two variables were obtained. "Passing time" was defined as the time taken by a termite to move through the entire tunnel length, from one chamber to the other. "Stop time" was defined as the time taken by a termite from the moment it entered one of the chamber and the moment it left the chamber. Passing times and stop times of each termite were measured for 10 min, and all of its instances of passing time and stop time were averaged, yielding a single value for passing time and stop time, respectively, for each termite. The passing time and stop time (variables) were compared among mating combinations using a permutation ANOVA and pairwise comparison as *post-hoc* at $\alpha = 0.05$. In the permutation ANOVA, the number of permutation was 9,999. In addition, rank distribution of passing and stop times were subjected to curve fitting with linear, logarithmic, power and exponential curves.

Experiment 3: Molting Ratio Experiment

Whole colonies (two-years-old) of HF, HG, FST and AST were processed and termites were placed in plastic boxes (17.5 × 12.5 × 7 cm^3) containing moistened sand (depth: 0.5 cm) at the bottom. Following the method of Raina et al. (2008) and modified by Xing et al. (2013) to identify termites in pre-molt and/or pre-ecdysis period, three cellulose filter papers (diameter: 9 cm) stained with Nile Blue A (0.1% wt:wt) were used as a dietary marker. Termites in the pre-molt and/or pre-ecdysis period stop feeding to void their guts prior to ecdysis and will not be stained. After 5 days, numbers of white termites and blue termites in the colonies were counted to calculate the percentages of termites, defined by number of white workers divided by total number of workers, in both pre-molt and pre-ecdysis stages. Also, mortality during the treatment of Nile Blue A was measured. In experiment 3, 3 different colonies of HF, HG, FST and AST were tested. Data were arcsine transformed and subjected to an one-way ANOVA ($\alpha = 0.05$) with mating combination as a factor, followed by Tukey's HSD for *post-hoc* analysis. All statistical analyses were carried out using PAST version 3 (Hammer et al., 2001) and IBM SPSS version 20 (IBM Corp.).

RESULTS

Experiment 1. Colony Census

We found no significant difference in total biomass ($F = 2.176$, $df = 3,8$, $p = 0.171$, body mass of queen ($F = 0.928$, $df = 3,8$, $p = 0.489$), and of king ($F = 2.073$, $df = 3,8$, $p = 0.170$) (**Table 1**). Statistical differences were detected in body mass of workers ($F = 6.228$, $df = 3, 8$, $p = 0.026$) and of soldiers ($F = 5.707$, $df = 3,8$, $p = 0.014$), revealing some degree of variability of biological traits among mating combinations (**Table 1**). FST workers displayed significantly higher individual body weight than AST, but neither hybrids (HF and HG) was different from FST nor AST, presented as intermediate trait. The average individual mass of soldiers was highest in HG and lowest in AST.

Experiment 2: Individual Behavior Assay

In individual movement bioassays, both FST and AST workers displayed significantly faster passing time ($F = 14.916$, $df = 3,176$, $p < 0.001$) than hybrid workers HF and HG (**Figure 2A**). In addition, HF and HG workers had statistically longer stop time than AST workers ($F = 6.019$, $df = 3,176$, $p < 0.001$), with FST being intermediate.

Exponential curve had the best fit for rank distribution of passing time for both hybrid species (HF: $R^2 = 0.965$, $F = 1,189.828$, $p < 0.001$; HG: $R^2 = 0.885$, $F = 332.139$, $p < 0.001$), whereas linear curves were the best fit for both FST and AST (FST: $R^2 = 0.955$, $F = 902.215$, $p < 0.001$; AST: $R^2 = 0.983$, $F = 2421.442$, $p < 0.001$) (**Figure 3A**). Such observation revealed that hybrid mating combination were slower on average than parental species and there was the presence of several outliers within the workers of both hybrid mating combinations with extremely slow individuals. In addition, similar patterns were observed in stop time that exponential curves showed the highest R^2 value among tested functions for both hybrids (HF: $R^2 = 0.875$, $F = 300.007$, $p < 0.001$; HG: $R^2 = 0.932$, $F = 586.752$, $p < 0.001$), however, rank

TABLE 1 | Census of 2-years-old hybrids (HF and HG) and conspecific colonies (FST and AST).

	Colony	Colony mass (g)	Mean ± S.D.	Queen mass (mg)	Mean ± S.D.	King mass (mg)	Mean ± S.D.	Worker mass (mg)*	Mean ± S.D.	Soldier mass (mg)*	Mean ± S.D.
HF	1	4.06	3.68 ± 0.41[a]	10.20	12.50 ± 1.71[a]	8.50	7.77 ± 0.89[a]	1.63	1.66 ± 0.03[ab]	2.47	2.26 ± 0.23[ab]
	2	3.87		13.00		6.50		1.69		2.38	
	3	3.12		14.30		8.30		1.68		1.94	
HG	1	2.57	2.81 ± 0.50[a]	10.40	14.53 ± 5.50[a]	7.10	7.03 ± 0.09[a]	1.96	1.79 ± 0.18[ab]	2.55	2.73 ± 0.24[b]
	2	3.50		22.30		6.90		1.87		3.08	
	3	2.36		10.90		7.10		1.54		2.57	
FST (*C. formosanus*)	1	2.92	4.53 ± 1.22[a]	13.20	13.20 ± 0.08[a]	7.50	9.03 ± 1.23[a]	1.96	1.90 ± 0.05[b]	2.40	2.50 ± 0.09[ab]
	2	5.89		13.10		10.50		1.90		2.49	
	3	4.76		13.30		9.10		1.84		2.62	
AST (*C. gestroi*)	1	3.86	4.08 ± 0.19[a]	10.10	9.83 ± 0.76[a]	8.50	7.13 ± 0.97[a]	1.42	1.47 ± 0.10[a]	1.86	2.01 ± 0.12[a]
	2	4.06		8.80		6.60		1.60		2.15	
	3	4.32		10.60		6.30		1.38		2.03	

For each mating type, colony biomass (Mean ± SD), body mass of queen, king, worker and soldier were measured from 3 different colonies. In case of workers and soldiers, biomass of 100 workers and 20 soldiers were recorded and divided by its numbers. Different letters in mean column denote significant differences according to Tukey's HSD (α = 0.05). HF (♀ AST × ♂ FST), HG (♀ FST × ♂ AST), FST (♀ FST × ♂ FST), and AST (♀ AST × ♂ AST). FST, Formosan subterranean termites; Coptotermes formosanus Shiraki; AST, Asian subterranean termites; C. gestroi (Wasmann).

*Average of three different samples per colony.

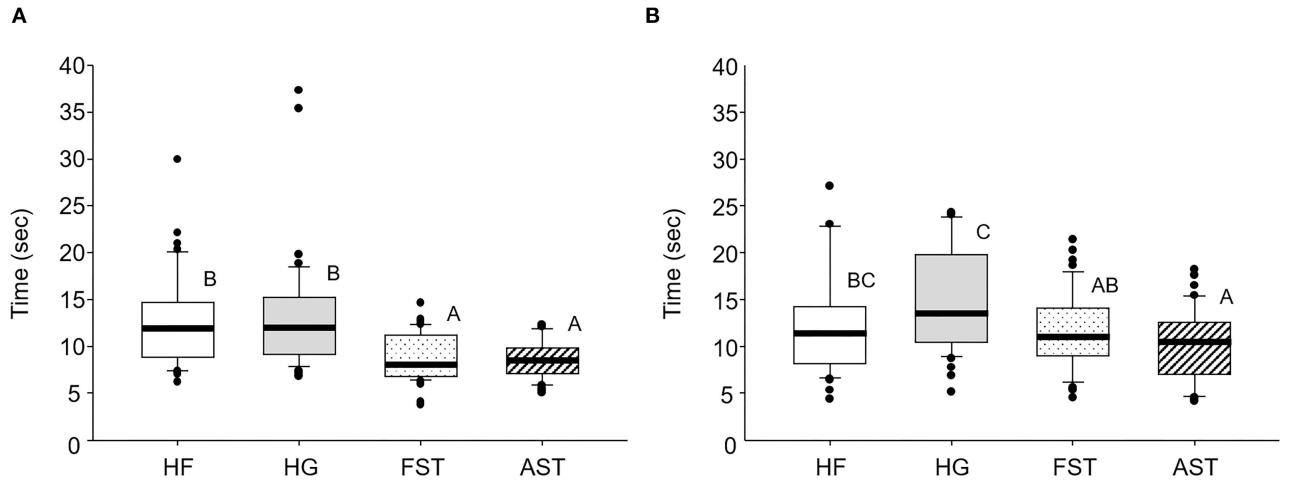

FIGURE 2 | Box plots of movement activities of 2 hybrids and conspecific colonies. **(A)** Passing time and **(B)** stop time. Different upper caste letters indicates statistical difference according to Tukey's HSD ($\alpha = 0.05$). Line on the box represents mean and circles above and below box indicate outliers. HF (♀ AST × ♂ FST), HG (♀ FST × ♂ AST), FST (♀ FST × ♂ FST), and AST (♀ AST × ♂ AST). FST, Formosan subterranean termites; *Coptotermes formosanus* Shiraki; AST, Asian subterranean termites; *C. gestroi* (Wasmann). In each colony, 15 termites were tested and 3 different colonies were used (*N*: 15 × 3 = 45).

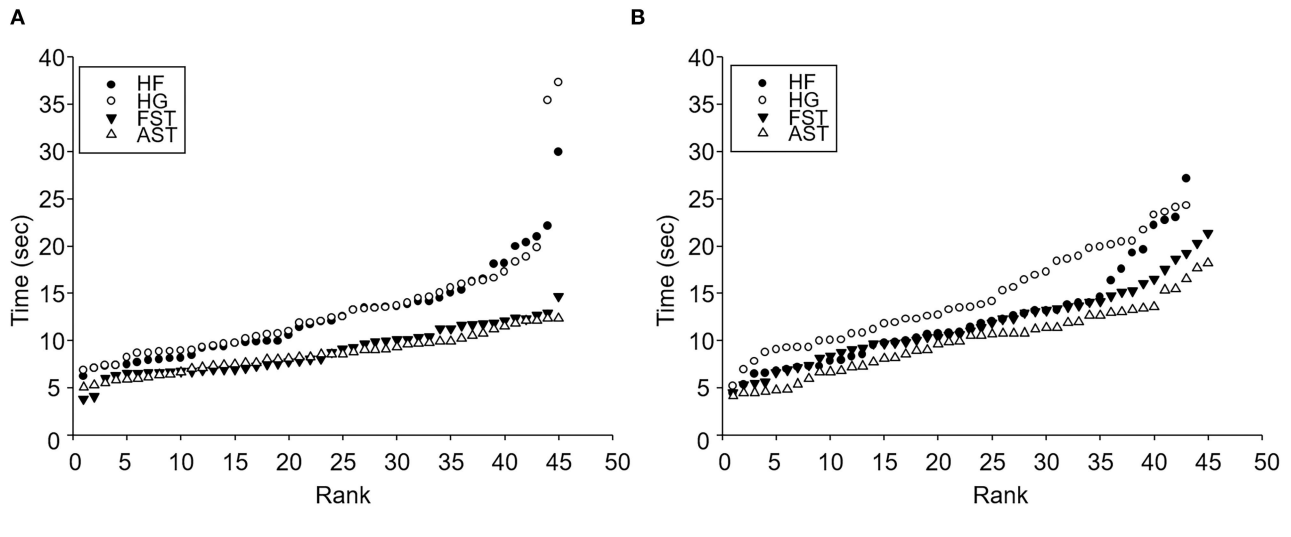

FIGURE 3 | Rank distribution of movement activities of 2 hybrids and conspecific colonies. **(A)** Passing time and **(B)** Stop time. Black circles, blank circles, black triangles, and blank triangles are HF, HG, FST, and AST. HF (♀ AST × ♂ FST), HG (♀ FST × ♂ AST), FST (♀ FST × ♂ FST), and AST (♀ AST × ♂ AST). FST, Formosan subterranean termites; *Coptotermes formosanus* Shiraki; AST, Asian subterranean termites; *C. gestroi* (Wasmann).

distribution of both FST and AST were fitted onto linear curves (FST: $R^2 = 0.957$, $F = 952.564$, $p < 0.001$; AST: $R^2 = 0.968$, $F = 1321.236$, $p < 0.001$) (**Figure 3B**), suggesting that hybrids species spend more time for stop.

Experiment 3: Molting Ratio Experiment

The mortality during Nile Blue A treatments was not significantly differed across mating types ($F = 1.171$, df =3,8, $p = 0.379$) and it was from 5 to 9% in 5 days. The percentages of termites in pre-molt fasting stage, right before molting, was significantly higher in FST and AST, 11.97 ± 1.98 and 10.46 ± 3.43%, respectively, than those of HF and HG 4.31 ± 1.98 and 2.34 ± 0.35%, respectively ($F = 10.535$, df =3,8, $p < 0.01$) (**Figure 4**).

DISCUSSION

Hybrid *Coptotermes* displayed faster growth during the 1st year after colony foundations than parent colonies (Chouvenc et al., 2015), but current study revealed that by year two, such differences in colony growth were no longer observed, and all mating combinations displayed similar colony size. Workers and soldiers in hybrid combination were approximately intermediate

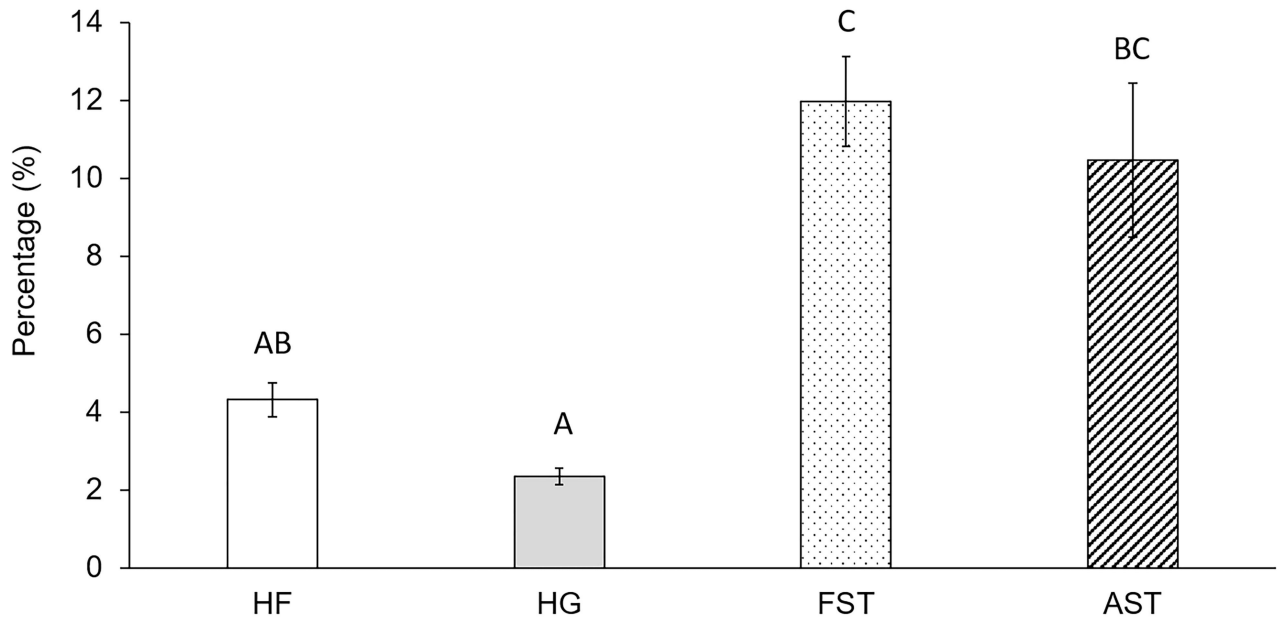

FIGURE 4 | Percentage of termites in pre-molting stage. Different upper case letters represent statistically different group according to Tukey's HSD ($\alpha = 0.05$). HF (♀ AST × ♂ FST), HG (♀ FST × ♂ AST), FST (♀ FST × ♂ FST), and AST (♀ AST × ♂ AST). FST, Formosan subterranean termites; *Coptotermes formosanus* Shiraki; AST, Asian subterranean termites; *C. gestroi* (Wasmann). For each mating types, 3 different colonies were used.

in body size and cuticular hydrocarbon profiles when compared to those of the parental species (Chouvenc and Su, 2017). These observations confirm that hybrid termites can display some intermediate phenotypic traits of their parental species.

We initially expected to obtain hybrid colonies with even larger colonies than parental species at 2 years of development. Our current study, however, showed no difference in growth rate between hybrid and parent species, which indicates that something happened in hybrid colonies between 1 and 2-year of colony growth that potentially slowed down their development. The movement assay revealed that hybrid workers were slower than workers of parental species. In addition, hybrid colonies contained a number of outlying individuals that were extremely slow, when compared with workers in parental species. These results therefore suggest that some individuals within hybrid colonies may suffer a reduced mobility, potentially impacting their overall efficiency and reducing their contribution toward the function of the colony, and ultimately reducing the vigor of the colony.

The physiological changes are currently unknown that underlie the slow movement of workers in hybrids colonies. Chouvenc and Su (2014) indicated that during this time frame of colony development, workers progressively have the ability to successfully molt into older instar, which implies an accumulation of older and more efficient workers within the colony. Molting in termite workers is a critical part of their development as their physiology require them to molt regularly (Korb and Hartfelder, 2008; Xing et al., 2013; Kakkar et al., 2016). Roisin (2016) argues that periodical molting in termite workers allow for individual to regularly shed an aging cuticle and dull mandibles, to a new, thicker cuticle and sharp mandibles. The intermolt period in a termite worker must therefore be bound in time and molting should be taking place at an optimal time to keep workers as efficient as possible within their molting cycle. This intermolt period is critical which is about 45 days in *C. formosanus* (Kakkar et al., 2016) and the molting takes usually 6 days in *C. formosanus*, as they stop feeding to void their guts around 6 days before ecdysis (Xing et al., 2013).

Our last experiment therefore aimed at investigating the molting rates of workers at the colony level, to determine if hybrid worker would molt more or less often than parental species, which would potentially impact the efficiency of their active intermolt period and their mobility. Daily molting frequencies of *C. formosanus* are determined to be ∼2% of all workers per day at 28°C (Kakkar et al., 2016), and in this study we confirmed such molting frequency in *C. formosanus* (2%/day), with similar molting frequencies in *C. gestroi.* However, the molting frequency in both hybrids were drastically lower (0.6%/day). The result implies that the intermolt period in hybrid workers is possibly longer than in parental species, resulting in the accumulation of individuals that have passed their optimal molting time with a reduced efficiency. Consequently, many worker termites in 2-year-old hybrid colonies may progressively lose the efficiency of their contribution to the colony, and potentially become a burden in the colony, ultimately slowing down the growth of the colony.

We here argue that, if hybrid termites of two *Coptotermes* species display a less than optimal molting cycle, with potential

complication in their development and their efficiency, it may be a significant hurdle for a potential establishment of hybrid colonies in the field and further potential gene flow among species. The molting process in termite workers is such a fundamental aspect of their biology (Nalepa, 2011) that a potential dysfunctional gene expression within hybrid termites could result in the accumulation of low vigor individuals, and raise questions about the potential production of functional and fertile alates in hypothetic mature colonies. However, it is challenging to observe the fertility of F_1 because it will take at least 5–8 years for a colony to reach maturity and produce winged adults (Chouvenc and Su, 2014).

Unlike social Hymenoptera, termites are diploid social insects, so hybridization will impacts on both females and males. In social Hymenoptera, such as ants, bees and wasps, hybridization can only affect female phenotypes because males develop from an unfertilized egg (Feldhaar et al., 2008). Currently, the effects on females and males in the hybridization of termites are unknown. Nevertheless, hybridization of termites can provide a framework to understand gene flow between males and females to their offspring. Only a few studies have reported hybridization of termites with cases of introgressive hybrids in the genus *Reticulitermes* (Lefebvre et al., 2008) and *Zootermopsis* (Aldrich and Kambhampati, 2009). Viable hybrids were observed from pairing *Nasutitermes corniger* (Motschulsky) and *N. ephratae* (Holmgren) in the laboratory (Hartke and Rosengaus, 2011). In heterospecific pairs, courtship and nest construction behavior as phenotypes of the colony were similar to conspecific pairs, and survival of heterospecific pairs was determined by females of pairings rather than males (Hartke and Rosengaus, 2011). There was no observable hybrid vigor, since each combination produced offspring either as similar as or less than conspecific pairs 60 days after colony foundation (Hartke and Rosengaus, 2011).

As pointed out by Chouvenc and Su (2017), hybrid colonies can provide unique opportunities and contribute not only to understanding of theoretical and fundamental questions, such as mode of transmission of endosymbiotic communities, but also practical aspects, such as controls against hybridization of two destructive subterranean termites. Future, hybridization between FST and AST is needed to address foraging activities, physiology and genetics.

In conclusion, it is premature to conclude consequences of hybridization of two destructive subterranean termites, since the life-cycle of these species takes more than 5 years. We only investigated 2-year-old hybrids and parental species in order to study consequences of hybrid vigor after the report by Chouvenc et al. (2015). Hybridization has significant effects on both the individual and colony level, and these effects are rather negative in terms of movement behavior and physiology, with no hybrid vigor after 2 years from colony foundation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

S-BL and TC developed the idea and designed experiments. S-BL conducted experiments and the statistical analyses. S-BL and JP collected the data. S-BL, N-YS, and TC interpreted the results and discussed the direction of the manuscript. All authors contributed to the writing and approved the manuscript.

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Can Differences in Symbiont Transmission Mode Explain the Abundance and Distribution of Fungus-Growing Termites in West Africa?

Judith Korb[1]*, *Simon Kolotchèlèma Silué*[1,2] and *N'golo Abdoulaye Koné*[2]

[1] Evolutionary Biology and Ecology, Institute of Biology i (Zoology), University of Freiburg, Freiburg im Breisgau, Germany,
[2] Natural Sciences Department (UFR-SN), Nangui Abrogoua University, Abidjan, Côte d'Ivoire

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***Correspondence:**
Judith Korb
judith.korb@biologie.uni-freiburg.de

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Fungus-growing termites (Isoptera: Macrotermitinae) dominate African savannah ecosystems where they play important roles in ecosystem functioning. Their ecological dominance in these ecosystems has been attributed to living in an ectosymbiosis with fungi of the genus *Termitomyces* (Lyophyllaceae). Evolutionary theory predicts that the transmission mode of a symbiont determines cooperation and conflict between host and symbiont with vertical transmission (co-transmission of host and symbiont offspring to the next generation) leading to less conflict than horizontal transmission (symbionts are acquired by the host from the environment). Thus, one can hypothesize associations with vertical transmission to be ecological more successful than those with horizontal transmission. We tested this by analyzing whether there is an association between transmission mode and fungus-growing termite species abundance and distribution in West-African savannah and forest ecosystems. We used data from a total of 78 study sites comprising protected National Parks as well as anthropogenically disturbed ecosystems, covering Benin, Côte d'Ivoire, and Togo. Our results showed that, in contrast to expectation, species with horizontal symbiont transmission were more common. We encountered more often species with horizontal than vertical transmission. This result might be due to the fact that only five out of the 25 identified fungus-growing termite species had vertical transmission. Yet, species with horizontal transmission also had higher relative abundances within study sites than those with vertical transmission. Thus, transmission mode is unlikely to explain abundance differences between fungus-growing termite species.

Keywords: cooperation, conflict, host, symbiosis, symbiont, termites, transmission mode

INTRODUCTION

Fungus-growing termites (Isoptera: Macrotermitinae) thrive in African savannah ecosystems where they often make up more than 50% of termites' species diversity and the large majority of termites (e.g., Hausberger et al., 2011; Hausberger and Korb, 2015, 2016; Schyra et al., 2019a; and reference therein) and where they play pivotal roles in ecosystem functioning (e.g., Bignell and Eggleton, 2000; and references therein). Within the ecological food web, they are main macro-detrivores, essential for decomposition of dead plant material, and are prey for a wealth of animals from invertebrates (such as ants) to vertebrates (such as birds and mammals). Some species are even specialized on termites as prey, like the ant *Megaponera analis* or aardwarks (*Orycteropus afer*), aardwolfs (*Proteles cristata*) or pangolins. Termites also provide important ecosystem services as their activity enhances soil quality, for instance by increasing soil fertility and improving water infiltration rates (Lee and Wood, 1971; Holt and Lepage, 2000; and references therein). Additionally, the "termitaria" of mound-building species present new habitats for plants and animals which are partly obligatorily dependent on them (e.g., Darlington, 1989; Erpenbach et al., 2017). Thus, fungus-growing termites foster biodiversity and are essential for its maintenance, especially in savannah ecosystems.

The ecological dominance of fungus-growing termites in savannah ecosystems, which are characterized by dry seasons, has been attributed to their ectosymbiosis with *Termitomyces* fungi (reviewed in Korb, 2020). The termites cultivate these fungal symbionts within their nests where they provide constant optimal conditions for fungal growth, including high humidity. This allows the fungal symbiont to be active all year round and relatively independent of environmental conditions, while other decomposing fungi and microbes are mainly restricted to the rainy season. Thus, *Termitomyces* symbionts flourish together with their termite hosts which benefit from reliable and more efficient plant decomposition compared to other termites (e.g., Rouland et al., 1991; Rouland-Lefèvre, 2000; Poulsen, 2015; da Costa et al., 2019). Yet, species of fungus-growing termites differ in distribution and abundance (e.g., Pomeroy et al., 1991; Bagine et al., 1994; Lepage and Darlington, 2000; Schyra et al., 2019a), and thus potentially in ecological importance. In this study, we tested whether the fungal transmission mode (i.e., how the symbiont is transmitted to the next generation) may contribute to explaining the ecological success of different fungus-growing termite species.

Evolutionary theory predicts that the transmission mode of a symbiont can be an important factor in explaining the degree of cooperation and conflict in symbiotic associations, and thus their "success" (Frank, 1994, 1996, 1997, 1998; Foster and Wenseleers, 2006; Leeks et al., 2019). Associations in which symbiont offspring are strictly co-transmitted with host offspring to the next generation (vertical transmission) are predicted to have less conflict than associations in which the symbiont can disperse independently of their host and infect new hosts (horizontal transmission). In the former, the fitness interests of host and symbiont are largely aligned and symbionts can mainly increase their fitness by enhancing host fitness. This leads to less potential conflict between host and symbiont. Additionally, evolutionary theory predicts that vertical transmission of symbionts, especially if uniparental (i.e., via a single host parent), further reduces potential conflict because it results in increased genetic homogeneity (i.e., increased relatedness) of symbionts within hosts. In the case of strict uniparental, vertical transmission, symbionts are expected to be clonally propagated. Thus, conflict among different symbiont strains within a host is prevented which would, for instance, reduce availability of resources to hosts.

In fungus-growing ants, fungal symbiont transmission is generally vertical and per default uniparental as colonies are founded by a single female (the queen) (Nobre et al., 2011c). This contrasts with fungus-growing termites (e.g., Korb and Aanen, 2003; Nobre et al., 2011c). Most association are characterized by horizontal transmission with *Termitomyces* fungi producing fruiting bodies (mushrooms) which release spores that are picked up by foraging termite workers (Johnson et al., 1981; Koné et al., 2011). The few exceptions are *Macrotermes bellicosus* and *Microtermes* species (Grasse and Noirot, 1955; Johnson, 1981; Johnson et al., 1981; Korb and Aanen, 2003; Nobre et al., 2011a, c). Strikingly, in both taxa vertical transmission is uniparental (in *M. bellicosus* the winged males carry fungal spores, in *Microtermes* the winged females) although the default option would be biparental as colonies are founded by a male (king) and a female (queen). This variation in transmission mode in fungus-growing termites offers the unique possibility to test for an effect of transmission mode on the ecological success of fungus-growing termites.

For a long time, research on fungus-growing termites was hampered by taxonomical problems. Except for a few, often iconic mound building species such as *Macrotermes bellicosus*, species could not be identified reliably using morphological means (Korb et al., 2019). Especially for the potentially species-rich genera *Microtermes* and *Odontotermes* this is very difficult (Korb et al., 2019). Morphological species identification is generally difficult in termites as species-specific markers are rare (e.g., Hausberger et al., 2011; Korb et al., 2019; and references therein), but it is even more problematic in Macrotermitinae. They have simplified guts which prevents using gut traits for identification, as successfully done in some other termites (Sands, 1998). The problem of species identification has been overcome by applying genetic markers (Korb et al., 2019 and references therein), so that we now have a good species list for West African termites (Hausberger et al., 2011; Schyra et al., 2019a). This allows us to do comparative analyses and test for an effect of transmission mode on the ecological success of fungus-growing termite species across West African savannah and forest regions.

MATERIALS AND METHODS

Data Sets

We used data on termite communities that we had published for disturbed and protected savannah ecosystems in Benin (Hausberger et al., 2011; Hausberger and Korb, 2015, 2016) and Togo (Schyra and Korb, 2019; Schyra et al., 2019a,b). They were supplemented with data from Côte d'Ivoire collected

in protected as well as disturbed areas belonging to different phytogeographical zones (i.e., from evergreen forest to Guinean and Sudano-Guinean savannah). In line with the published studies (Hausberger and Korb, 2016), we had three disturbance regimes: (1) protected (i.e., well protected National Parks); (2) intermediate anthropogenic disturbance (e.g., old fallows, National Parks with cattle grazing, low protection status); (3) strong anthropogenic disturbance (e.g., young fallows, plantations, fields).

In all studies, sampling was done using the standardized belt transect protocol, first developed for sampling termites in forests (Jones and Eggleton, 2000) and then adapted to savannahs (Hausberger et al., 2011). In short, a transect is established of 50–100 m lengths and 2 m width which is subdivided into 5 m × 2 m sections. Within each section, a thorough systematic search for termites of dead plant material on the ground, on and in trees and mounds is done for a standardized period of time by a trained person. This search is supplemented by soil scrapes measuring around 15 cm × 15 cm × 10 cm to specifically collect termites in the soil. Whenever we found/encountered termites during the search within a transect section, we collected a few specimens in a vial (5–10 individuals; mainly soldiers). Then we continued searching within the section and when we encountered termites again they were placed in a separate vial. The number of resulting vials for a study site (i.e., the sum over all transect sections for all replicate within a site) was used as encounter rate. This is used as a surrogate of species abundance (Davies, 2002). Samples were stored in 99% ethanol for species identification and subsequent analysis.

Some details in sampling effort differed between the published data and the newly included data for Côte d'Ivoire. Transect lengths in Togo and Benin was 50 m (i.e., 10 sections) while it was 100 m (i.e., 20 sections) for Côte d'Ivoire. The time that one person spent searching for termites in a section was 15 min for Togo and Benin, while it was four persons each 10 min for Ivorian savannah sites and 15 min for Ivorian forest sites. The number of soil scrapes was eight in the former studies and 12 in Côte d'Ivoire. To characterize a study site, one transect was done in Benin, three transects in Togo, and five in Côte d'Ivoire. Sampling effort differed to adjust for varying termite abundance between countries/regions and habitats. Varying sampling effort, however, did not bias this study because we do not compare termite composition across study sites. This study is a global analysis that uses all data combined to compare the encounter rates and relative abundance (see below) of fungus-growing termites with horizontal vs. vertical transmission across all regions.

Species Identification

All Macrotermitinae species were identified morphologically using keys for African termites by Bouillon and Mathot (1965, 1966, 1971) and Webb (1961), illustrations by Josens (1972) and descriptions by Grassé (1984, 1986). Additionally, samples were genetically identified as described elsewhere (Hausberger et al., 2011; Schyra and Korb, 2019), using particularly a fragment of *cytochrome oxidase II* (COII) as "barcode," which turned out to be especially suitable for West African termite species identification. Species names were assigned consistently across all studies.

Statistical Analyses

We analyzed the total number of encounters of all fungus-growing termite species as well as their relative abundance among the Macrotermitinae within a study site to compare species with horizontal vs. vertical transmission. As additional variables, we tested for effects of "habitat" (savannah vs. forest) and "disturbance" (protected, intermediate disturbance, strong disturbance) on the occurrence of fungus-growing termites. All analyses were done with IBM SPSS 26. All tests were two-tailed with an alpha-value of 0.05 which was adjusted for multiple testing using the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995), whenever necessary. For more details on specific tests, see Results.

RESULTS

Total Encounters Across All Sites

In total, we analyzed the occurrence of Macrotermitinae in 78 study sites distributed across three countries. We found 25 species, belonging to seven genera (*Allodontotermes*, *Ancistrotermes*, *Macrotermes*, *Microtermes*, *Odontotermes*, *Protermes*, and *Pseudacanthotermes*), in 3,812 encounters with Macrotermitinae (**Figure 1**; for more details see **Supplementary Table 1**). Five species (*Macrotermes bellicosus*, *Microtermes subhyalinus*, *Microtermes osborni*, *Microtermes* sp. 2, *Microtermes* sp. 4) had vertical symbiont transmission (**Figure 1**, **Supplementary Table 1**).

The three most common species (with more than 500 encounters each) were *Ancistrotermes cavithorax*, *Ancistrotermes guineensis* and *Microtermes osborni* with 21.7, 15.7, and 13.4% of all samples, respectively (**Figure 1**). The former two species are supposed to transmit their fungal symbiont horizontally, while the last species has supposed vertical transmission (Grasse and Noirot, 1955; Johnson, 1981; Johnson et al., 1981; Korb and Aanen, 2003). Two species were encountered only once: *Pseudacanthotermes* sp. A and *Odontotermes* sp. 4 (**Figure 1**, **Supplementary Table 1**).

In contrast to expectation, when a termite was encountered it was significantly less likely to be a species with vertical than with horizontal transmission of the fungal symbiont ($chi^2_1 = 785.13$, $p < 0.001$). It was almost three times more likely to encounter a species with horizontal (73%) than vertical (27%) transmission (**Figure 2**). Encounter frequencies were significantly affected by habitat ($chi^2_1 = 111.22$, $p < 0.001$) (**Figure 2**). In forests, encounters of species with horizontal transmission were more than four times more likely than encounters with vertically transmitting species (80.6 vs. 19.4%), while it was only around twice as likely that an encounter in the savannah was with a horizontally vs. vertically transmitting species (65.4 vs. 34.6%).

More detailed analyses within habitats showed that disturbance had an effect on the likelihood to encounter species with horizontal vs. vertical transmission in forests ($chi^2_2 = 28.55$, $p < 0.001$), but not the savannah ($chi^2_2 = 0.46$, $p = 0.796$) (**Figure 3**). In the forests, the proportion of species with horizontal transmission increased on disturbed sites while it did not change in the savannah (**Figure 3**).

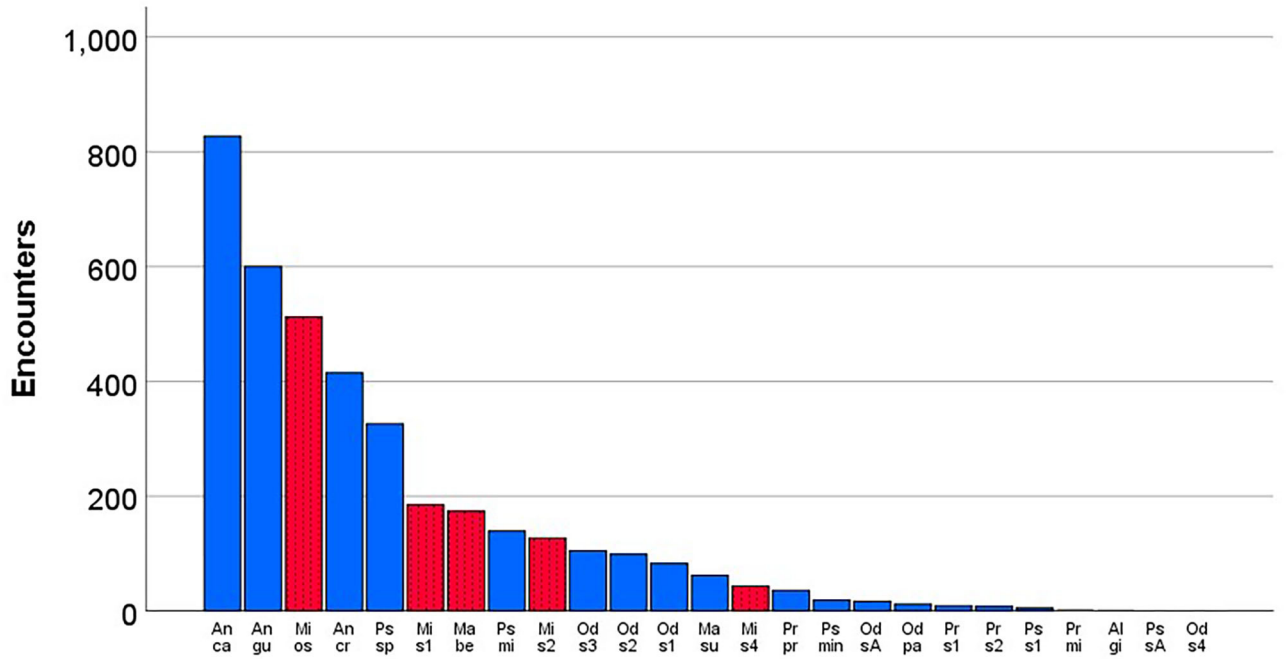

FIGURE 1 | Total number of encounters of different fungus-growing termite species studied in 78 savannah and forest study sites from Benin, Côte d'Ivoire, and Togo. Species with horizontal transmission are indicated in blue and those with vertical transmission in red (with dots). Al gi, *Allodontotermes giffardi*; An ca, *Ancistrotermes cavithorax*; An cr, *Ancistrotermes crucifer*; An gu, *Ancistrotermes guineensis*; Ma be, *Macrotermes bellicosus*; Ma su, *Macrotermes subhyalinus*; Mi os, *Microtermes osborni*; Mi su, *Microtermes subhyalinus*; Mi s2, *Microtermes* sp. 2; Mi s4, *Microtermes* sp. 4; Od sA, *Odontotermes* sp.A; Od pa, *Odontotermes* sp. (aff. pauperans); Od s1, *Odontotermes* sp. 1; Od s2, *Odontotermes* sp. 2; Od s3, *Odontotermes* sp. 3; Od s4, *Odontotermes* sp. 4; Pr mi, *Protermes minutus*; Pr pr, *Protermes prorepens*; Pr s1, *Protermes* sp. 1; Pr s2, *Protermes* sp. 2; Ps mi, *Pseudacanthotermes militaris*; Ps min, *Pseudacanthotermes minor*; Ps sp, *Pseudacanthotermes spiniger*; Ps sA, *Pseudacanthotermes* sp.A; Ps s1, *Pseudacanthotermes* sp. 1.

Only five out of the 25 identified fungus-growing termite species had vertical transmission. This biases interpretation of the results on total encounter frequencies. Therefore, we did additional analyses in which we compared species-specific encounter frequencies (i.e., the proportion of a species among all encounters; hereafter "encounter proportions;" **Figure 1**) and tested whether these values differed between species with horizontal vs. vertical symbiont transmission. Across all study sites, encounter proportions did not differ between the five species with vertical transmission and the 20 species with horizontal transmission (Mann Whitney U-test: $N_1 = 20$, $N_2 = 5$, $U = 24.00$, ns after FDR). This shows that (absolute) encounters with horizontally transmitting species were more common mainly because we had more species with horizontal transmission.

Relative Abundance Within Sites

The results of the total encounter frequencies were biased due to the fact that there were more Macrotermitinae species with horizontal than vertical transmission (20 vs. 5). Thus, we did another set of analyses in which we determined the relative abundance (i.e., the number of encounters of a species within a study site divided by the total number of encounters with fungus-growing termites in this site; this measure only included species that occurred in a site) of each Macrotermitinae species in a study site and tested whether species with horizontal vs. vertical transmission differed in their relative abundance across all sites.

The species that occurred in the most study sites were *Microtermes osborni* (55 out of 78 study sites) and *Microtermes subhyalinus* (51 out of 78 study sites), both have vertical transmission. Rare species that only occurred in a single plot were *Pseudacanthotermes* sp. A, *Odontotermes* sp. 4 and *Protermes* sp., all with horizontal transmission.

Ancistrotermes cavithorax had the highest relative abundance within study sites (mean ± SE: 0.29 ± 0.027), followed by *Pseudacanthotermes militaris* (0.20 ± 0.049), *Pseudacanthotermes spiniger* (0.20 ± 0.047), and *Pseudacanthotermes minor* (0.19 ± 0.075). All four species have horizontal transmission. Species with the lowest relative abundances within plots were *Odontotermes* sp. 4 (0.01, $N = 1$) and *Pseudacanthotermes* sp. 1 (0.01 ± 0.002), both with horizontal transmission.

Similar as with total encounter frequencies (**Figure 2**) and in contrast to expectation, species with horizontal transmission had significantly higher relative abundances within study sites than species with vertical transmission (Mann-Whitney test: $N = 439$, $U = 20676.5$, $P = 0.024$) (**Figure 4**). This effect was less strong than when using encounter frequencies (**Figure 2**) because also some species with horizontal transmission had low relative abundances (e.g., *Odontotermes* sp. 4, *Pseudacanthotermes* sp. 1; **Figure 4**). The significant effect disappeared when we split

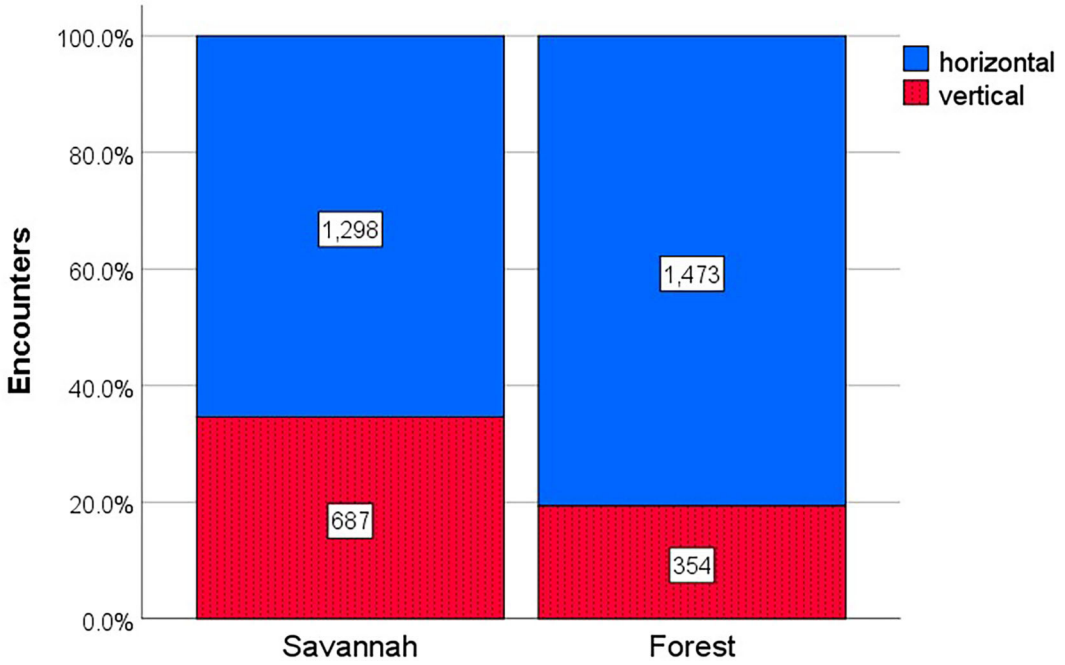

FIGURE 2 | Encounter frequencies of fungus-growing termites with different symbiont transmission modes across habitats. Shown is the proportion of encounters (with absolute numbers inside the bars) of termites with horizontal (blue) and vertical transmission (red, dotted) in 78 savannah and forest study sites from Benin, Côte d'Ivoire, and Togo.

our data set and analyzed it separately for forest and savannah (savannah: $N = 260$, $U = 7494.0$, $P = 0.219$; forest: $N = 179$, $U = 2483.5$, $P = 0.389$), supporting the notion that the effect size was small.

In the savannah as well as the forest, there was a trend that species with horizontal transmission had higher relative abundances than species with vertical transmission at intermediate disturbance levels (savannah: $N = 60$, $U = 305.00$, $P = 0.067$; forest: $N = 54$, $U = 178.50$, $P = 0.075$), while this was not the case in protected study sites (savannah: $N = 145$, $U = 248.00$, $P = 0.601$; forest: $N = 78$, $U = 400.00$, $P = 0.763$) and sites with strong anthropogenic disturbance (savannah: $N = 55$, $U = 329.00$, $P = 0.818$; forest: $N = 47$, $U = 212.00$, $P = 0.830$) (**Figure 5**). Combining both habitats, species with horizontal transmission had significantly higher relative abundances than species with vertical transmission at intermediate anthropogenic disturbance ($N = 114$, $U = 1103.50$, $p = 0.005$), but not in protected ($N = 223$, $U = 5462.0$, $P = 0.251$) or strongly disturbed study sites ($N = 102$, $U = 1267.5$, $P = 0.835$).

Which Factors Influence the Relative Abundance of a Fungus-Growing Termite?

To analyse the global effects on the occurrence of fungus-growing termite species we run a generalized linear mixed model (GLMM) with gamma error distribution, using "relative abundance" as dependent variable and "transmission mode," "habitat," and "disturbance" as fixed factors and "species ID" as random factor. The analysis revealed that transmission mode had a strong and significant effect on the relative abundance of a species (**Table 1**) with species with horizontal transmission having higher abundances within study sites than species with vertical transmission (**Figure 6**). Noteworthy is also the trend for an interaction between transmission mode × disturbance (**Table 1**). The relative abundances of species with horizontal transmission increased under disturbed conditions. In contrast to the former analyses, this GLMM controlled for species identity by using it as random factor.

DISCUSSION

Ecological Dominance and Transmission Mode of Fungal Symbiont

In contrast to expectation, we did not find fungus-growing termites with vertical transmission to be more abundant. On the contrary, species with horizontal transmission were more likely to be encountered (**Figure 2**) and also had higher relative abundances (**Figures 4**, **6**). While the results for the encounter frequencies have been biased by the fact that there were more species with horizontal than vertical transmission (which itself may tell something about the evolutionary success of species with horizontal transmission), this effect was accounted for in the relative abundance analyses across species (**Figure 4**) and also when including species identity in the GLMM (**Figure 6**). There was only one result which might indicate that species with vertical transmission are more common: the two species that occurred in most study sites, *Microtermes subhyalinus* and *M. osborni*,

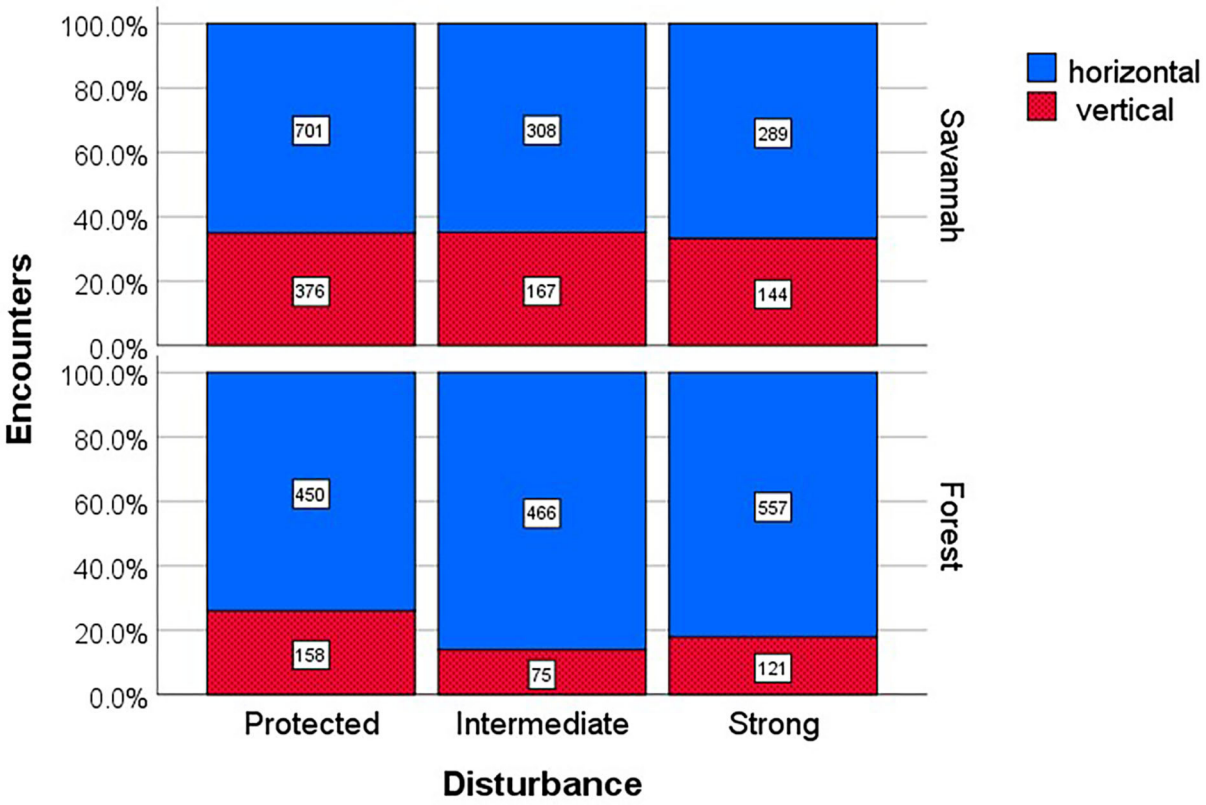

FIGURE 3 | Encounter frequencies of fungus-growing termites with different symbiont transmission modes across disturbance regimes and habitats. Shown is the proportion of encounters (with absolute numbers inside the bars) of termites with horizontal (blue) and vertical transmission (red, dotted) across anthropogenic disturbance regimes for savannah and forest study sites.

had vertical symbiont transmission. These two species were also among the three most common encountered species (**Figure 1**, **Supplementary Table 1**). This may imply that unknown species-specific traits (e.g., broad ecological niche, good dispersal abilities, or fungal identity) rather than transmission mode account for their wide-spread occurrence. Overall, however, our results suggest that species with horizontal transmission were more abundant.

Transmission Mode, Habitat Type, and Disturbance

We found some striking effects of disturbance on the occurrence of fungus-growing species with horizontal vs. vertical transmission mode. Species with horizontal transmission increased with disturbance. This was revealed for encounter rates (**Figure 3**) and also in the relative abundance analyses (**Figure 5**), the latter confirming that it is not only an effect of more species with horizontal than vertical transmission. Also the GLMM results, which included species identity, supports this notion with a trend for the interaction between transmission mode and disturbance to be significant (**Supplementary Table 1**). This implies that species with horizontal transmission thrive better under disturbed conditions than those with vertical transmission. An explanation for this may be that humans directly favor species with horizontal transmission, for instance, because they collect and eat mushrooms and spread spores. This is contrary to the common understanding that, at least nowadays, human consumption of *Termitomyces* mushrooms constrain the occurrence of Macrotermitinae (Koné et al., 2013). Humans may also indirectly favor horizontal transmitting species because they might be more resistant to disturbance, for example, as they can re-acquire fungal spores during colony establishment when they get lost due to disturbance. By contrast, species with vertical transmission are confined to the single inoculum that the king or queen carries at colony foundation.

Besides disturbance, habitat also seemed to have an effect on transmission mode. In the forest, encounter rates of species with horizontal transmission were around four times (80.6%) more common than those with vertical transmission, while they were only twice (65.4%) as likely in the savannah (**Figure 2**). However, this effect can be explained by the different proportions of species with horizontal vs. vertical transmission in both habitats. In the forest, 19 out of 22 species (86.3%) had horizontal transmission, while it were 11 out of 16 (68.7%) species in the savannah (**Supplementary Table 1**). In line with this interpretation, we did not find habitat effects in the relative abundance analyses. The occurrence of more species with horizontal transmission in the forest than in the savannah may be explained by the fact

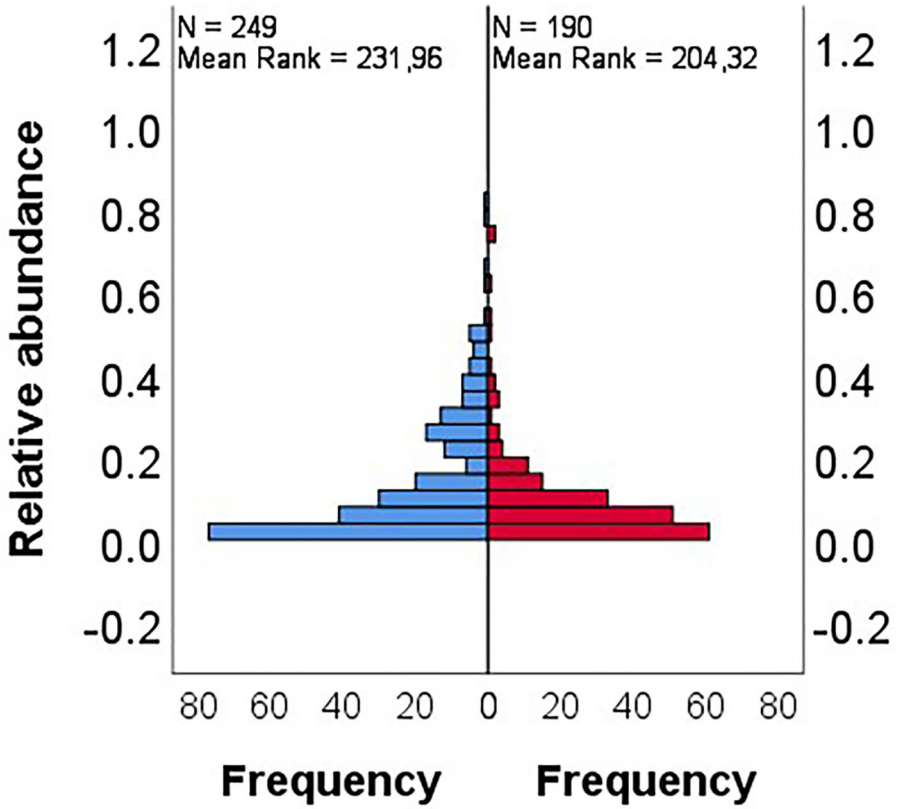

FIGURE 4 | Relative abundances of fungus-growing termite species with horizontal (blue) and vertical (red) transmission. Shown are frequency distributions of relative abundances of species within study sites.

that—despite their strong prevalence in savannahs nowadays—fungus-growing termites evolved in the African rainforest (Aanen and Eggleton, 2005) and that horizontal transmission was most likely the ancestral mode of fungal transmission (Aanen et al., 2002; Nobre et al., 2011b). We also can hypothesize that species with vertical transmission especially evolved in the savannah. This derived trait is associated with species (*M. bellicosus*, *Microtermes*) that mainly or exclusively occur in the savannah. This hypothesis can easily be tested when considering data for whole Africa.

Why Are Species With Horizontal Transmission so Common?

Theory predicts that there are two main reasons why associations with uniparental, vertical symbiont transmission should have less conflict than those with horizontal transmission (Frank, 1994, 1995, 1996, 1997, 1998; Foster and Wenseleers, 2006; Leeks et al., 2019), and hence may be more successful. First, uniparental, vertical transmission results in symbionts being closely related (generally clones). Thus, 'intra-symbiont' conflict is reduced. Second, strict vertical transmission leads to an alignment of the fitness interests of symbiont and host because symbionts can only increase their fitness by enhancing host fitness. Thus symbiont-host conflict is reduced. A recent modeling and simulation study implies that both, relatedness and fitness alignment, influence the level of cooperation evolving between hosts and symbionts but that relatedness is more important than fitness alignment to explain symbiont cooperation (Leeks et al., 2019).

What is the relatedness of fungal symbionts within a colony? Studies for three common fungus-growing termite species from Southern Africa have shown that the fungus garden of established colonies consists always of a single fungal cultivar, despite horizontal transmission (Aanen et al., 2009). Aanen and colleagues (Aanen, 2006; Aanen et al., 2009) explained this by positive feedback mechanisms within colonies in which the most productive fungal cultivar is positively selected through re-current inoculation of new fungus combs and genetic bottlenecks, after a colony had been inoculated by different cultivars at the incipient stage. This mechanism is in line with lab-experiments that simulated with-nest propagation of the fungal cultivar (Aanen et al., 2009). Thus, intra-symbiont conflict is reduced within termite colonies by generating high relatedness within the fungal cultivar, which stabilizes the association.

However, there is still conflict between host and symbiont in associations with horizontal transmission, for instance, as the fungus is selected to spread independently from its host colony by producing mushrooms with sexual spores that can be picked up by other colonies (Vreeburg et al., 2020; Wisselink et al., 2020). These mushrooms have a high biomass (Yorou et al.,

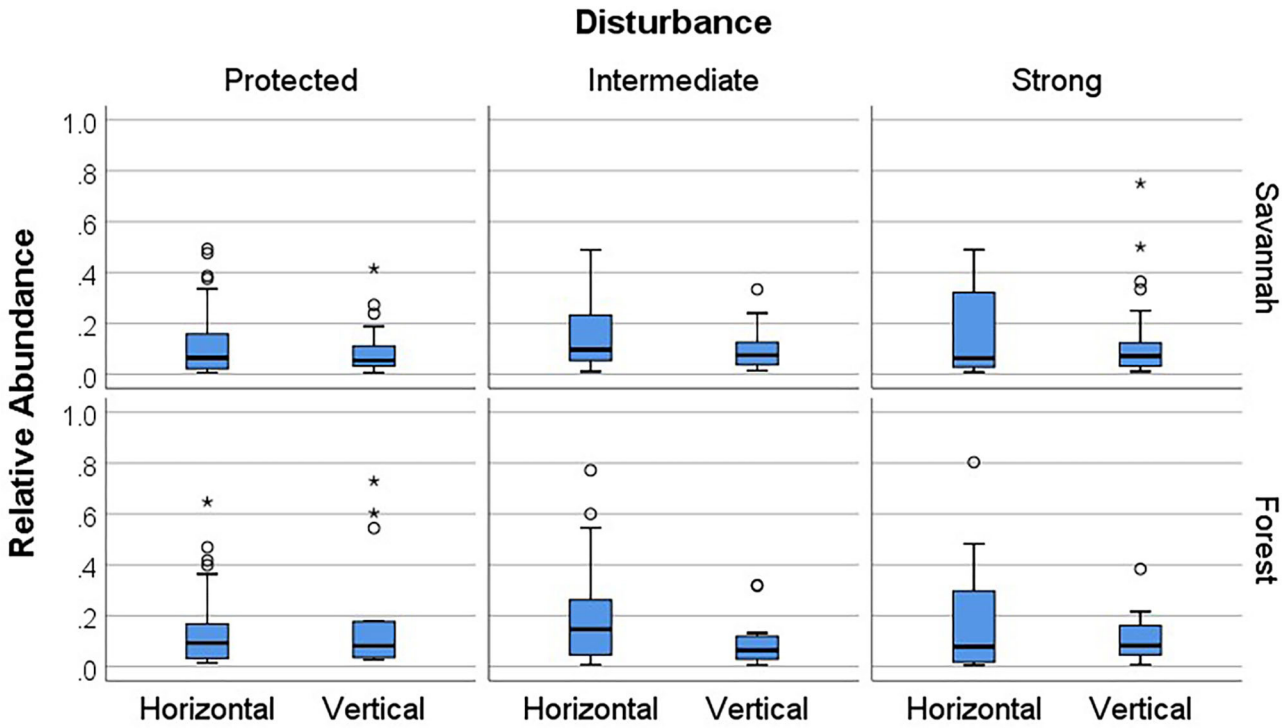

FIGURE 5 | Boxplots of the relative abundances of fungus-growing termite species with horizontal vs. vertical transmission in the savannah and forest across different anthropogenic disturbance regimes. ◦ are outliers, defined as data that fall not within the whiskers. * are extreme outliers, defined as data that have values more than three times the height of the boxes.

TABLE 1 | Results of a GLMM analyzing the effect of transmission mode, disturbance, and habitat (all fixed factors) on the relative abundance of fungus-growing termites, using species ID as random factor.

	F	df_1,df_2	P
Transmission mode	8.77	1,427	0.003
Disturbance	0.49	2,427	0.612
Habitat	3.34	1,427	0.068
Transmission × Disturbance	3.00	2,427	0.051
Transmission × Habitat	1.27	1,427	0.260
Disturbance × Habitat	2.74	2,427	0.066
Transmission × Disturbance × Habitat	2.04	2,427	0.132

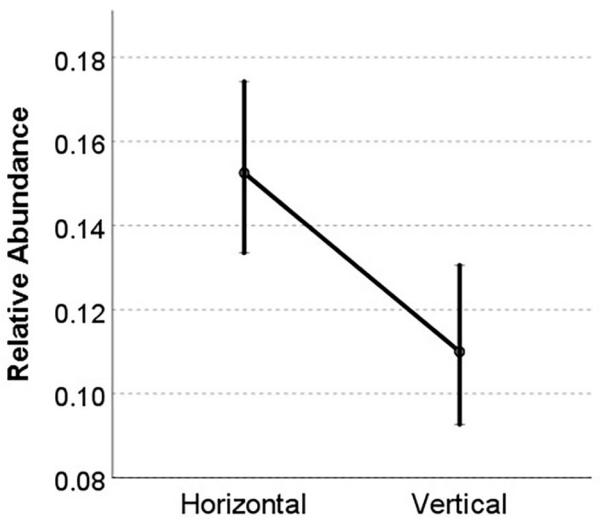

FIGURE 6 | Relative abundances of fungus-growing termite species with horizontal vs. vertical transmission. Shown are estimated marginal means with standard errors as revealed by the GLMM.

2014). Following three nests of a *Pseudacanthotermes* species during their fructification period, revealed a mean biomass of 27 kg/colony/year (N.A. Koné, unpublished data). This means a large amount of food lost for consumption by the termite colony. Therefore, there must be other costs associated with vertical transmission to account for its lack of ecological dominance.

What Remains to Be Done?

In order to understand the contribution of the fungal symbiont for the varying ecological success of different fungus-growing species, it will be essential to know which fungi associate with which termite species. This would allow us to test, for instance, whether specific 1:1 associations ("specialists") are ecologically more successful due to co-evolutionary fine-tuning of plant degrading pathways. Alternatively, termites with a wide range of fungal symbionts may be more ecological dominant because this

may allow their host termite species to occupy broader niches, depending on which symbiont they actually are associated with. Lastly, the success of the termite might depend only on symbiont identity when there are some *Termitomyces* species which are just more competitive or efficient in degrading plant material than others. The fungal symbiont might also shape whole termite community composition through niche differentiation as a recent study hypothesized (Schyra et al., 2019b).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JK conceptionalized the manuscript and did statistical analyses. SS, JK, and NAK collected samples. SS identified all new samples. JK and NAK wrote the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.600318/full#supplementary-material

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Soil Physical Quality and Relationship to Changes in Termite Community in Northwestern Colombian Amazon

Ervin Humprey Duran-Bautista[1,2]*, *Yolima Muñoz Chilatra*[1], *Juan Diego Galindo*[1], *Tania Alexandra Ortiz*[1] and *María Fernanda Bermúdez*[3,4]*

[1] Programa de Ingeniería Agroecológica, Universidad de la Amazonia, Florencia, Colombia, [2] Centro de Investigaciones Amazónicas CIMAZ Macagual Cesar Augusto Estrada González, Florencia, Colombia, [3] Laboratorio de Entomología, Grupo de Investigación en Entomología, Universidad de la Amazonia, Florencia, Colombia, [4] Maestría en Ciencias–Entomología, Universidad Nacional de Colombia–Sede Medellín, Medellín, Colombia

Conversion from Amazon forest to low-management pasture or agriculture causes not only degradation of aboveground vegetation but also negative changes in soil properties and ecosystem services. This study aimed to evaluate the impact of physical soil degradation on termite community changes in three contrasting land uses (natural regeneration, rubber plantations, and silvopastoral systems). Soil physical quality was assessed through a set of physical variables, such as bulk density, porosity, soil macro-aggregation state, Visual Evaluation of Soil Structure (VESS) and penetration resistance, which were summarized in an overall synthetic indicator of physical quality. Besides, transects of 20 × 2 m were established in each land use; each transect was divided into four sections of 5 m to search and collect termites during 1 hour in each section; likewise, termites were collected from blocks of soil 25 × 25 × 10 cm (length, width, and depth, respectively) adapted from the Tropical Soil Biology and Fertility (TSBF) method. In total, 60 transects were evaluated, 20 in each land use. A total of 41 species were collected across the three land uses evaluated: natural regeneration presented 60% of the collected species (25 species), silvopastoral systems 53% (22 species), and rubber plantations 39% (16 species). Additionally, composition species from the silvopastoral, agroforestry systems, and natural regeneration were different, and a close association between these last land uses was observed. Soil physical characteristics showed significant variations between land uses. The rubber plantations presented lowest values of soil physical quality, while the natural regeneration showed high soil physical quality. These changes affected termite community and lead to changes in its composition with disproportionate loss of some species; however, there are some that can acclimate well to the decline in the soil physical quality.

Keywords: land use, soil compaction, soil moisture, soil-feeders termites, deforested Amazonia

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***Correspondence:**
Ervin Humprey Duran-Bautista
ervinduranb@gmail.com
María Fernanda Bermúdez
mafezz_12@hotmail.com

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INTRODUCTION

The Amazon region plays an essential role to the Earth, harboring about 10–15% of land biodiversity (Hubbell et al., 2008; Nobre et al., 2016). It serves as a sink for greenhouse gasses by absorbing up to 5% of annual CO_2 emissions (∼2 billion tons) (Saatchi et al., 2011), and it has been well-recognized as one of the key components of the Earth's climate system (Malhi et al., 2008).

Despite its relevance, this ecosystem is under increasing human pressures related to agriculture and livestock farming, timber extraction, mining, illicit crops, among others, which resulted in unprecedented rates of land cover changes due to forest clearing, degradation, and fragmentation (Sonter et al., 2017; Roque et al., 2018). In Colombia, the Amazon covers 42% of the country's territory (Etter et al., 2006). Despite many efforts to enhance forest landscape conservation and its biodiversity (Furumo, 2020), this region concentrated 70.1% (1,381.8 km^2 $year^{-1}$) of deforestation from a national total in 2018. Deforestation in the department of Caquetá alone accounted for 23.7% (467.6 km^2 $year^{-1}$), becoming an important hotspot of deforestation in Colombian Amazon (SMByC 2020).

Forest clearing not only generates the loss of biological diversity at all levels but also leaves the soil exposed to processes of erosion and degradation. For example, in deforested areas for crops and degraded pastures by domestic livestock, a reduced soil moisture, increase in the bulk density and a decrease in total porosity at soil surface has been observed due to animal grazing, compaction of agricultural machinery, and the impact of the raindrops (Cherubin et al., 2016; de Queiroz et al., 2020).

In tropical rainforests, termites constitute an important part of the soil fauna biomass (Bignell and Eggleton, 2000). Some studies suggest that termites may represent 40–65% of overall soil macrofaunal biomass in some biotopes (Loveridge and Moe, 2004). They contribute to ground decomposition of litter, plant growth, and overall biodiversity (Jouquet et al., 2011; Bottinelli et al., 2015).

Specifically, in tropical and subtropical soils, the role of termites has been widely documented demonstrating its impact on soil structure and properties, such as soil formation and aeration, bioturbation, water content and hydraulic proprieties, soil organic matter, and nutrient cycling (Bignell, 2006; Jouquet et al., 2011; Harit et al., 2017). These alterations are caused by the development of their biogenic structures (nests, foraging tunnels, and formation of galleries) and feeding strategies (ingesting and egesting soil) (Jouquet et al., 2016a).

However, the effect generated by changes in soil characteristics on termite's community distribution has been poorly studied, and it is limited to some efforts aimed to analyze the influence of soil chemical properties on termites (Bourguignon et al., 2015; Jouquet et al., 2015). In this regard, we evaluate the impact of soil physical quality on changes in termite communities and hypothesized that soil physical quality affects different termite communities.

MATERIALS AND METHODS

Study Area

The study area was located in the Department of Caquetá southeastern Colombia between 1°30′N and 1°10′N, and between 75°35′W and 76°0′W (**Figure 1**). It has an average yearly temperature of 25°C, yearly rainfall of 3,600 mm, concentrated between April and November and a light dry season from December to March.

Sampling in this area was carried out during September 2019 in the following land uses: natural forest (NF), rubber plantations (RP), degraded pasture (DP) according to the methodology Corine Land Cover adapted by the Geographic Institute Agustín Codazzi (IGAC) and the Institute of Hydrology, Meteorology and Environmental Studies (IDEAM) for Colombia (**Table 1**). A brief characterization of the chemical properties of the soil in the evaluated areas is presented in **Table 2**.

Termite Sampling

Termites were collected from transects of 20 × 2 m (adapted from Swift and Bignell, 2001) located in the central part of each land use type avoiding the edge effects; a minimum distance of 30 m between transects was guaranteed. In the silvopastoral system, the transects were located under the *Gliricidia sepium* tree line, while in the agroforestry system, they were established under the *Hevea brasiliensis* and *Theobroma cacao* intercalated line; a total of 60 transects were evaluated, 20 in each land use.

The transects were divided into four sections of 5 m. In each section, termites were searched for in any specific microhabitat, such as nests, dead wood, trunks, roots, and under rocks, 1 hour per person. When a colony was encountered, collection time could not exceed 3 min to avoid overestimation of the dominant species. Also, termites were collected from extracted blocks of soil by using a 25 × 25 × 10 cm (length, width, and depth, respectively) metallic frame adapted from the International Organization for Standardization (ISO) and the Tropical Soil Biology and Fertility (TSBF) methods (Anderson and Ingram, 1993; ISO, 2011).

Identification was made at genus and species levels, using taxonomic guides and reviews (Constantino, 2002a; Constantino et al., 2006; Constantino and Carvalho, 2012; Rocha et al., 2012; Constantini and Cancello, 2016). When identification was not possible, individuals were separated into morphospecies according to morphological differences. Individuals of the Apicotermitinae subfamily were separated at the species and morphospecies levels by dissecting the enteric valve and comparing its morphology with that described in the literature (Bourguignon et al., 2010, 2013, 2016). Termite samples are kept in 2-ml vials containing 80% ethanol, in the Colección Entomológica, Laboratorio de Entomología, Universidad de la Amazonia (LEUA).

Soil Physical Parameters Determination

A set of five physical variables of the soil was evaluated, which was considered key to have a general idea of the physical quality of the soil namely:

- Bulk density
- Soil moisture
- Soil resistance to penetration
- Soil macro-aggregation state
- Visual evaluation of soil structure

Undisturbed soil samples were collected in the center of the 0–10, 10–20, and 20–30 cm layers using a metallic ring (5 × 5 cm 98 cm^2) and disturbed samples from the same soil depths. In the laboratory, undisturbed samples were weighed, dried in a forced-air oven at 105°C for 48 hours, and weighed again. Bulk density (BD, Mg m^{-3}) was calculated

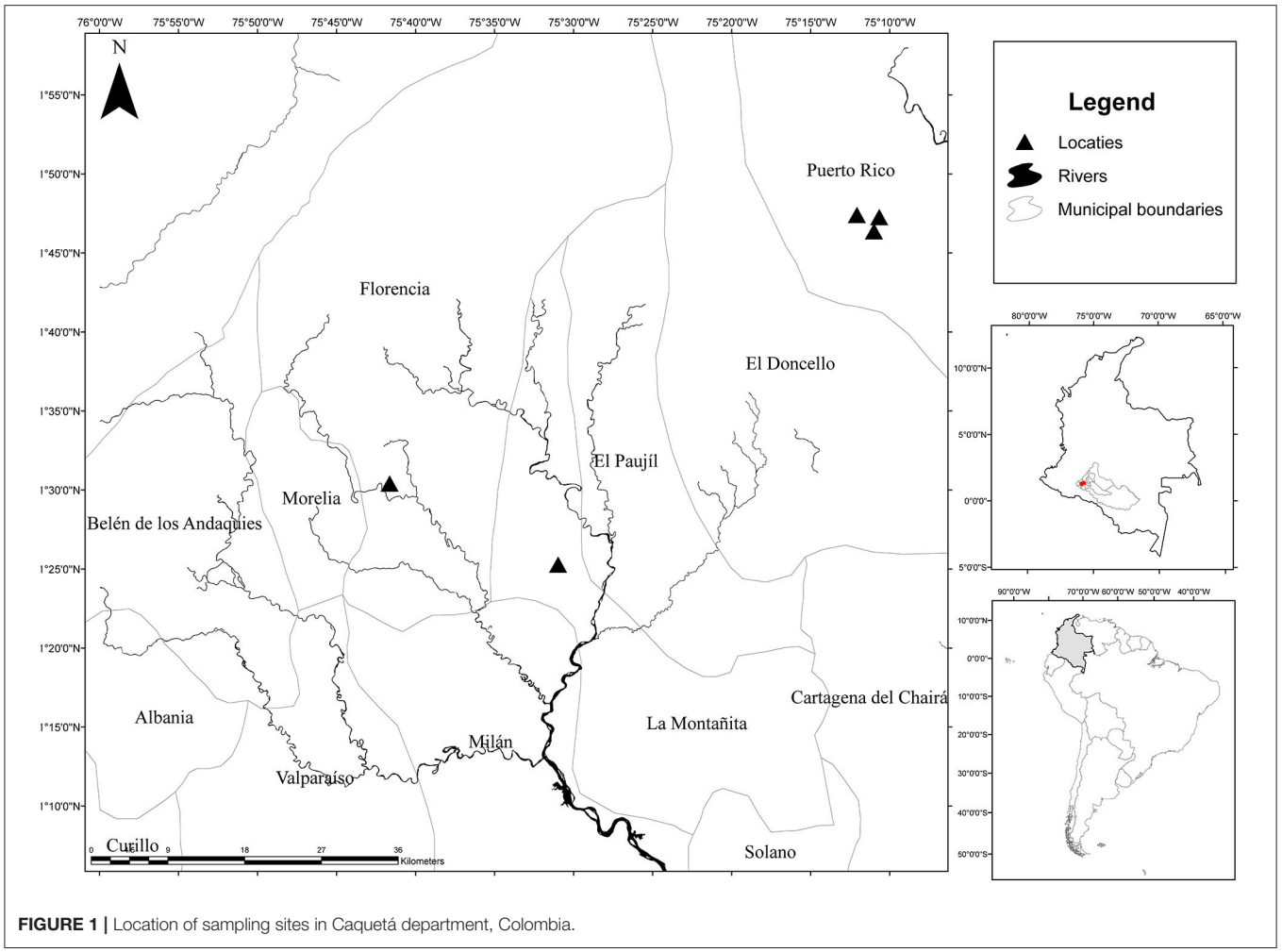

FIGURE 1 | Location of sampling sites in Caquetá department, Colombia.

by dividing the soil dry mass by the volume of the cylinder, whereas soil moisture (%) was determined by the equation: soil moisture = [(dry soil mass/wet soil mass) – 1] × 100.

Measurements of Soil Resistance to Penetration (SRP) were performed using a hand penetrometer (Eijkelkamp) around the soil sampling trenches down to 30 cm with angle and surface area of a cone of 60° and 3.3 cm^2, respectively. Also, total porosity was calculated by using bulk density and particle density of soil through Equation (1):

$$TP = 1 - \left(\frac{Bd}{Pd}\right) \quad (1)$$

where TP is the total porosity, Bd and Pd are the bulk density and particle density, respectively.

The state of soil macro-aggregation was determined using the methodology described by Velasquez et al. (2007). In each transect, we also collected a small block of soil (10 × 10 × 10 cm) and separated macroaggregates based on the visual separation of macroaggregates (>4 mm) according to biogenic aggregates produced by macroinvertebrates, or root aggregates made of soil stuck to the roots, physical aggregates produced by physical processes, and non-macroaggregated soil. Separation was done by gently breaking the soil apart into its natural constituents.

The Visual Evaluation of Soil Structure (VESS) was performed following the methodology proposed by Ball et al. (2017). In each transect, a soil block of 20 × 10 × 25 cm (width, thickness, and depth, respectively) was extracted, and each block was divided into two layers: topsoil (0–10 cm) and subsoil (10–25 cm). This method involves the removal and gentle breakup of a spadeful of topsoil by hand to reveal the main structural units and any layers of contrasting aggregation. Each layer is compared to the photographs with identified dimensions and descriptions in a colored chart and allocated to one of five soil quality scoring categories (Sq) from Sq 1 = best to Sq 5 = worst topsoil quality (VESS) and Ssq 1 = best to Ssq 5 = worst subsoil quality (SubVESS).

An overall weighted Sq score was calculated for each sample based on the individual score and thickness of each contrasting soil layer, according to Equation (2)

$$VESS\ Sq_{score} = \sum_{i=1}^{n} \frac{SqiTi}{TT} \quad (2)$$

TABLE 1 | Description of land use types evaluated; the photos are representative of the sampled area.

Land use type	
Natural regeneration Deforested area that was abandoned ∼15 years ago to recover soil, differing from the forests in species composition (dominated by *Cecropia* spp.) and in structure (canopy <10 m).	
Rubber plantations Rubber crops planted in late 1990s composed of rubber tree as the main species and some cocoa trees (*Theobroma cacao* L.) planted since 2015.	
Silvopastoral systems An area that combines pastures (*Brachiaria brizantha* or *Brachiaria humidicola*) subject to cattle grazing and strips of trees (*Gliricidia sepium*) isolated and without trampling planted since 2005.	

where VESS Sqscore is the overall VESS score of the sample, Sqi and Ti are the score and thickness of each identified soil layer, respectively, and TT is the total thickness of the soil sample.

Data Analysis

Termite Community Analysis

The relative abundance of each termite species was expressed as the number of encounters established by recording the presence of each species only once in each plot. Encounters and richness species data per transect were analyzed using a mixed generalized linear model (GLMM) with negative binomial and Poisson distribution for abundance and richness, respectively, stating land use as a fixed effect and farms as a random effect. We use the "lme4" package (Bates et al., 2018). Fisher multiple comparison tests ($\alpha = 0.05$) were also applied for fixed effect using the "multcomp" package (Hothorn et al., 2008). To compare alpha- and beta-diversity across sites, the Shannon exponential (eH') (Jost, 2006), and Simpson's inversed (1-D) indices, as well as rarefaction and extrapolation curves of richness were calculated using iNext (Hsieh et al., 2020). To test the hypothesis of differences in Shannon exponential and Simpson's inversed between land uses, we performed a generalized linear model (GLM). Non-metric multidimensional scaling (NMDS) based on the Bray–Curtis index of dissimilarity was used to compare composition termite species between land uses; species with a single encounter in all sampling were not considered to avoid the zero-inflated effect. Besides, an analysis of similarities (ANOSIM) was performed with 999 permutations to test statistically whether there were significant differences between land uses. A similarity percentage analysis (SIMPER) was used to identify which species contributed the most to the differences in termite community structure between land use types. These analyses were performed with "vegan" package (Oksanen et al., 2019).

Analysis Between Physical Soil Variables and Land Use Groups

Physical soil variables were analyzed with a generalized linear model (GLM) and a principal component analysis (PCA) associated with a Monte Carlo test from the "ade4" package to assess differences among land use and soil physical characteristics, and their statistical significance (Dray and Dufour, 2007). Besides, physical soil variable dataset was summarized in overall synthetic indicator of physical quality, following the methodology adapted from Velasquez et al. (2007). A principal component analysis (PCA) was used to determine the parameters that best-captured variance within a dataset. We selected those variables with a significant contribution (>50% of the explained variance) in either of the first two main axes. The selected variables were then combined into a single value indicator and scaled to a number ranging from 0.1 to 1.0 using a homothetic transformation.

Analysis Between Physical Soil Variables and Termite Species

To examine the relationship of termite species abundance to physical soil variables, canonical correspondence analysis (CCA) was performed. This analysis was run with two matrices: a dependent matrix containing 24 termite species whose abundance was >1% of the total number of individuals collected, species with abundance lower than 1% were grouped in the category "others" and considered in the analysis. The independent matrix contained the soil physical variables, which we did not keep in the analysis variables that had variance inflation factor (VIF) values higher than 10. The significance of these associations was verified using a permutation test from the "vegan" package (Oksanen et al., 2019); neither were they considered variables that were not significant using permutation tests. All analyses were performed using the R language software, version 3.5.3 (R Core Team, 2018).

RESULTS

Species Richness and Community Structure

A total of 41 species from seven subfamilies and 24 genera were collected across the three land uses evaluated (**Table 3**) in 135 encounters. Likewise, Nasutitermitinae and Syntermitinae were the most abundant subfamilies with 43 and 24% of the total species collected, respectively. In contrast, Amitermitinae, Coptotermitinae, and Heterotermitinae recorded only one species each in the sampling. The genera with the largest number of species were *Nasutitermes*, *Anoplotermes*, and *Syntermes* containing 10 and 3 morphospecies, respectively, representing 38% of the total specific richness.

The most frequent species in natural regeneration were *Silvestritermes* cf. *lanei* (Canter, 1968; 10 encounters), *Nasutitermes guayanae* (Holmgren, 1910; 5 encounters), and *Anoplotermes* sp. 1 (5 encounters). In the silvopastoril system, the most frequent species were *Aparatermes silvestrii* (Emerson, 1925; seven encounters), *Nasutitermes* sp. 7 (six encounters), and *Embiratermes neotenicus* (Holmgren, 1906; four encounters). Finally, in the rubber plantations, the more frequent species were *Nasutitermes guayanae* (six encounters), *Silvestritermes* cf. *lanei* (six encounters), and *Heterotermes tenuis* (Hagen, 1858; four encounters).

There were no statistically significant differences according to GLMM for species richness by transect among land uses; neither were there statistically significant differences according to GLM in the Shannon exponential (H) and Simpson's inverse (1-D) diversity indices per transect between land uses. When analyzing the accumulated species richness in each land use, natural regeneration presented 60% of the collected species (25 species), silvopastoral systems 53% (22 species), and rubber plantations 39% (16 species). Furthermore, sampling coverage curve including interpolation and extrapolation values for each land use averaged 72% of completeness and suggests that the sampling appropriately represent the termite diversity in the study area (**Table 4**).

TABLE 2 | Chemical characteristics of the soil in the evaluated areas.

Land use	pH	CIC (meq/100 g)	OM (%)	P (ppm)	Ca (meq/100 g)	K (meq/100 g)	Mg (meq/100 g)	Al (meq/100 g)	Sand (%)	Silt (%)	Clay (%)
Natural regeneration	4.18 ± 0.16	11.63 ± 0.49	2.75 ± 0.51	5.12 ± 1.09	0.21 ± 0.02	0.24 ± 0.02	0.14 ± 0.01	2.82 ± 0.13	44.54 ± 1.68	15.06 ± 0.83	40.41 ± 1.33
Rubber plantations	4.78 ± 0.13	15.11 ± 0.80	2.95 ± 0.27	4.43 ± 0.89	0.65 ± 0.06	0.23 ± 0.04	0.19 ± 0.2	2.95 ± 0.17	32.66 ± 3.06	21.81 ± 2.57	45.53 ± 2.96
Silvopastoral systems	4.56 ± 0.15	11.42 ± 0.66	2.15 ± 0.41	2.36 ± 0.43	0.25 ± 0.02	0.15 ± 0.01	0.30 ± 0.03	3.88 ± 0.34	25.19 ± 1.45	24.32 ± 1.90	50.48 ± 1.26

TABLE 3 | Termite species encounters per transect (or morpho-species) at individual sampling points, in Natural Regeneration (RN), silvopastoral systems (SPS), rubber plantations (RP) in Colombian Amazon region.

Taxon	RP	NR	SPS
Rhinotermitidae	**4**	**4**	**3**
Coptotermitinae	**0**	**1**	**0**
Coptotermes testaceus (Linnaeus, 1758)	0	1	0
Heterotermitinae	**4**	**3**	**3**
Heterotermes tenuis (Hagen, 1858)	4	3	3
Termitidae	**32**	**52**	**40**
Amitermitinae	**2**	**0**	**0**
Microcerotermes arboreus Emerson, 1925	2	0	0
Apicotermitinae	**3**	**7**	**8**
Anoplotermes sp. 1	2	5	1
Anoplotermes sp. 2	0	1	0
Anoplotermes sp. 3	1	0	0
Aparatermes silvestrii (Emerson, 1925)	0	1	7
Nasutitermitinae	**12**	**19**	**16**
Araujotermes zeteki (Snyder, 1924)	1	1	0
Atlantitermes snyderi (Emerson, 1925)	0	1	0
Atlantitermes stercophilus Constantino and DeSouza, 1997	0	0	1
Coatitermes clevelandi (Snyder, 1926)	1	0	0
Coatitermes cf. *kartaboensis* (Emerson, 1925)	0	1	0
Diversitermes sp.1	0	1	0
Nasutitermes guayanae (Holmgren, 1910)	7	5	3
Nasutitermes sp. 1	0	1	1
Nasutitermes sp. 2	0	2	0
Nasutitermes sp. 3	0	2	0
Nasutitermes sp. 4	1	1	1
Nasutitermes sp. 5	1	0	0
Nasutitermes sp. 6	0	1	1
Nasutitermes sp. 7	0	0	6
Nasutitermes sp. 8	0	2	0
Nasutitermes sp. 9	1	0	1
Rotunditermes bragantinus (Roonwal and Rathore, 1976)	0	1	0
Subulitermes sp. 1	0	0	2
Syntermitinae	**10**	**19**	**10**
Cornitermes pugnax Emerson, 1925	0	1	0
Curvitermes odontognathus (Silvestri, 1901)	0	0	1
Cyrilliotermes angulariceps (Mathews, 1977)	1	4	0
Embiratermes festivellus (Silvestri, 1901)	0	0	1
Embiratermes neotenicus (Holmgren, 1906)	3	3	4
Labiotermes guasu Constantino and Acioli, 2006	0	0	1
Silvestritermes cf. *lanei* (Canter, 1968)	6	10	1
Syntermes molestus (Burmeister, 1839)	0	0	1
Syntermes territus Emerson, 1924	0	0	1
Syntermes sp.1	0	1	0
Termitinae	**5**	**7**	**6**
Crepititermes verruculosus (Emerson, 1925)	1	0	0
Cylindrotermes capixaba Rocha and Cancello, 2007	1	4	1
Neocapritermes opacus (Hagen, 1858)	0	0	2
Neocapritermes utiariti Krishna and Araujo, 1968	0	1	0
Spinitermes longiceps Constantino, 1991	0	0	2
Termes hispaniolae (Banks, 1918)	3	2	1
Total morphospecies	16	25	22
Total encounters	36	56	43

Values in bold correspond to families and subfamilies.

The NMDS diagram based on the Bray–Curtis index of dissimilarity showed termite species composition differences (**Figure 2**). In general, 12 species were found exclusively from natural regeneration, nine from silvopastoral systems, and five in the rubber plantation. Eight termite species were found in all land uses. The ANOSIM demonstrated a significant statistical difference between the land uses evaluated (Global $R = 0.29$, $P = 0.002$). The species from the silvopastoral system was different from the species in agroforestry systems and natural regeneration. Moreover, a close association between these last land uses was suggested by the analysis (**Figure 2**).

The similarity percentage analysis (SIMPER) allowed us to identify which termite species mainly contributed to the dissimilarity in the species composition between land uses. The SIMPER showed that species of the genus *Nasutitermes*, the xylophages species *Heterotermes tenuis*, *Microcerotermes arboreus*, and *Nasutitermes guayanae* and *Aparatermes silvestrii* were the main species responsible for the differences between land uses (**Table 5**). *Nasutitermes* was the more common species in natural regeneration, with a 12.2% contribution; while *Heterotermes tenuis*, *Microcerotermes arboreus*, and *Nasutitermes guayanae* were the more abundant species in rubber plantation, with a 21.3% contribution, and *Aparatermes silvestrii* dominant in silvopastoral systems, with a 13.25% contribution.

Physical Soil Variables

Physical soil characteristics showed significant variations between land uses according to GLM. Rubber plantations presented lowest values of the overall synthetic indicator of physical quality (0.41 ± 0.04; **Figure 3A**) associated with highest values for the parameters of bulk density (1.24 ± 0.06 Mg m^{-3}; **Figure 3B**), soil resistance to penetration (1.17 ± 0.09 Mpa; **Figure 3C**), and visual evaluation of soil structure (1.49 ± 0.16 Sq score; **Figure 3F**). Meanwhile, natural regeneration showed high physical soil quality with highest values for soil porosity ($57.16 \pm 2.69\%$; **Figure 3D**) and moisture ($44.66 \pm 2.28\%$; **Figure 3E**).

The variance of physical soil properties was explained by 21.24% by land use according to the PCA. Axes 1 and 2 of the PCA represent 36.2 and 19.2%, respectively. Axis 1 separated on one side the sites with high porosity, macro-aggregation status, and soil moisture (**Figure 4A**) to which secondary vegetation was associated, at the other end of Axis 1 are the sites with high values for the parameters of bulk density and soil resistance to penetration like rubber plantations (**Figure 4B**). The formation of Axis 2 was mainly contributed on one side by sites with high remaining material from aggregate morphology and on the other side by high Sq score visual evaluation of the soil structure.

Soil macro-aggregation on average, 21.5% of the soil volume was not macro-aggregated, biogenic aggregates contributing 21.8% of the total soil mass and physical aggregates comprising 20.9% (**Figure 5**). Important differences between land uses were suggested in soil macro-aggregation, with high fraction from remaining material representing 33.4% of soil under rubber plantations, while natural regeneration possessed much larger percentages of biogenic aggregates (28.1%).

TABLE 4 | Means and standard error for number of species and encounters per transect, Shannon and Simpson indices, as well as total number of species and genus, sample coverage from estimated species richness for Natural Regeneration (RN), silvopastoral systems (SPS), and rubber plantations (RP) in Colombian Amazon region.

	NR	SPS	RP	Model *P*-value	Land use effect *P*-value
Species per transect	9.02 ± 1.81[a]	7.8 ± 1.65[a]	5.85 ± 1.37[a]	<0.0001	0.3003
Encounters per transect	13.13 ± 2.0[a]	10.65 ± 1.79[a]	10.41 ± 1.76[a]	<0.0001	0.4717
Shannon exponential (H) index	5.64 ± 1.19[a]	5.39 ± 1.19[a]	4.91 ± 1.19[a]	0.0016	0.8300
Simpson's index	4.51 ± 1.1[a]	4.68 ± 1.19[a]	4.35 ± 1.19[a]	0.0028	0.9490
Total species	25	22	16	–	–
Total genera	18	15	12	–	–
Sample coverage estimate (q0)	75.77%	68.35%	72.67%	–	–

Means followed by the same letter in the rows did not differ among themselves according to Fisher's test ($P < 0.05$).

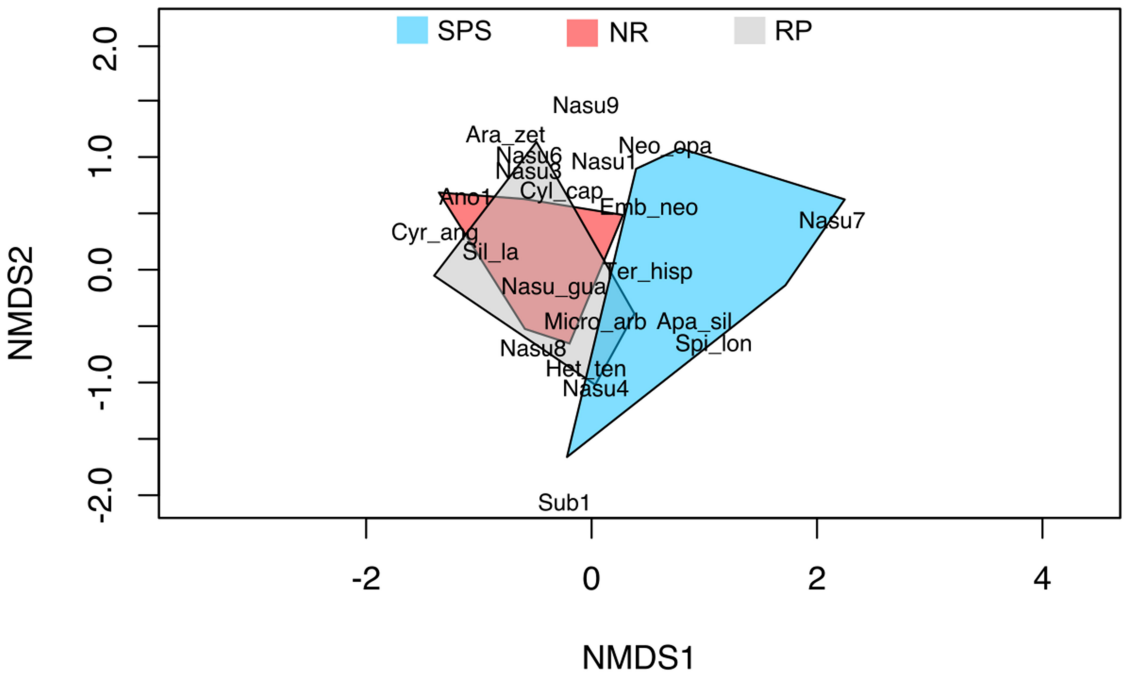

FIGURE 2 | Nonmetric multidimensional scaling (NMDS) composition of termite species collected in natural regeneration (RN), silvopastoral systems (SPS), and rubber plantations (RP) in Colombian Amazon region. This ordination analysis was calculated from the Bray–Curtis dissimilarity index's (Stress = 0.07). Ano1, *Anoplotermes* sp. 1; Apa_sil, *Aparatermes silvestrii*; Ara_zet, *Araujotermes zeteki*; Cyl_cap, *Cylindrotermes capixaba*; Cyr_ang, *Cyrilliotermes angulariceps*; Emb_neo, *Embiratermes neotenicus*; Het_ten, *Heterotermes tenuis*; Micro_arb, *Microcerotermes arboreus*; Nasu_gua, *Nasutitermes guayanae*; Nasu1, *Nasutitermes* sp. 1; Nasu3, *Nasutitermes* sp. 3; Nasu4, *Nasutitermes* sp. 4; Nasu6, *Nasutitermes* sp. 6; Nasu7, *Nasutitermes* sp. 7; Nasu8, *Nasutitermes* sp. 8; Nasu9, *Nasutitermes* sp. 9; Sil_la, *Silvestritermes* cf. *lanei*; Spi_lon, *Spinitermes longiceps*; Sub1, *Subulitermes* sp. 1; Ter_hisp, *Termes hispaniolae*.

Effect of Physical Soil Variables on Termite Species Abundance

The CCA explained 16.1% of the variation in termite species ($P < 0.001$) and included variables related to soil physical variables (bulk density, soil resistance to penetration, total porosity, visual evaluation of soil structure, and soil moisture). The first two axes of the biplot accounted for 48.9 and 45.0%, respectively, of the variance explained by the CCA (**Table 6**).

Some termite species were associated with the physical soil variables, for example, *Embiratermes neotenicus* associated with high soil moisture, *Curvitermes odontognathus* and *Cyrilliotermes angulariceps* with high total porosity, a species group made up of the species *Cylindrotermes capixaba*, *Microcerotermes arboreus*, and *Termes hispaniolae* was related to high values of visual evaluation of soil structure, finally soil resistance to penetration was correlated positively with some species of the genus *Nasutitermes* and negatively with total species richness (**Figure 6**).

DISCUSSION

Termite Community

Although the number of species per transect did not present significant differences, the total number of species did show important variations, the area of natural regeneration to be on the way of recovery of termite diversity with 25 species collected

TABLE 5 | Similarity-percentage analysis (SIMPER) comparing species composition of termites collected in natural regeneration (RN), silvopastoral systems (SPS), and rubber plantations (RP) in Colombian Amazon region.

Land use	Specie	Average dissimilarity	Cumulative contribution (%)	Average abundance		*P*-value
Natural regeneration vs. Rubber plantation	*Anoplotermes* sp. 1	0.08	12.26	49.37	2.85	0.087
Natural regeneration vs. Silvopastoral systems	*Silvestritermes* cf. *lanei*	0.2	21.82	106.5	0.75	0.008
	Anoplotermes sp. 1	0.08	31.09	49.37	0.62	0.018
	Nasutitermes sp. 7	0.08	40.0	0.0	40.37	0.056
	Nasutitermes sp. 6	0.05	46.1	22.37	2.87	0.045
	Nasutitermes sp. 3	0.03	49.86	16.12	0.0	0.017
	Nasutitermes sp. 8	0.02	52.23	9.37	0.0	0.014
Rubber plantation vs. Silvopastoral systems	*Aparatermes silvestrii*	0.12	13.5	0.0	62.87	0.029
	Nasutitermes guayanae	0.11	26.08	51.28	10.25	0.033
	Nasutitermes sp. 7	0.1	36.99	0.0	40.37	0.005
	Termes hispanolae	0.06	44.4	32.28	4.62	0.030
	Heterotermes tenuis	0.04	49.36	21.85	4.87	0.024
	Microcerotermes arboreus	0.04	54.15	20.0	0.0	0.009

Here, we only show statistically significant termite species according to the permutation test.

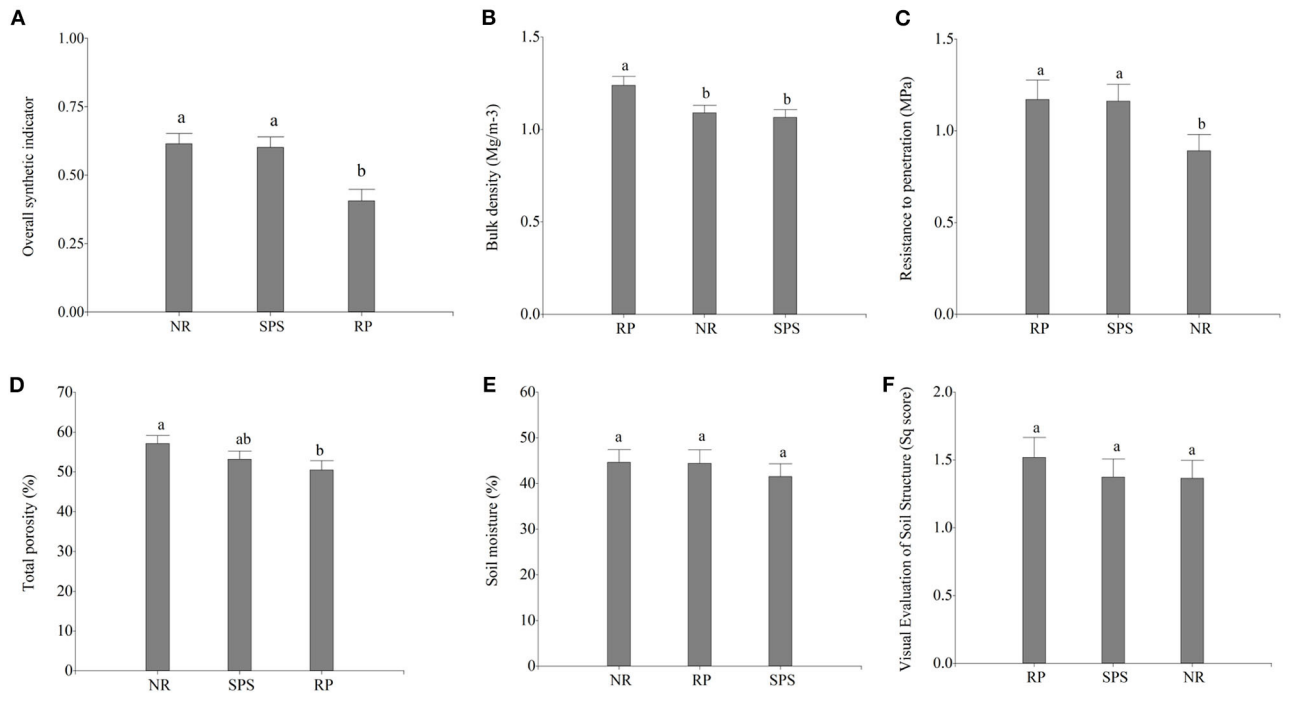

FIGURE 3 | Physical soil characteristics: **(A)** overall synthetic indicator of physical quality, **(B)** bulk density, **(C)** soil resistance to penetration, **(D)** total porosity, **(E)** soil moisture content, and **(F)** visual evaluation of soil structure in Natural Regeneration (RN), silvopastoral systems (SPS), and rubber plantations (RP) in Colombian Amazon region. The bars represent standard deviation, and the different letters indicate statistically significant differences according to the Fisher Test ($P < 0.05$).

in total, if it is compared with previous studies carried out in the Amazon that report 52 species for forest (Ackerman et al., 2009). The species richness found in the rubber plantation in our study (16 species) was greater compared to others' studies in Colombia for commercial rubber plantation (10 species; Pinzon et al., 2012).

Despite the present study was not evaluated, in the area of natural forest, the total diversity observed was similar to studies carried out by Pinzon et al. (2017) who reported the presence of 40 termite species in riparian forests of the eastern plains of Colombia. However, these data are not completely comparable because of the differences between land uses and the study areas; it can be used as a reference.

Several authors have reported a negative effect on termite assemblages according to the level of habitat disturbance and

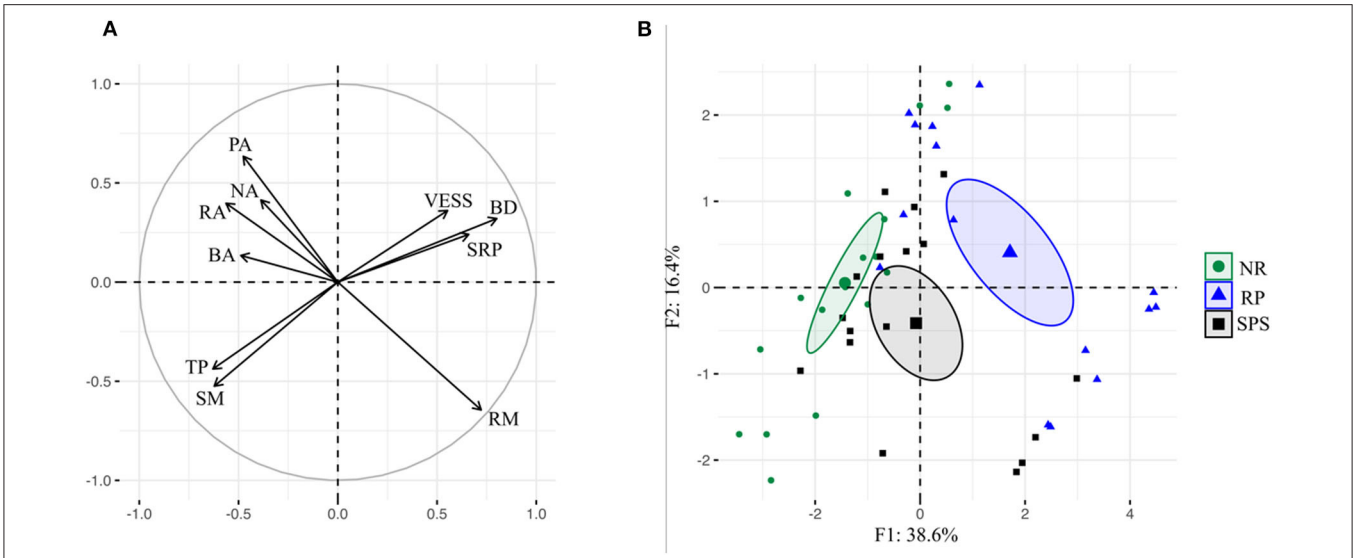

FIGURE 4 | Projection of physical soil characteristics and the 60 sampling points according to land uses **(A)**, and in the plane formed by Axes 1 and 2 of PCA **(B)** in Colombian Amazon region. Ellipses show sampling points grouped from a given land use centered on their respective bari-centers ($P < 0.01$: Monte Carlo permutation test, Explained variance: 21.24%). BA, biogenic aggregates; BD, bulk density; NA, soil not macro-aggregate; PA, physical aggregates; RA, root aggregates; RM, remaining material; SRP, soil resistance to penetration; TP, total porosity; VESS, visual evaluation of soil structure.

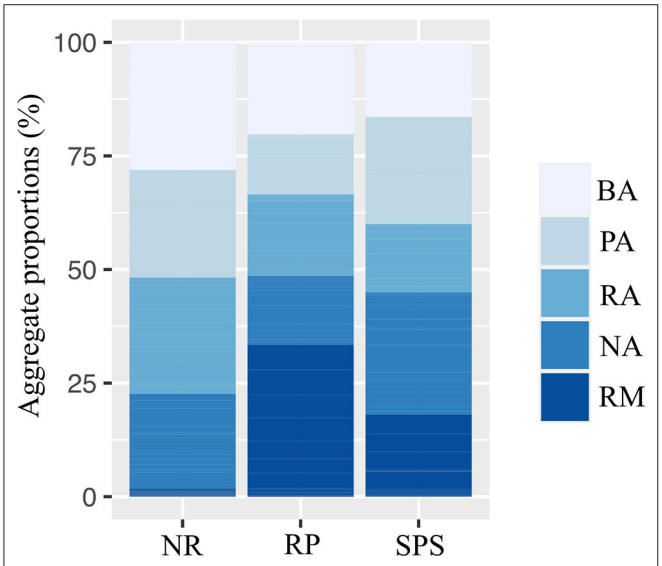

FIGURE 5 | Proportions of land aggregates classified according to their origin in Natural Regeneration (RN), silvopastoral systems (SPS), and rubber plantations (RP) in Colombian Amazon region. BA, biogenic aggregates; NA, soil not macro-aggregate; PA, physical aggregates; RA, root aggregates; RM, remaining material.

TABLE 6 | Canonical correspondence analysis (CCA) permutation test results examining the association between termite species composition with physical soil variables.

Physical soil variables	Explains (%)	VIF	Pseudo-F
Bulk density	2.89	2.7	1.6*
Total porosity	3.45	1.95	1.93**
VESS	3.08	1.56	1.72**
Soil moisture	2.61	1.7	1.46*
Soil resistance to penetration	3.82	1.35	2.1**

*P-value ** = 0.01, * = 0.05.*

trampling, with a reduction in termite diversity and abundance (Barros et al., 2004; Vasconcellos et al., 2010). Nevertheless, the inclusion of trees in pastures and the isolation of these to prevent livestock from entering have benefited the diversity of termites in our study site (22 species) exceeding the number of species reported for degraded pastures (Carrijo et al., 2009; Duran-Bautista et al., 2020).

The changes in species composition observed in this study confirm the results of previous work that suggests that simplified habitats are associated with a decrease in termite richness (Vasconcellos et al., 2008; Luke et al., 2014). Besides, the exclusive presence and high dominance of species, such as *Microcerotermes arboreus* in the rubber plantations, suggest the creation of favorable environments that could turn them into pests (Constantino, 2002b). Likewise, in Colombia, the *Hetereotermes* spp. have been reported in high densities in rubber crops of Puerto López-Meta (Pinzon et al., 2012) and as a pest in rubber plantations in the Amazon region (Sterling et al., 2011).

The dominance of *Aparatermes silvestrii* in silvopastoral systems may be associated with selective low regarding the origin of organic matter they feed on, which may explain their presence and significant abundance in artificial pastures (Bourguignon et al., 2009). The dominance of some species of the genus *Nasutitermes* in the area of natural regeneration shows that this land use is in the process of recovery and is providing the necessary resources for the nesting and feeding of these species.

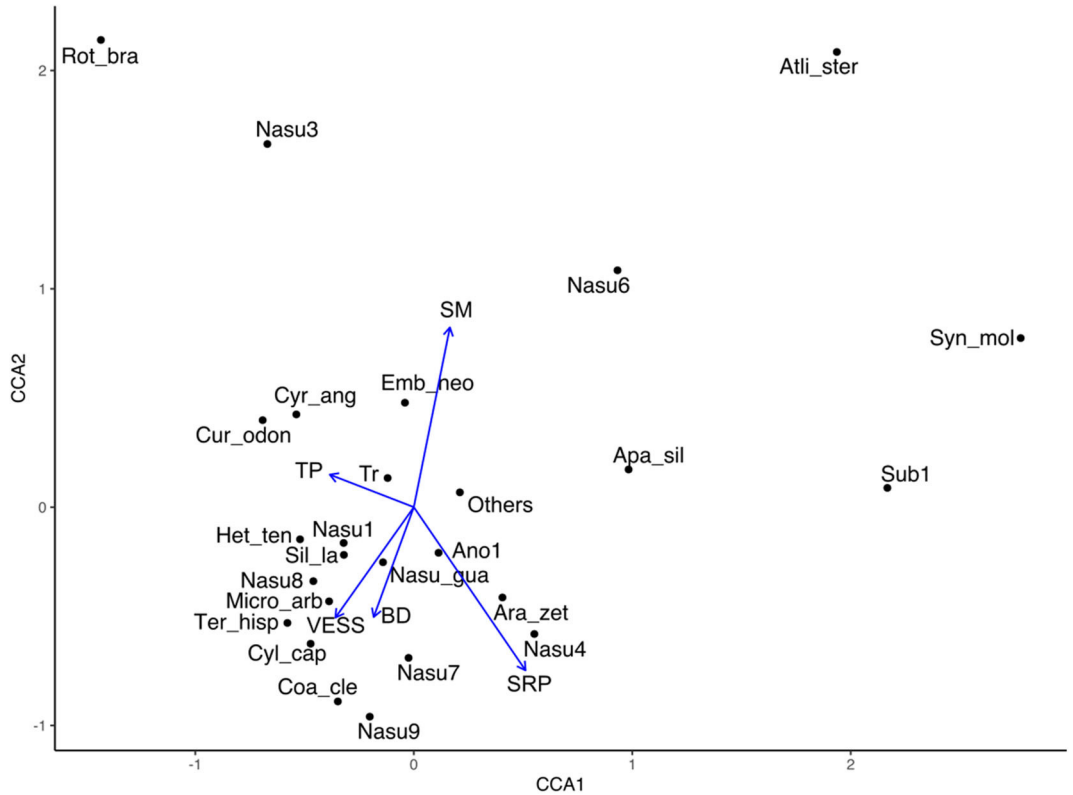

FIGURE 6 | Canonical correspondence analyses (CCA) ordination plot for termite species composition and physical soil variables with a significant effect in Colombian Amazon region. The analysis deals with 24 termite species related to five physical soil variables. SRP, soil resistance to penetration; TP, total porosity; VESS, visual evaluation of soil structure; Ano1, *Anoplotermes* sp. 1; Apa_sil, *Aparatermes silvestrii*; Ara_zet, *Araujotermes zeteki*; Atli_ster, *Atlantitermes stercophilus*; Coa_cle, *Coatitermes clevelandi*; Cur_odon, *Curvitermes odontognathus*; Cyl_cap, *Cylindrotermes capixaba*; Cyr_ang, *Cyrilliotermes angulariceps*; Emb_neo, *Embiratermes neotenicus*; Het_ten, *Heterotermes tenuis*; Micro_arb, *Microcerotermes arboreus*; Nasu_gua, *Nasutitermes guayanae*; Nasu1, *Nasutitermes* sp. 1; Nasu3, *Nasutitermes* sp. 3; Nasu4, *Nasutitermes* sp. 4; Nasu6, *Nasutitermes* sp. 6; Nasu7, *Nasutitermes* sp. 7; Nasu8, *Nasutitermes* sp. 8; Nasu9, *Nasutitermes* sp. 9; Rot_bra, *Rotunditermes bragantinus*; Sil_la, *Silvestritermes* cf. *lanei*; Sub1, *Subulitermes* sp. 1; Syn_mol, *Syntermes molestus*; Ter_hisp, *Termes hispaniolae*; Tr, total richness.

Previous work has shown that *Nasutitermes* spp are frequent in areas with regeneration process for 5 years (Viana-Junior et al., 2014) as well as in reforestation sites with native species (de Paula et al., 2016).

Land Use Effect on Physical Soil Variables

In general, all land uses presented a good soil physical quality, and the natural regeneration presented the best values. In this sense, it is proposed that changes in forest land use to more intense land uses, such as agroforestry or silvicultural systems generate changes due to disturbance that alters the chemical and physical composition of the soil (Tellen and Yerima, 2018). The time of abandonment and non-intervention in the areas of natural regeneration seems to have generated a positive effect that lead to soil physical characteristic protection (Lisboa et al., 2009), reflected in high values of total porosity and soil moisture, low resistance to penetration, and consequently better overall quality of physical soil characteristics.

However, important differences were observed in rubber plantations with higher values of apparent density and resistance to penetration even surpassing silvopastoral systems. This could be explained by two mechanisms: (1) the silvopastoral systems evaluated were recently established, and (2) the samples were taken below the tree line where the cattle did not have access. de Souza Braz et al. (2013) propose that in areas where grazing pressure is low, it avoids the negative effects on physical soil characteristics generated by cattle trampling, such as increasing bulk density and soil resistance to penetration, reducing soil aeration, aggregate stability, and water infiltration that have been widely documented (Arevalo et al., 1998; Sharrow, 2007; Drewry et al., 2008).

Concerning the above, a study found that rubber agroforestry systems with low diversification, present higher values of apparent density and resistance to penetration than more diversified agroforestry systems due to high abundance of roots mixed with a soft layer composed primarily of porous and rounded aggregates present in these systems (Cherubin et al., 2018). Likewise, an assessment of the effect of different land uses on soil quality suggests that rubber trees are the plant cover causing the smallest change in terms of soil quality (Moreira et al., 2011).

Physical Soil Variables and Relationship With Termite Species

Soil compaction indicated by resistance to penetration had a negative relationship with total termite species richness. In this regard, Jones et al. (2003) suggested that compaction of soil surface and an increase in soil bulk density might have had a negative impact on soil-dwelling termites. This may be due to creation of tunnel and foraging galleries during termite's feeding and foraging activities by moving soil particles of different sizes (Jouquet et al., 2011, 2016b). These activities are difficult by increased soil compaction. Tucker et al. (2004) found that construction speed of tunnel network varied with soil compaction, being much slower in the more compacted soil (1.35 g/cm^3). Although this study was conducted under laboratory conditions using a different substrate and moisture content, and it cannot be directly compared with the present study, however, clearly the same relationship was observed.

Nevertheless, some species of the genus *Nasutitermes* manage to support well in conditions of high resistance to penetration, and this would be related to the ability of these species to adapt to disturbed environments. For example, they can be pests in sugarcane crops (Constantino, 2002b; Miranda et al., 2004) where the physical quality of the soil is low by compaction due to intensive mechanization (Cherubin et al., 2016). The relationship of *E. tenicus* with high-humidity soil could be explained by two mechanisms: the first is the nesting habits developed for this specie as it builds an epigeal earthen nest of variable shape, sometimes at the bases of trees (Constantino, 1992a,b), and the second is one of the more frequently found termite species in primary and secondary forests (Bandeira et al., 2003; Davies et al., 2003; Souza et al., 2012) where soil moisture is higher.

Cyrilliotermes angulariceps was positively related to total porosity; the species of this genus are soil feeders (Constantino and Carvalho, 2012), and specifically, *C. angulariceps* feeds on soil particles richer in organic matter (Bourguignon et al., 2009) where soil porosity is generally higher. Although little information is available on the influence of termite foraging activity on soil porosity (Jouquet et al., 2016a), some authors have demonstrated that termite mediated increases in soil porosity (Lavelle, 1997). The relation of *Cylindrotermes capixaba*, *Microcerotermes arboreus*, and *Termes hispaniolae* with high values of soil structure can be explained because these species were presented in high density in the rubber plantations where this parameter presented the highest values. These results coincide with those from Thoumazeau et al. (2019), which reported a VESS gradient from intensive cash crops, the most disturbed system, through different ages of rubber planting to forest as the least disturbed system.

In general, changes in physical soil quality presented in different land uses affect termite communities and lead to changes in its composition with disproportionate loss of some species; however, there are some who can adapt well to the decline in physical soil quality, especially in rubber plantations and silvopastoral systems. Among the parameters evaluated, the resistance to penetration was the one that generated the greatest negative impact, and soil moisture was positively related to the presence of some species.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they were financed by the University of the Amazon. Requests to access the datasets should be directed to Ervin Humprey Duran-Bautista, ervinduranb@gmail.com.

AUTHOR CONTRIBUTIONS

ED-B: conceiving and designing analyses, data collection, data analysis, and wrote paper. YM, JG, TO, and MB: data collection, data analysis, and wrote paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Review of Termite Pheromones: Multifaceted, Context-Dependent, and Rational Chemical Communications

Yuki Mitaka and Toshiharu Akino*

Applied Entomology Laboratory, Department of Bioresource Field Sciences, Kyoto Institute of Technology, Kyoto, Japan

Termite colonies, composed of large numbers of siblings, develop an important caste-based division of labor; individuals in these societies interact via intra- or intercaste chemical communications. For more than 50 years, termites have been known to use a variety of pheromones to perform tasks necessary for maintenance of their societies, similar to eusocial hymenopterans. Although trail-following pheromones have been chemically identified in various termites, other types of pheromones have not been elucidated chemically or functionally. In the past decade, however, chemical compositions and biological functions have been successfully identified for several types of termite pheromones; accordingly, the details of the underlying pheromone communications have been gradually revealed. In this review, we summarize both the functions of all termite pheromones identified so far and the chemical interactions among termites and other organisms. Subsequently, we argue how termites developed their sophisticated pheromone communication. We hypothesize that termites have diverted defensive and antimicrobial substances to pheromones associated in caste recognition and caste-specific roles. Furthermore, termites have repeatedly used a pre-existing pheromone or have added supplementary compounds to it in accordance with the social context, leading to multifunctionalization of pre-existing pheromones and emergence of new pheromones. These two mechanisms may enable termites to transmit various context-dependent information with a small number of chemicals, thus resulting in formation of coordinated, complex, and rational chemical communication systems.

Keywords: termite, pheromone, parsimony, antimicrobial, cuticular hydrocarbon, social context

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****Correspondence:***
Yuki Mitaka
ymitaka02@gmail.com

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INTRODUCTION

Termites are eusocial insects that live in colonies with large numbers of siblings, where both males and females perform largely equal roles with respect to colony maintenance. Their societies develop a caste-based division of labor; each caste is morphologically and physiologically specialized for different tasks (Eggleton, 2011). For example, reproductive castes (kings and queens) concentrate on reproduction, and soldiers and workers are engaged in other tasks necessary for colony maintenance (e.g., foraging; colony defense; nest construction; hygiene control; and caring for reproductive castes and eggs) (Eggleton, 2011).

The sophisticated colony organization of termites and other eusocial insects is characterized by an efficient communication system based mainly on pheromones. In termites and social hymenopterans, it has long been hypothesized that pheromones might be involved in all social activities (Wilson, 1965). However, termite pheromonal studies have mainly focused on trail-following pheromones and sex-pairing pheromones. A trail pheromone was first identified in the Eastern subterranean termite *Reticulitermes virginicus* (Rhinotermitidae) (Matsumura et al., 1968); trail-following pheromones and sex-pairing pheromones were subsequently identified in various termite species (detailed below). However, the identification of other pheromone types did not progress until the 1990s, although the existence of primer pheromones regulating differentiation into supplementary alates or soldiers had been suggested (Krishna et al., 2013a,b,c,d,e). Since the 2000s, however, several other pheromone types have been chemically and functionally identified in succession.

In this review, we first focus on the functions and components of each type of pheromone. We then discuss the origins of pheromone components; the evolutionary process of multifunctionalization; the development of intra- and intercaste communication; and the interactions among termites, pathogens, predators, and inquilines via termite pheromones.

SEX-PAIRING PHEROMONES

Sex-pairing pheromones in termites are used by alates (i.e., adult individuals with wings) for attracting sexual partners during mating behavior (Bordereau and Pasteels, 2011). Termite nuptial flight usually occurs once per year in a certain season, whereby virgin female and male alates come together in pairs to establish new colonies. Numerous alates disperse from their colonies by flight, then land on the ground or in trees; subsequently, they shed their wings (becoming dealates) and search for mating partners (Bordereau and Pasteels, 2011). In many species, female dealates perform calling behavior (raising the tip of the abdomen and emitting the sex-pairing pheromone from the tergal glands or the sternal gland) to attract male dealates (Bordereau and Pasteels, 2011). In a few species, such as *Zootermopsis nevadensis* and *Zootermopsis angusticollis*, both female and male dealates emit the sex-pairing pheromone (Bordereau et al., 2010). The range of action of the pheromone in natural conditions depends on the termite species; it ranges from a few centimeters (Leuthold, 1975) to a few meters (Leuthold and Bruinsma, 1977). When a male succeeds in finding the female, the male follows after it (tandem running). The leading female seeks a suitable nesting site, while the following male continues to antennate (or lick) the posterior pleural membranes of the female. During the tandem running, short-range or contact chemical stimuli by the sex-pairing pheromones play an important role in maintaining the tandem formation (Bordereau and Pasteels, 2011). After the pair of male and female dealates finds a suitable site, they establish a new colony and begin to produce offspring as the primary king and queen, respectively.

Thus far, sex-pairing pheromones have been fully identified in 17 termite species belonging to three families (Archotermopsidae, Rhinotermitidae, and Termitidae); these pheromones mainly consist of aliphatic aldehydes, alcohols, and/or diterpenes (**Table 1**). In *Z. nevadensis* and *Z. angusticollis* (Archotermopsidae), which belong to a relatively ancestral family (Lo and Eggleton, 2011), the female and male dealates use different compounds; the females use (5*E*)-2,6,10-trimethyl-5,9-undecadienal, while the males use 4,6-dimethyldodecanal (Bordereau et al., 2010). However, in more derivative termite families (Rhinotermitidae and Termitidae), only female dealates emit the pheromone, namely, (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol, which is common among many species, although some termitid species use mixtures of two or three compounds for the pheromone (**Table 1**). In most species, all components are required for high attraction activity of the pheromone. In *Odontotermes formosanus*, however, the two components act in synergy at long distance, whereas (*Z*,*Z*)-3,6-dodecadien-1-ol can act alone at a short distance (Wen et al., 2012).

The number of chemical components in termite sex-pairing pheromones varies among termite species; for example, *Cornitermes bequaerti* uses only (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol, whereas *C. cumulans* uses both (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol and (*E*)-nerolidol. Furthermore, *C. silverstrii* uses a mixture of three compounds including (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol, (*E*)-nerolidol, and (*Z*)-3-dodecen-1-ol (**Table 1**). The addition of species-specific minor components to a pre-existing pheromone might facilitate species recognition among sympatric species, as is often suggested of insect sex pheromones (Weiss et al., 2013; Allison and Cardé, 2016; Chen et al., 2018; Valterová et al., 2019). However, the development of species-specific pheromones among sympatric species varies considerably among species (Bordereau and Pasteels, 2011).

TRAIL-FOLLOWING PHEROMONES

When foraging individuals (workers, pseudergates [individuals performing works while remaining developmentally flexible], or soldiers) find new food, they deposit trail-following pheromones from the sternal gland while returning to the nest; the pheromones elicit trail-following behavior in nestmates, leading them to the food resource (Traniello, 1981; Traniello and Busher, 1985; Czaczkes et al., 2015; Almeida et al., 2016). Trail-following pheromones are one of the most studied pheromone types, especially in ants and termites. Therefore, the biochemical, physiological, ecological, and evolutionary aspects of trail-following pheromones have been well characterized in previous studies (Wilson, 1971; Hölldobler and Wilson, 1990; Morgan, 2009; Bordereau and Pasteels, 2011; Wyatt, 2014; Czaczkes et al., 2015; Leonhardt et al., 2016).

Thus far, termite trail-following pheromones are known in 68 species (**Table 2**). Their chemical components are often common within a taxonomical group. In particular, (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol is used in Rhinotermitidae (12 species) and Termitidae (24 species). While the trail-following pheromones of lower termite species belonging to families

TABLE 1 | List of sex-pairing pheromones studied in termites.

Termite family and species	Sex	Component	Type of Evidence	References
Archotermopsidae				
Zootermopsis nevadensis	F	(5*E*)-2,6,10-trimethyl-5,9-undecadienal	BS	Bordereau et al., 2010
	M	4,6-Dimethyldodecanal	BS	Bordereau et al., 2010
Zootermopsis angusticollis	F	(5*E*)-2,6,10-trimethyl-5,9-undecadienal	BS	Bordereau et al., 2010
	M	4,6-Dimethyldodecanal	BS	Bordereau et al., 2010
Hodotermopsis sjoestedti	F	(5*E*)-2,6,10-trimethyl-5,9-undecadienal	E	Lacey et al., 2011
	M	4,6-Dimethylundecanal	E	Lacey et al., 2011
Rhinotermitidae				
Prorhinotermes simplex	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Hanus et al., 2009
Reticulitermes flavipes	F	*n*-Tetradecyl propionate	BS	Clément et al., 1989
Reticulitermes santonensis	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Laduguie et al., 1994
Coptotermes formosanus	F	Unknown	BE	Raina et al., 2003
Psammotermes hybostoma	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Sillam-dussès et al., 2011
Termitidae				
Odontotermes formosanus	F	(*Z*, *Z*)- 3,6-dodecadien-1-ol and (*Z*)-3-dodecen-1-ol	BS	Wen et al., 2012
Ancistrotermes pakistanicus	F	(*Z*,*Z*)- 3,6-dodecadien-1-ol	BS	Robert et al., 2004
Ancistrotermes dimorphus	F	(*Z*,*Z*)- 3,6-dodecadien-1-ol	BS	Wen et al., 2015
Macrotermes annandalei	F	Unknown	BE	Peppuy et al., 2004
Macrotermes barneyi	F	Unknown	BE	Peppuy et al., 2004
Pseudacanthotermes spiniger	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Bordereau et al., 1991
Pseudacanthotermes militaris	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Bordereau et al., 1993
Cornitermes bequaerti	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol	BS	Bordereau et al., 2002
Cornitermes cumulans	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol and (*E*)-nerolidol	BS	Bordereau et al., 2011
Cornitermes silverstrii	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol and (*E*)-nerolidol and (*Z*)-3-dodecen-1-ol	BS	Bordereau et al., 2011
Embiratermes neotenicus	F	(*Z*,*Z*,*E*)- 3,6,8-dodecatrien-1-ol and (*Z*)-3-dodecen-1-ol	BS	Dolejšová et al., 2018
Silvestritermes minutus	F	(*Z*,*Z*)- 3,6-dodecadien-1-ol and (*Z*)-3-dodecen-1-ol	BS	Dolejšová et al., 2018
Silvestritermes heyeri	F	(*Z*,*Z*)- 3,6-dodecadien-1-ol	BS	Dolejšová et al., 2018
Nasutitermes ephratae	F	Neocembrene and Neocembrene isomers and Trinervitatriene	E	Buděšínský et al., 2005

Sex, F, Female; M, Male; type of evidence, E, Estimation by chemical analyses (but the function is unproved); BE, Bioassay with termite extracts; BS, Bioassay with synthesized compound (truly identified).

other than the Termitidae consist of only one compound, some higher termite species use a plurality of compounds; for example, *O. formosanus* uses two compounds, (Wen et al., 2014) and *Nasutitermes corniger* uses three compounds (Sillam-Dussès et al., 2010; **Table 2**). In *O. formosanus*, one of the compounds, (*Z*)-dodec-3-en-1-ol, elicits orientation behavior of termites exploring the environment, and the other (*Z*,*Z*)-dodeca-3,6-dien-1-ol has both orientation and recruitment effects on termites when food is discovered (Wen et al., 2014). Furthermore, workers of *O. formosanus* change the mixture ratio of the pheromone components depending on their ongoing behaviors, and the threshold of response to the pheromone components also change depending on behavioral contexts (Wen et al., 2014).

In 10 species, the constituents are completely (or partially) identical between the trail pheromone and the sex-pairing pheromone (**Table 2**). For example, (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol is used alone as both the trail pheromone and the sex-pairing pheromone in *Reticulitermes santonensis* (Laduguie et al., 1994), *Pseudacanthotermes militaris* (Bordereau et al., 1993), *Pseudacanthotermes spiniger* (Bordereau et al., 1991, 1993), and *C. bequaerti* (Bordereau et al., 2002; Sillam-Dussès et al., 2020b). In contrast, in *C. cumulans*, this compound is used alone as the trail pheromone (Sillam-Dussès et al., 2020b), but used together with (*E*)-nerolidol as the sex-pairing pheromone (Bordereau et al., 2011).

AGGREGATION PHEROMONES

Aggregation pheromones elicit an aggregation behavior that involves attraction of conspecific individuals from a distance, followed by arrest of those individuals at the pheromone source (Kennedy, 1978). When foraging individuals discover a new food source, they gather their nestmates to that area for exploitation and subsequent colonization of the new area. This aggregation behavior is beneficial to the individuals because it facilitates allogrooming (removal of cuticular stains and pathogens by grooming each other) and trophallaxis (exchange of nutrients, gut symbionts, and immune substances by stomodeal and proctodeal feeding), which enables workers to survive oligotrophic

TABLE 2 | List of trail-following pheromones in termites.

Family	Species	Component	References
Mastotermitidae	*Mastotermes darwiniensis*	(*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol	Sillam-Dussès et al., 2007
Archotermopsidae	*Porotermes adamsoni*, *Stolotermes victoriensis*	(*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol	Sillam-Dussès et al., 2007
	Hodotermopsis sjoestedti	4,6-Dimethylundecan-1-ol	Lacey et al., 2011
	*Zootermopsis nevadensis**, *Z. angusticollis**	4,6-Dimethyldodecanal	Bordereau et al., 2010
Serritermitidae	*Glossotermes oculatus*, *Serritermes serrifer*	(*Z,Z*)- 10,13-nonadecadien-2-one	*G. oculatus* (Hanus et al., 2012), *S. serrifer* (Sillam-Dussès et al., 2020a
Kalotermitidae	*Kalotermes flavicollis*, *Cryptotermes brevis*, *Cry. pallidus*, *Cry. darlingtonae*, *Procryptotermes falcifer*, *Proc. leewardensis*, *Neotermes holmgreni*, *Incisitermes tabogae*, *I. minor*, *Postelectrotermes howa*	(*Z*)-3-dodecen-1-ol	*I. minor*: (Chrysanti and Yoshimura, 2012), Others: (Sillam-Dussès et al., 2009
Rhinotermitidae	*Prorhinotermes canalifrons*, *Pror. simplex*	Neocembrene	Sillam-Dussès et al., 2005
	Heterotermes tenuis, *Reticulitermes flavipes*, *Re. speratus*, *Re. santonensis**, *Re. lucifugus grassei*, *Re. virginicus*, *Re. hesperus*, *Coptotermes formosanus*, *Cop. gestroi*, *Rhinotermes marginalis*, *Schedorhinotermes lamanianus*, *Psammotermes hybostoma**	(*Z,Z,E*)-3,6,8-dodecatrien-1-ol	*H, tenuis* and *Cop. gestroi*: (Arab et al., 2004), *Re. flavipes*: (Tai et al., 1969), *Re. speratus*: (Yamaoka et al., 1987; Tokoro et al., 1990), *Re. santonensis*: (Laduguie et al., 1994), *Re. lucifugus grassei*: (Wobst et al., 1999), *Re. virginicus*: (Matsumura et al., 1968), *Re. hesperus*: (Saran et al., 2007), *Cop. formosanus*: (Tokoro et al., 1989), *Re. marginalis* and *Sc. lamanianus*: (Sillam-Dussès et al., 2006), *Psam. hybostoma*: (Sillam-dussès et al., 2011
Termitidae	*Ancistrotermes pakistanicus**	(*Z,Z*)- 3,6-dodecadien-1-ol	Robert et al., 2004
	Microcerotermes exiguous, *Pseudacanthotermes militaris**, *Pseu. spiniger**, *Cubitermes sp.*, *Drepanotermes perniger*, *Termes hispaniolae*, *Cornitermes bequaerti**, *Cor. cumulans**, *Cor. snyderi*, *Syntermes grandis*, *Cyrilliotermes angulariceps*, *Embiratermes neotenicus**, *Labiotermes labralis*	(*Z,Z,E*)-3,6,8-dodecatrien-1-ol	*Mic. exiguus* (Lubes and Cabrera, 2018), *Pseu. militaris* and *Pseu. spiniger*: (Bordereau et al., 1993), *Cub. sp.*, *D. perniger* and *Te. hispaniolae*: (Sillam-Dussès et al., 2006), Others: (Sillam-Dussès et al., 2020b
	Macrotermes annandalei, *Mac. barneyi*, *Mac. bellicosus*, *Mac. subhyalinus*, *Odontotermes hainanensis*, *O. maesodensis*	(*Z*)- 3-dodecen-1-ol	Peppuy et al., 2001
	*Odontotermes formosanus**	(*Z,Z*)- 3,6-dodecadien-1-ol, (*Z*)-3-dodecen-1-ol	Wen et al., 2014
	Amitermes evuncifer, *Nasutitermes ephratae*, *N. guayanae*, *N. kemneri*, *N. lujae*, *N. voeltzkowi*, *N. exitiosus*, *Constrictotermes cyphergaster*, *Trinervitermes geminatus*, *Trin. trinervoides*	(*Z,Z,E*)-3,6,8-dodecatrien-1-ol, Neocembrene	*A. evuncifer*: (Kotoklo et al., 2010), *Con. cyphergaster* (Cristaldo et al., 2014), Others: (Sillam-Dussès et al., 2010
	Silvestritermes euamignathus	(*Z,Z*)- 3,6-dodecadien-1-ol, Neocembrene	Sillam-Dussès et al., 2020b
	Nasutitermes corniger	(*Z,Z,E*)-3,6,8-dodecatrien-1-ol, Neocembrene, Trinervitatriene	Sillam-Dussès et al., 2010
	Nasutitermes graveolus, *N. walker*, *Trinervitermes bettonianus*	Neocembrene	*N. graveolus* and *N. walkeri*: (Birch et al., 1972), *Tr. bettonianus*: (McDowell and Oloo, 1984

Species with an asterisk indicate that the same compound(s) is used both for the sex-paring pheromone and the trail pheromone. All of the listed compounds were identified by the bioassay with the authentic standards.

and microbe-rich environments (Eggleton, 2011). Thus, rapidly formed yet long-lasting aggregation is needed for foraging individuals.

A recent study revealed that *Reticulitermes speratus* workers release an aggregation pheromone at their nesting site, and that this pheromone induces rapid and long-lasting aggregation of workers (Mitaka et al., 2020; **Table 3**). The pheromone is a mixture of 2-phenylundecane, pentacosane, heptacosane, palmitic acid, *trans*-vaccenic acid, and cholesterol. It is estimated that the aggregation pheromone functions as a signal to indicate nestmate and/or food presence, and as an arresting agent that causes the attracted workers to remain at the pheromone source for a long period.

TABLE 3 | List of pheromones used by non-reproductive castes in termites.

Pheromone type	Termite family and species	Emitting caste	Type of evidence	Component	Function	References
Alarm pheromone						
	Mastotermes darwiniensis	Workers and Soldiers	BS	*p*-Benzoquinone	Increasing worker's walking speed and soldier's mandible opening.	Delattre et al., 2015
	Rhinotermitidae					
	Prorhinotermes canalifrons	Soldier	BS	(*E*,*E*)-α-farnesene	Triggering alarm behavior in psudagates and soldiers	Šobotník et al., 2008
	Termitogeton planus	Soldier	BE	A mixture of monoterpenes	Triggering alarm behavior in psudagates and soldiers.	Dolejšová et al., 2014
	Termitidae					
	Nasutitermes rippertii	Soldier	BS	α-Pinene, Limonene	Triggering alarm behavior in workers and soldiers.	Vrkoc et al., 1978
	Nasutitermes costalis	Soldier	BS	Carene, Limonene	Triggering alarm behavior in workers and soldiers.	Vrkoc et al., 1978
	Nasutitermes princeps	Soldier	BS	α-Pinene	Triggering alarm behavior in workers and soldiers.	Roisin et al., 1990
	Constrictotermes cyphergaster	Soldier	BS	α-Pinene, Myrcene, (*E*)-β-ocimene	Triggering alarm behavior in workers and soldiers with vibration.	Cristaldo et al., 2016b
	Velocitrmes velox	Soldier	BS	α-Pinene, Limonene	Triggering alarm behavior in workers and soldiers.	Valterová et al., 1988
Soldier pheromone	**Rhinotermitidae**					
	Reticulitermes flavipes	Soldier	BE	γ-Cadinene	Promoting workers' differentiation into soldiers.	Tarver et al., 2011
			BE	γ-Cadinenal	Inhibiting workers' differentiation into soldiers.	Tarver et al., 2011
	Reticulitermes speratus	Soldier	BS	(–)-β-Elemene	Inhibiting workers' differentiation into soldiers. Arresting workers. Fungistatic activities against entomopathogenic fungi	Mitaka et al., 2017b
					Soldier's age indicator	Mitaka and Matsuura, 2020
Phagostimulanting pheromone	**Mastotermitidae**					
	Mastotermes darwiniensis	Worker	BS	Hydroquinone	Promoting worker's feeding behavior	Reinhard et al., 2002
Aggregation pheromone	**Rhinotermitidae**					
	Reticulitermes speratus	Worker	BS	2-Phenylundecane, Pentacosane, Heptacosane, *trans*-Vaccenic acid, Palmitic acid, Cholesterol	Attracting and arresting workers.	Mitaka et al., 2020

Type of evidence, BE, Bioassay with termite extracts; BS, Bioassay with synthesized compound.

PHAGOSTIMULANT PHEROMONES

A previous study showed that hydroquinone is present in the labial glands of workers in several termite species; those findings suggested that this compound acts as a phagostimulant pheromone, which elicits worker feeding behavior, in various termites (Reinhard et al., 2002; **Table 3**). However, the pheromonal activity has been demonstrated only in *Mastotermes darwiniensis* (Reinhard et al., 2002). Therefore, the generality of termite phagostimulant pheromones has not been fully elucidated (Raina et al., 2005).

ALARM PHEROMONES

In termites, colony defense is performed by soldiers and workers (Roisin et al., 1990; Reinhard and Clement, 2002; Šobotník et al., 2010; Ishikawa and Miura, 2012; Yanagihara et al., 2018). Alarm pheromones elicit alarm behaviors including orientation to the pheromone source, nestmate recruitment, and attack on enemies in termite soldiers and workers (or pseudergates), although the details of the behavioral sequences are different among species. For example, in European *Reticulitermes* species (*R. santonensis*, *R. lucifugus*, *R. grassei*, and *R. banyulensis*), chemical alarm signals from soldier's head induce zigzag running, antennation with nestmates, body shaking behavior, and orientation to the odor source in both soldiers and workers (Reinhard and Clement, 2002). Soldiers also perform mandible snapping and head-banging on the substrate. While workers are attracted by the odor within a few seconds, soldiers are attracted much later (Reinhard and Clement, 2002). In *Nasutitermes princeps*, when the alarm pheromone is secreted from soldiers, soldiers and workers are attracted by the pheromone, and then soldiers emit their sticky secretion to immobilize and incapacitate enemies. After that, older large workers eliminate the enemies (Roisin et al., 1990).

Termites use vibratory signals in combination with chemical signals, resulting in complex alarm communication. For example, in *M. darwiniensis*, soldiers actively face the disturbance source with repeating mandible openings and secreting defensive secretion, while workers run away from the disturbance source and spread the alarm signals to the nestmates using body vibrations (Delattre et al., 2015). Also, in *R. flavipes*, the alarm pheromone increases the vibratory communication among soldiers and workers (Delattre et al., 2019). Furthermore, the evoked alarm responses depending on the dose of alarm pheromone. In *Constrictotermes cyphergaster*, the soldiers also actively face the source of disturbance and then emit the alarm pheromone; lower doses increase body shaking movements of nestmates, and higher doses induced long-term running of them (Cristaldo et al., 2016b).

Thus far, alarm pheromones (comprising monoterpenes and/or sesquiterpenes) have been identified in six species (**Table 3**): *p*-benzoquinone in *M. darwiniensis* (Delattre et al., 2015), (*E*,*E*)-α-farnesene in *Prorhinotermes canalifrons* (Šobotník et al., 2008); α-pinene in *N. princeps* (Roisin et al., 1990); α-pinene, myrcene, and (*E*)-β-ocimene in *C. cyphergaster* (Cristaldo et al., 2016b); carene and limonene in *Nasutitermes costalis* (Vrkoc et al., 1978); and α-pinene and limonene in *Nasutitermes rippertii* (Vrkoc et al., 1978) and *Velocitermes velox* (Valterová et al., 1988). Although a mixture of terpenes is estimated to be used as the alarm pheromone in *Termitogeton planus* (Dolejšová et al., 2014), *R. santoensis*, *R. grassei*, *R. lucifugus*, *R. banyulensis* (Reinhard et al., 2003), and *R. flavipes* (Delattre et al., 2019), the compositions of the alarm pheromones has not been clarified. The secretion gland of the alarm pheromones is restricted to the frontal grand of the soldier head in Rhinotermitidae and Termitidae (Šobotník et al., 2010), but in *M. darwiniensis*, the alarm pheromone is secreted from the labial glands of soldiers and workers (Delattre et al., 2015).

SOLDIER PHEROMONES

In termites, soldiers are differentiated from workers (or pseudergates). For more than 35 years, it was presumed that the soldier's head extract contains a primer pheromone that inhibits soldier differentiation (Lefeuve and Bordereau, 1984). Until recently, there was no proof of the existence of this pheromone. A previous study reported that γ-cadinene and γ-cadinenal, which were isolated from the soldiers of *R. flavipes*, showed stimulatory and inhibitory effects, respectively, on soldier differentiation (Tarver et al., 2011); however, these pheromonal activities were not demonstrated using authentic standards.

Recently, it was revealed that the soldiers of *R. speratus* secrete (−)-β-elemene as the soldier pheromone; this compound functions as the primer pheromone that inhibits soldier differentiation, as a releaser pheromone that arrests workers, and as a fungistatic agent that protects against entomopathogenic fungi (e.g., *Metarhizium anisopliae* and *Beauveria bassiana*) (Mitaka et al., 2017b; **Table 3**). Furthermore, the amount of (−)-β-elemene a soldier holds increases with age, resulting in functioning that serves as a signal to indicate the soldier's age (Mitaka and Matsuura, 2020). The nests of *R. speratus* are divided into chambers connected by small openings; the royal chamber (i.e., location of reproductive castes) is located deep inside the nest wood. In this species, younger soldiers gather around the royal chamber to protect kings and queens, while older soldiers are distributed in the periphery of the nest wood to defend the nest entrances (Yanagihara et al., 2018). When workers decide whether to move among chambers, they examine the amount of soldier pheromone held by the soldier standing guard at the chamber entrance; they do not move to the next chamber if the amount of soldier pheromone is very small (that is, if the soldier is very young) (Mitaka and Matsuura, 2020).

EGG RECOGNITION PHEROMONES

Egg protection is one of the most fundamental social activities performed by social insects (Ayasse and Paxton, 2002). In

termites, workers recognize the eggs laid by queens, pile the eggs in nursery rooms, and smear their saliva on the eggs' surfaces to protect them from desiccation and infection by pathogens (Matsuura et al., 2000, 2007). In *R. speratus*, lysozyme, an antibacterial enzyme expressed in the eggs and the workers' salivary glands, is used as an egg recognition pheromone; it elicits egg-carrying and -piling behaviors in workers (Matsuura et al., 2007; **Table 4**). Moreover, *R. speratus* workers simultaneously use β-glucosidase, a cellulose-digesting enzyme expressed in the eggs, as well as in the workers' salivary glands and hind gut, as an egg recognition pheromone (Matsuura et al., 2009; **Table 4**). Although each enzyme itself has sufficient pheromonal activity, the two enzymes act synergistically to elicit strong egg-carrying and -piling behaviors (Matsuura et al., 2009). It is speculated that *Reticulitermes* spp. commonly use lysozymes and β-glucosidase as egg recognition pheromones because of their broad cross-species activities (Matsuura et al., 2007, 2009); however, the salivary gland extracts derived from workers of *Hodotermopsis sjostedti*, *Cryptotermes brevis*, and *Coptotermes formosanus* also show strong pheromone activity (Matsuura et al., 2009). In addition, termites use C-type lysozymes as an egg recognition pheromone, although recent transcriptomic analyses revealed that *Z. nevadensis*, *C. formosanus*, and *R. speratus* also have I-type and P-type lysozymes; particularly in *R. speratus*, I-type and P-type lysozymes are highly expressed in soldiers (Mitaka et al., 2017a). The functions of these lysozymes are unknown; thus, further studies are needed.

QUEEN PHEROMONES

In social insects, reproduction is primarily monopolized by queens; the number of fertile queens is regulated by communication between reproductive and non-reproductive individuals, often through queen pheromones (Van Oystaeyen et al., 2014). Although the queen pheromones of social hymenopteran species, which inhibit worker differentiation into supplementary queens, have been suggested or identified for over 50 years (Van Oystaeyen et al., 2014), studies of termite queen pheromones have made little progress.

Thus far, *R. speratus* is the only termite species in which the queen pheromone has been identified (Matsuura et al., 2010; **Table 4**). The queen pheromone is a volatile pheromone that consists of butyl butyrate and 2-methyl-1-butanol (mixture ratio 2:1); it is emitted from secondary queens and eggs as an honest signal of queen presence and fertility (Matsuura et al., 2010). This pheromone has multifunctional roles: inhibition of the differentiation of workers into supplementary queens (Matsuura et al., 2010), enhancement of workers' egg-carrying and -piling behaviors (Matsuura et al., 2010), regulation of egg production in secondary queens (Yamamoto and Matsuura, 2011), promotion of lysozyme production in workers' salivary glands (Suehiro and Matsuura, 2015), and performance of antifungal activities against entomopathogenic and parasitic fungi (Matsuura and Matsunaga, 2015). Although 2-methyl-1-butanol is an optically active alcohol [(*R*)- and (*S*)-isomers], both enantiomers have the same pheromonal activities (Yamamoto et al., 2012). For the workers' egg-carrying and -piling behaviors, the egg recognition pheromones (lysozyme and β-glucosidase) and the volatile queen pheromone act synergistically to enhance these behaviors (Matsuura et al., 2010).

In addition, queen-specific volatiles have been discovered in other termite species (**Table 4**): 2-phenylethanol in *Nasutitermes takasagoensis* (Himuro et al., 2011); (*5Z*,*9S*)-tetradec-5-en-9-olide in *Silvestritermes minutus* (Machara et al., 2018); and (*3R*,*6E*)-nerolidol in *Embiratermes neotenicus*, *S. heyeri*, *Labiotermes labralis*, and *Cyrilliotermes angulariceps* (Havlíèková et al., 2019). Furthermore, a non-volatile (contact) queen pheromone is suspected in *Cryptotermes secundus*; this pheromone consists of cuticular hydrocarbons (CHCs) including 4-methylnonacosane, 3-methylnonacosane, hentriacontene, dotriacontene, tritriacontene, and pentatriacontadiene [the positions of double bond(s) are unknown in all of these unsaturated hydrocarbons], and another thirteen alkanes/alkenes (Hoffmann et al., 2014). However, the pheromonal activities of these compounds have not yet been demonstrated (**Table 4**).

ROYAL RECOGNITION PHEROMONES

It has long been hypothesized that when termite nestmates recognize royal castes (i.e., kings and queens), they contact the body surfaces of royals and then perceive royal-specific chemicals (i.e., royal recognition pheromones) (Korb, 2018; Hefetz, 2019). Royal recognition pheromones are predicted to be non-volatile, because volatile compounds easily saturate the colony space, and thus hamper the nestmates' perception due to sensory habituation (Korb, 2018; Hefetz, 2019). Indeed, neotenic kings and queens of *Z. nevadensis* have some royal-specific CHCs, including 6,9-nonacosadiene and three long-chain hydrocarbons (Liebig et al., 2009); similarly, neotenic kings and queens of *Kalotermes flavicollis*, *Prorhinotermes simplex*, and *R. santonensis* have royal-specific proteins on their body surfaces (Hanus et al., 2010), although the pheromone activities of these compounds have not been demonstrated (**Table 4**).

A recent study revealed that one royal-specific CHC, heneicosane, functions as a royal-recognition pheromone under the presence of workers' cuticular extract in *R. flavipes* (Funaro et al., 2018; **Table 4**). The pheromone elicits strong royal recognition behaviors (antennation and shaking of the body) in both workers and soldiers that come into contact with it (Funaro et al., 2018). Even when the pheromone is combined with foreign workers' CHCs, the royal recognition behaviors are elicited both in workers and soldiers (Funaro et al., 2019). However, these studies analyzed the CHC profiles of the neotenic kings and queens but not of the primary ones, i.e., adult reproductives. It is therefore still unknown whether the "royal-specific" CHC would be common between primary and neotenic reproductives. Additionally, the relative proportion of each component of CHCs in termite kings and queens can change with aging (Gordon et al.,

TABLE 4 | List of pheromones involving in reproduction.

Pheromone type	Termite family and species	Emitting caste	Type of evidence	Component	Function	References
Egg recognition	**Rhinotermitidae**					
pheromone	*Reticulitermes speratus*	Egg, Worker	BS	Lysozyme, β-Glucosidase	Eliciting worker's egg piling behavior.	Matsuura et al., 2007, 2009
Volatile queen	**Rhinotermitidae**					
pheromone	*Reticulitermes speratus*	SQ Egg	BS	Butyl butyrate 2-methyl-1-butanol	Inhibiting female workers' differentiation into neotenic queens. Promoting worker's egg piling behavior.	Matsuura et al., 2010
					Inhibiting egg production of neotenic queens	Yamamoto and Matsuura, 2011
					Promoting lysozyme production in worker's salivary gland	Suehiro and Matsuura, 2015
					Antifungal activity against entomopathogenic fungi, termite balls, and its related fungi	Matsuura and Matsunaga, 2015
	Termitidae					
	Nasutitermes takasagoensis	PQ	E	2-Phenylethanol	Unproved	Himuro et al., 2011
	Silvestritermes minutus	PQ	E	(5*Z*,9*S*)-tetradec-5-en-9-olide	Unproved	Machara et al., 2018
	Embiratermes neotenicus	SQ	E	(3*R*, 6*E*)-Nerolidol	Unproved	Havlíèková et al., 2019
	Silvestritermes heyeri	SQ	E	(3*R*, 6*E*)-Nerolidol	Unproved	Havlíèková et al., 2019
	Labiotermes labralis	SQ	E	(3*R*, 6*E*)-Nerolidol	Unproved	Havlíèková et al., 2019
	Cyrilliotermes angulariceps	SQ	E	(3*R*, 6*E*)-Nerolidol	Unproved	Havlíèková et al., 2019
Contact queen	**Kalotermitidae**					
pheromone	*Cryptotermes secundus*	SQ	E	4-Methylnonacosane, 3-Methylnonacosane, Hentriacontene, Dotriacontene, Tritriacontene, Pentatriacontadiene, Other thirteen alkanes/alkenes	Unproved	Hoffmann et al., 2014
Royal recognition	**Archotermopsidae**					
pheromone	*Zootermopsis nevadensis*	SK, SQ	E	6,9-Non-acosadiene, Three long-chain hydrocarbons	Unproved	Liebig et al., 2009
	Kalotermes flavicollis	SK, SQ	E	Unidentified proteins	Unproved	Hanus et al., 2010
	Rhinotermitidae					
	Prorhinotermes simpex	SK, SQ	E	Unidentified proteins	Unproved	Hanus et al., 2010
	Reticulitermes santonensis	SK, SQ	E	Unidentified proteins	Unproved	Hanus et al., 2010
	Reticulitermes flavipes	SK, SQ	BS	Heneicosane, Common CHCs	Eliciting antennation and shaking behavior of workers and soldiers	Funaro et al., 2018

Emitting caste, PQ, Primary queen; SK, Secondary king; SQ, Secondary queen. Evidence, E, Estimation by chemical analyses; BS, Bioassay with synthesized compound.

2020). Further study will be needed to elucidate how termites share the information of the royal CHC profiles among nestmates for a long period of time.

INTERSPECIFIC INTERACTION VIA TERMITE PHEROMONES

Termite chemical communications are often eavesdropped or mimicked by termitophagous predators and inquilines. For example, the termite-raiding ant *Odontoponera transversa* eavesdrops the trail-following pheromones of fungus-growing termites (*Odontoponera yunnanensis*, *Macrotermes yunnanensis*, and *Ancistrotermes dimorphus*) (Wen et al., 2017). The termitophilous rove beetles (Staphylinidae) mimic CHC profiles of the host termite species: *Trichopsenius frosti* mimics the CHC profile of *R. flavipes* (Howard et al., 1980); *Trichopsenius depressus*, *Xenistusa hexagonalis*, and *Philotermes howardi* mimic that of *R. virginicus* (Howard et al., 1982); *Corotoca melantho* mimics that of *C. cyphergaster* (Rosa et al., 2018). The termite *Inquilinitermes microcerus*, which is an obligatory inquiline of *C. cyphergaster*, does not have its own trail pheromone, but this inquiline termite follows the trail pheromone of its host (Cristaldo et al., 2014). Also, *C. cyphergaster* responds to only its own alarm signal, while *I. microcerus* responds both to its own alarm signal and to an alarm signal from its host (Cristaldo et al., 2016c).

Inter-colonical chemical interactions can be affected by food resource availability and previously exposed odor. In *Nasutitermes* aff. *coxipoenens*, although the workers from colonies under low or high resource availability do not discriminate between foreign trails leading into rich and poor food resources, the workers from colonies under intermediate resource availability discriminate between foreign trails leading into rich and poor resources (Cristaldo et al., 2016a). Moreover, the individuals of *N.* aff. *coxipoensis* are attracted to allocolonial odor cues to which they were previously exposed (Ferreira et al., 2018).

The termite egg-mimicking fungus "termite ball" represents one of the most remarkable cases of fungal inquiline. Because termite nests are well sanitized by secretion of antimicrobial agents (Chen et al., 1998; Rosengaus et al., 2000, 2004; Zhao et al., 2004; Matsuura et al., 2007), it is difficult for microbes to intrude into the nest. However, the termite balls succeed in intruding termite nests by mimicking termite eggs; termite workers take care of the termite balls in a manner identical to that of eggs to prevent them from desiccation and pathogen infection (Matsuura et al., 2000). The termite balls are the sclerotia of an athelioid fungus of the genus *Fibularhizoctonia* (Matsuura et al., 2000), which morphologically mimics the eggs of *Reticulitermes* termites by matching the diameter and the smooth surface texture of the eggs (Matsuura et al., 2000; Matsuura, 2006; Yashiro and Matsuura, 2007; Ye et al., 2019). Furthermore, the termite ball chemically mimics the eggs by expressing β-glucosidase, a component of the termite egg recognition pheromone (Matsuura et al., 2009). The termite balls grow on the termite nest wood and obtain nutrition and energy by digesting cellulose contained in the wood. Accordingly, it has been speculated that the termite balls originally had the potential to produce the same substance as the termite egg recognition pheromone component; this facilitated the evolution of termite egg mimicry (Matsuura et al., 2009). Furthermore, the soldier pheromone of *R. speratus*, which has fungistatic activities against entomopathogenic fungi, is unable to inhibit the mycelial growth of termite balls (Mitaka et al., 2017b), suggesting that termite balls newly acquired resistance to termite fungistatic compounds (Mitaka et al., 2019). Thus far, the volatile queen pheromone of *R. speratus* is the only known antifungal agent that inhibits the germination and growth of termite balls (Matsuura and Matsunaga, 2015). However, the inhibitory effect of the queen pheromone differs among termite ball strains, suggesting that some termite ball strains may develop resistance even to the queen pheromone (Matsuura and Matsunaga, 2015).

EVOLUTION OF TERMITE PHEROMONES

Many termite species live inside predator- and microbe-rich habitats, such as rotten wood and soil; thus, they develop a wide variety of defensive substances including CHCs, terpenes, and antimicrobial molecules (Howard and Blomquist, 2005; Stow and Beattie, 2008; Šobotník et al., 2010; Rosengaus et al., 2011). Recent studies outlined in the above sections suggested that termites parsimoniously use these substances for pheromones involved in caste recognition and caste-specific roles.

For example, CHCs are presumed to originally have been used by insects to withstand desiccation and pathogen invasion (Blomquist and Bagnères, 2010; Menzel et al., 2017). Most insects have diversified their CHC compositions, such that the CHCs are species-specific; these CHCs often serve as species recognition cues for mating (Howard and Blomquist, 1982). In eusocial insects including termites, the compositional ratios of CHCs significantly differ between reproductive and non-reproductive castes in each colony; queens (and kings in termites) develop royal-specific CHC profiles (Van Oystaeyen et al., 2014; Hefetz, 2019). The termite kings and queens in each colony begin to produce *de novo* CHCs (or to increase production of a certain existing CHC component, compared to non-reproductive castes), accompanied by enhancement of juvenile hormone titer followed by sexual maturation (Brent et al., 2016). Ultimately, the royal-specific compounds are utilized as royal recognition pheromones (Le Conte et al., 2008; Leonhardt et al., 2016).

In parallel with the above CHC divergences, soldiers in Rhinotermitidae and Termitidae species developed the production ability of a variety of terpenes in the frontal glands of their heads, and they use the terpenes for defensive compounds such as repellents, poisons, antimicrobials, and sticky substances for immobilizing predators (Šobotník et al., 2010). However, some termite species use terpenes not only

for such defensive substances but also for pheromones, which are associated with nestmate recruitment for colony defense and soldier differentiation (Vrkoc et al., 1978; Valterová et al., 1988; Roisin et al., 1990; Šobotník et al., 2008, 2010; Dolejšová et al., 2014; Cristaldo et al., 2016b; Mitaka et al., 2017b).

Other antimicrobial molecules are also used for chemical communication in termite societies. Workers and eggs produce the antibacterial enzyme for egg recognition pheromone (Matsuura et al., 2007), while queens use the queen-specific antifungal volatiles for the pheromone indicating the queen fertility, resulting in acquiring multifaceted roles associated with promoting egg production and survivorship, and regulating queen differentiation (Matsuura, 2012; Matsuura and Matsunaga, 2015).

Even when the same set of compounds is used, the pheromone function can change according to the emitter, and the dose. For example, in some termites, the same set of compounds is used both for sex-pairing pheromones secreted from alates and for trail-following pheromones secreted from workers (**Table 2**). Also, different concentrations of an alarm pheromone induce different alarm behaviors of nestmates (Cristaldo et al., 2016b).

These facts strongly suggest that multifaceted usage of the same set of compounds could have been the driving force behind sophistication of termite pheromone communication. Because the capacity of *de novo* biosynthesis of chemical compounds is limited and costly, evolutional pressures have led to the reuse of existing compounds for chemical communication. This phenomenon is called semiochemical parsimony (Blum, 1996), which also occurs in many other insects (Blum and Brand, 1972; Blum, 1996; Ruther et al., 2001; Allison et al., 2004; Nojima et al., 2005; de Bruijn et al., 2006; Le Conte et al., 2008; Geiselhardt et al., 2009; Chung and Carroll, 2015; Takata et al., 2019). Therefore, it has been considered that semiochemical parsimony may account for the birth of pheromones and the multifunctionalization in social insects (Blum and Brand, 1972; Matsuura, 2012). Moreover, pheromones can be developed by adding new compound(s) to pre-existing semiochemicals, according to the social context. In *R. speratus*, two major components of the workers' CHCs are also used as the aggregation pheromone, in combination with another four compounds. This usage suggests that workers inform nestmates of both the presence of other nestmates and locations suitable for foraging/nesting (Mitaka et al., 2020). It is hypothesized that both the parsimonious usage of the same compound(s) and the addition of supplementary compounds to a pre-existing semiochemical depending on the social context enable termites to process considerable quantities of context-dependent information with a small number of chemicals, thus forming a coordinated and reasonable chemical communication system. However, the rationalization of chemical communications does not always induce the increase of types of pheromones in a termite society, because the obligatory inquiline termite *I. microcerus* takes advantage of the host's trail and alarm pheromones instead of using its own pheromones (Cristaldo et al., 2014, 2016c). Such a usage of allelochemicals provides an extended pheromone communication to inquiline species as another level of semiochemical parsimony. Evolution of pheromone communication system may be largely affected by social structure, lifestyle, and habitat environment in termites.

CONCLUSION AND PROSPECTS

Ongoing development of methods and devices for chemical analyses has facilitated increased pheromone identification in recent years (Wyatt, 2014). Since the identification of the first termite pheromone [i.e., the trail pheromone of *R. virginicus* in 1968 (Matsumura et al., 1968)], many pheromone compounds have been identified in various termite species. In particular, the number of termite pheromone studies has been rapidly increasing since 2000 (∼84% of termite pheromone papers were published from 2000 to 2020) (**Tables 1–4**). Notably, trail-following pheromones are the most popular in termite pheromone studies and have been identified in 67 species; however, recent studies have made *R. speratus* the most well-studied species with respect to pheromones, such that five types of pheromones have been identified (as of July 2020) including the trail pheromone (Yamaoka et al., 1987; Tokoro et al., 1990), aggregation pheromone (Mitaka et al., 2020), soldier pheromone (Mitaka et al., 2017b), egg recognition pheromone (Matsuura et al., 2007, 2009), and volatile queen pheromone (Matsuura et al., 2010). Some pheromones gained multifunctional roles by parsimoniously using the same set of compounds for multiple purposes or by adding new compounds to pre-existing semiochemicals depending on the situation; these multifunctional pheromones can enable more informative communication in social insects. However, there remain predicted but unidentified pheromones, such as a king pheromone that regulates caste differentiation (Wilson, 1965) and a cement pheromone that evokes nest-building behaviors (Bonabeau et al., 1998; Mizumoto et al., 2015; Green et al., 2017). In addition, minimal research has been performed regarding the biosynthesis (Prestwich et al., 1981; Hojo et al., 2007, 2011; Blomquist, 2010; Šobotník et al., 2010; Bordereau and Pasteels, 2011; Beran et al., 2019) and molecular-level chemoreceptive mechanisms (Poulsen et al., 2014; Terrapon et al., 2014; Mitaka et al., 2016; Harrison et al., 2018) in termite pheromones. Future interdisciplinary research—including chemical ecology, genetics, physiology, and biochemistry—will provide important insights into the molecular and evolutionary mechanisms of the development of intracolonial, intercolonial, and interspecies chemical communications in termite societies.

AUTHOR CONTRIBUTIONS

YM conducted literature retrieval. YM and TA wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Regulatory Mechanisms Underlying the Differentiation of Neotenic Reproductives in Termites: Partial Release From Arrested Development

Kohei Oguchi[1,2]*, *Kiyoto Maekawa*[3] *and Toru Miura*[1]

[1] *Misaki Marine Biological Station, School of Science, The University of Tokyo, Miura, Japan,* [2] *National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan,* [3] *Faculty of Science, Academic Assembly, University of Toyama, Gofuku, Japan*

Eusocial insects exhibit reproductive division of labor, in which only a part of colony members differentiates into reproductives. In termite colonies, the division of labors is performed among multiple types of individuals (i.e., castes), such as reproductives, workers, and soldiers to organize their society. Caste differentiation occurs according to extrinsic factors, such as social interactions, leading to developmental modifications during postembryonic development, and consequently, the caste ratio in a colony is appropriately coordinated. In particular, when the current reproductives die or become senescent, some immature individuals molt into supplementary reproductives, also known as "neotenics," that take over the reproductive task in their natal colony. Neotenics exhibit variety of larval features, such as winglessness, and thus, immature individuals are suggested to differentiate by a partial release from arrested development, particularly in the reproductive organs. These neotenic features, which have long been assumed to develop via heterochronic regulation, provide us opportunities to understand the developmental mechanisms and evolutionary origin of the novel caste. This article overviews the accumulated data on the physiological and developmental mechanisms that regulate the neotenic differentiation in termites. Furthermore, the evolutionary trajectories leading to neotenic differentiation are discussed, namely the acquisition of a regulatory mechanism that enable the partial release from a developmentally arrested state.

Keywords: termite, caste differentiation, neotenic, modularity, heterochrony, developmental arrest

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***Correspondence:**
Kohei Oguchi
k-oguchi@aist.go.jp;
k.ohgreen226@gmail.com

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INTRODUCTION: CASTE DIFFERENTIATION IN TERMITES

The acquisition of the caste system in social insects is a critical step in eusocial evolution, in which colony tasks are allocated to different types of individuals with caste-specific morphological features (Lin and Michener, 1972). Regulatory mechanisms underlying the development of caste-specific characteristics should be deeply associated with eusociality (Miura, 2005). In particular, the reproductive division of labor between the reproductive and sterile castes is one of the most distinctive features in eusocial insects (Wilson, 1971, 1975). Among eusocial insects, termites, which are distantly related to hymenopterans (Noirot, 1969; Korb, 2015), exhibit highly sophisticated and

complex societies. It is believed that the termite sociality first emerged in the Early Cretaceous, approximately 50 million years before the appearance of eusocial hymenopterans (Grimaldi and Engel, 2005; Roisin and Korb, 2011; Engel, 2015). Although regulatory mechanisms that control the differentiation of reproductive and sterile castes are thought to differ between hymenopterans and termites, most of the involved mechanisms are unclear, particularly in termites.

Generally, in hemimetabolous insects (e.g., cockroaches, locusts, etc.), adult-specific characteristics, such as compound eyes, wings, and reproductive organs, gradually develop during postembryonic development via moltings, and the dramatic morphological modifications occur at the final molt, i.e., imaginal molt (Anderson, 1972). In termites, caste-specific morphological characteristics are created through moltings (Noirot, 1969; Roisin, 2000), in which the developmental patterns differ depending on the caste fate (Miura, 2005; Korb and Hartfelder, 2008). Therefore, the regulatory mechanisms of molting and metamorphosis contribute to caste-specific development. Concerning the differentiation into alates, i.e., winged reproductives that form new colonies, imaginal characteristics, such as compound eyes, wings, and gonads, dramatically develop (Nutting, 1969; Weesner, 1969; Nii et al., 2019). Thus, the differentiation into alates is considered homologous to the imaginal development in other hemimetabolous insects (Nalepa and Bandi, 2000). In particular, termite colonies include numerous sexually immature individuals engaged in various tasks such as nursing and foraging (Thorne, 1996; Korb and Hartfelder, 2008). In these individuals, the development of imaginal organs like compound eyes, wings, and gonads is restricted or arrested during postembryonic development (Nalepa and Bandi, 2000; Bourguignon et al., 2016).

As caste differentiation fates are determined during postembryonic development in termites, the patterns of caste differentiation are often depicted as differentiation pathways. Among termite families, two major patterns of caste differentiation pathways are recognized, i.e., linear and bifurcated pathways (**Figure 1**; Noirot, 1969; Roisin, 2000; Bourguignon et al., 2014). Bifurcated pathways have been described in Mastotermitidae, Hodotermitidae, Termitidae, and some species of Rhinotermitidae, in which the differentiation point for alates and apterous individuals occurs at a relatively early stage of postembryonic development, i.e., first or second larval instars (Roisin, 2000). Workers in the bifurcated pathways cannot develop into alates, and thus, they are called "true workers," although they still have the potential to develop into reproductives (Korb and Hartfelder, 2008; Bourguignon et al., 2016). In contrast, in the linear pathways observed in Archotermopsidae, Stolotermitidae, Kalotermitidae, and Serritermitidae, in addition to some species of Rhinotermitidae, elder larval individuals serve as "workers" while possessing the potential to differentiate into alates, and thus, they are called pseudergates, meaning "false workers" (Grassé and Noirot, 1947; Korb and Hartfelder, 2008).

In addition to these developmental stages, other derived pathways, i.e., soldier and neotenic differentiation pathways,

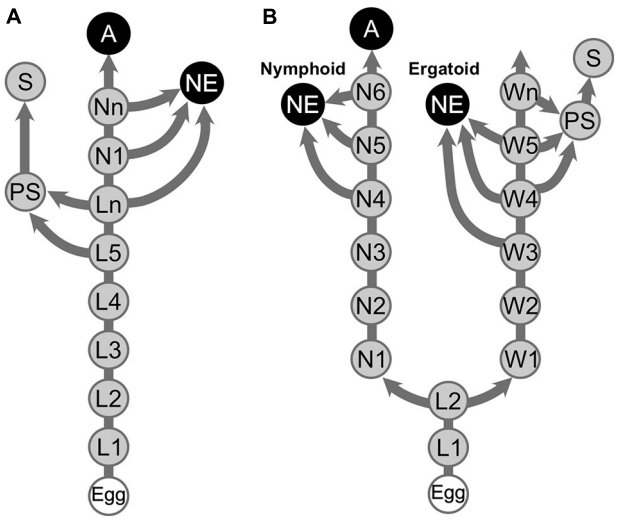

FIGURE 1 | Representative caste developmental patterns in termites. **(A)** Linear pathway. **(B)** Bifurcated pathway. Arrows indicate hatching and molts. A, Alate; L, Larva; N, Nymph; NE, Neotenic reproductive; PS, Presoldier; S, Soldier; W, Worker. Elder larvae (Ln) in the linear pathway are referred to as "pseudergates" in this article.

have also been investigated by many researchers. Both soldier and neotenic castes are novel developmental stages only present in termites, and these castes represent synapomorphies of the termite lineage, as they are present in all termite species with few exceptions (Noirot, 1969; Korb, 2015). Soldiers differentiate from workers or pseudergates through two molting events via a presoldier stage (Noirot, 1969; Roisin, 2000; Korb and Hartfelder, 2008). Because soldier differentiation can be artificially induced by the application of juvenile hormone (JH), several studies have described soldier developmental process in different species (Hrdý and Křeček, 1972; Howard and Haverty, 1979; Scharf et al., 2003). During soldier differentiation, the body plan of termites, particularly the anterior parts that are used for defensive behaviors, are largely modified through a specific morphogenesis (Miura and Matsumoto, 2000; Koshikawa et al., 2003; Watanabe and Maekawa, 2008; Toga et al., 2013). The morphogenetic process occurs downstream of hormonal and morphogenic factors, which are well documented (Cornette et al., 2008; Sugime et al., 2019; Miura and Maekawa, 2020). However, until recently, because methods for inducing neotenic differentiation had not been established, studies on neotenic differentiation are relatively sparse compared with those of soldier differentiation (Shimoji et al., 2017; Oguchi et al., 2020).

Neotenic reproductives are seen in nearly all the termite families, although they are occasionally lost in some genera (Myles, 1999; Bourguignon et al., 2016). Neotenics, also known as secondary reproductives, can also be referred to as replacement or supplementary reproductives, depending on situations of occurrence (Korb and Hartfelder, 2008). At the time when primary reproductives die or become senescent, they differentiate from immature individuals and

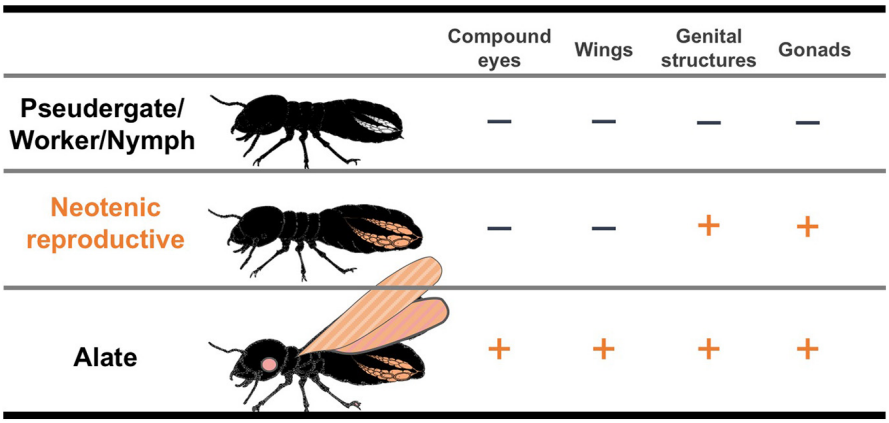

FIGURE 2 | Schematic illustrations of the developmental degree of imaginal organs in each caste. "+" indicates well-developed imaginal organs like alates. "–" indicates imaginal organs with suppressed or interrupted developments compared with those of alates.

exclusively engage in reproduction in their natal nests (**Figure 1**; Lüscher, 1961; Thorne, 1996; Korb and Hartfelder, 2008). Some species produce neotenics not only as replacements but also as backup against the loss of primary reproductives (Matsuura et al., 2009; Hellemans et al., 2019). Upon the differentiation of neotenic reproductives, adult-specific characteristics related to reproductive organs develop, whereas other imaginal features, such as wings, do not (**Figure 2**). Thus, it is suggested that the neotenic-specific morphology is accomplished by a heterochronic regulation, in which the development is accelerated or arrested depending on body modules (Nalepa and Bandi, 2000).

The differentiation of neotenic reproductives is controlled by genetic, maternal, and environmental factors. In *Reticulitermes speratus* and several other species, for instance, genetic, and maternal factors influenced by sex-linked genetic mechanisms, i.e., the asexual queen succession (AQS) system, are responsible for the neotenic differentiation (Hayashi et al., 2007; Matsuura et al., 2009; Matsuura et al., 2018; Hellemans et al., 2019), although environmental factors during the postembryonic development also affect the caste fate determination (Hayashi et al., 2007, 2013). In contrast, environmental factors during postembryonic development, such as social interactions between reproductives and non-reproductive castes, are also determinant force for the neotenic differentiation (Lüscher, 1974; Shimoji et al., 2017; Sun et al., 2017; Masuoka et al., 2021). Recently, neotenic differentiation was studied by applying induction methods in species with bifurcated and linear caste differentiation pathways (Saiki and Maekawa, 2011; Shimoji et al., 2017), focusing on morphogenetic processes during differentiation and the underlying physiological regulation (Saiki et al., 2015; Oguchi and Miura, 2019; Oguchi et al., 2020). By reviewing accumulated knowledge on the physiological and developmental regulation of neotenic differentiation in termites, the evolutionary processes toward the acquisition of neotenic reproductives are hypothesized.

MODULARITY: REPRODUCTIVE ORGAN-SPECIFIC MORPHOGENESIS

Neotenic reproductives in termites exhibit the juvenile characteristics of hemimetabolous insects, and thus, they are termed "neotenic" (Thorne, 1996). Meanwhile, they lack imaginal characteristics such as well-developed wings and compound eyes (Maekawa et al., 2008; Saiki and Maekawa, 2011). Neotenics can be roughly classified into two types: "nymphoids" that differentiate from nymphs and "ergatoids" that differentiate from workers (Korb and Hartfelder, 2008). Depending on the developmental stages from which neotenics derive, the developmental degree of imaginal characters differs (e.g., nymphoids have wing buds and slightly developed eyes, whereas ergatoids lack these features). However, in all cases, neotenics possess well-developed gonads that are comparable to those of primary reproductives (Thorne, 1996; Korb and Hartfelder, 2008; Saiki and Maekawa, 2011; Oguchi et al., 2016). This suggests that gonadal development is accelerated in neotenics in contrast to other body parts. This acceleration might be a case of "paedomorphosis," which is a type of heterochrony that involves a delay of specific organ development (Reilly et al., 1997; Nalepa and Bandi, 2000). The adaptive significance of neotenic differentiation, in which only some imaginal characters develop, might be that it permits individuals to immediately become reproductives through fewer moltings. In most cases, one molting event is required for neotenic differentiation from the previous undifferentiated stages, such as pseudergates, workers, or nymphs (Korb and Hartfelder, 2008), except in some species with bifurcated caste differentiation pathways of *Reticulitermes labralis, Nasutitermes corniger*, and *Nasutitermes aquilinus*, in which the pre-neotenic stages and two molts are required (Thorne and Noirot, 1982; Su et al., 2017; da Silva et al., 2019). The fact that the neotenic differentiation requires moltings suggests that morphological alterations are required for reproductive activities such as copulation and oviposition. In the case of neotenic molt (only female individuals) in a dampwood

termite *Hodotermopsis sjostedti*, which possesses a linear caste differentiation pathway, the loss of styli at the abdominal tip and expansion of the seventh sternites are observed (Oguchi and Miura, 2019). Therefore, the differentiation of neotenic reproductives may be accomplished by a partial release of immature individuals from arrested development, particularly in the reproductive organs. In other words, the pre-existing imaginal developmental processes (i.e., a late differentiation processes) are partially involved in neotenic differentiation; therefore, neotenic differentiation may be regarded as a case of "modular heterochrony." Thus, the neotenic reproductive is a distinctive phenotype (caste) that morphologically differs from primary reproductives (alates) (**Figure 2**). These mosaic-like phenotypes were not only observed in termites but also in eusocial hymenopteran species, particularly in ants (Wheeler and Nijhout, 1981; Abouheif and Wray, 2002; Miyazaki et al., 2010; Yang and Abouheif, 2011). Of note, "ergatoid queen" in ants, which has both the queen and worker traits, was believed to have emerged from the combination of the caste developmental systems (Miyazaki et al., 2010; Yang and Abouheif, 2011; Molet et al., 2012). Therefore, modular and heterochronic regulation by tinkering with the pre-existing developmental systems are thought to be important and ubiquitous evolutionary mechanisms for the acquisition of novel castes (cf. Cohen, 1999).

HETEROCHRONIC SHIFTS VIA PHYSIOLOGICAL CONTROL

Generally in insects, molting and metamorphosis are coordinated by JH and molting hormone (i.e., ecdysone) (Belles and Santos, 2014). It is well known that the imaginal molt is induced by the lowering of JH titers in an insect (Romaña et al., 1995; Treiblmayr et al., 2006). A similar physiological pattern is also present during alate differentiation in termites (**Figure 3A**; Nijhout and Wheeler, 1982; Cornette et al., 2008). However, detailed physiological analyses, especially on JH dynamics, have yet to be conducted concerning the differentiation into neotenic reproductives, although previous studies predicted that the shift from low to high JH titers during the intermolt period would induce neotenic differentiation (reviewed in Nijhout and Wheeler, 1982; Korb, 2015). Some studies actually reported an extraordinary high JH titer in the female neotenics (e.g., Elliott and Stay, 2007; Maekawa et al., 2010; Korb et al., 2012; Saiki et al., 2015). Similar tendencies of high JH titers were observed after female alate differentiation (Cornette et al., 2008; Maekawa et al., 2010). The high JH titers in female reproductives probably contribute to the development of female reproductive systems, including ovaries (Korb, 2015; Saiki et al., 2015). Furthermore, in many insects including termites, JH is sequestered in fat bodies and engaged in yolk protein synthesis (Nijhout, 1994), suggesting that this function of JH is also driven at the time of neotenic differentiation. However, the JH function in the ovarian development is only applicable to the differentiation of female neotenics, and thus, the JH action leading to the differentiation of male neotenics should also be elucidated in future studies.

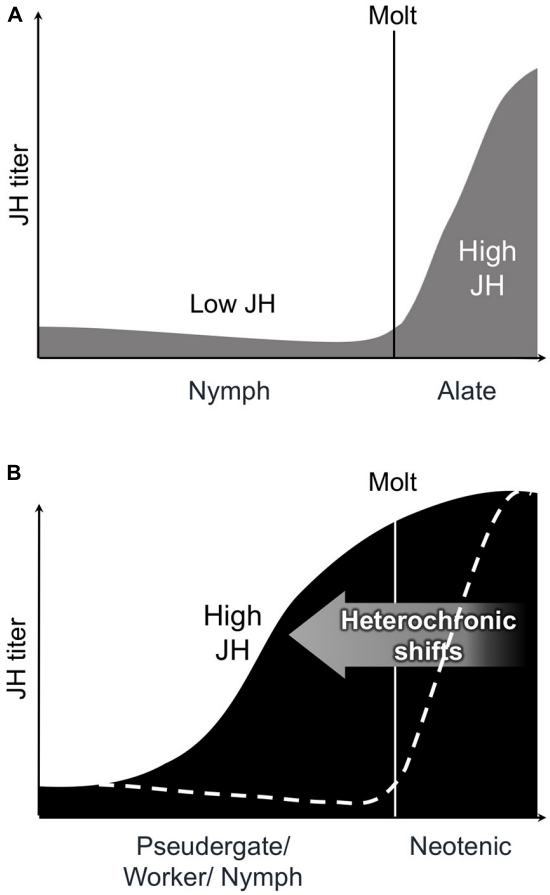

FIGURE 3 | Schematic illustrations of models on the transitions of juvenile hormone (JH) titer (vertical axis) during alate or neotenic differentiation. **(A)** Consistently-lowered JH titer lead alate molt from nymphal instar. After alate molt, the JH titer rise in female alates. Figures were modified as per Cornette et al. (2008, Figure 7). **(B)** JH titer ransition from low to high leads the neotenic differentiation. Dotted line indicates the JH titer during alate molt.

Recently, the physiological regulation at the time of neotenic differentiation was unraveled in the dampwood termite *H. sjostedti* (Oguchi et al., 2020). This research applied an induction method for neotenic differentiation in which the sex caste ratio was artificially manipulated (Shimoji et al., 2017). Under these experimental conditions, the existence of only a male (or female) neotenic reproductive promotes the differentiation of female (or male) neotenics from pseudergates. Their findings illustrated that the JH titer of female pseudergates in the presence of a male neotenic without a female neotenic continuously lowered and then increased immediately before the molt (Oguchi et al., 2020). Thus, this study supports the hypothesis by Nijhout and Wheeler (1982), at least in terms of the female neotenic differentiation, considering that neotenic differentiation requires a transition of JH titers from low to high (**Figure 3B**). The similar tendencies and models have been shown in other species, such as *R. speratus* (Saiki et al., 2015) and *Cryptotermes secundus* (Korb et al., 2012), suggesting that this model can be applicable to other

termite lineages, at least lower termite species. Moreover, the JH analog application of female pseudergates inhibits the reproductive organ development and neotenic molt (Oguchi et al., 2020). This role of JH is similar to the antimetamorphic action of insects and lowering JH titer, which are required for the imaginal molts (Nijhout, 1994). Therefore, the fact that female neotenic differentiation requires low JH titers suggests that the neotenic molt can be regarded as a type of imaginal molt and rapid change of JH titers might be heterochronic shifts (**Figures 3A,B**). In other words, the neotenic reproductive can be regarded as a "physiological imago." Recently, the master regulatory pathway controlling molting and metamorphosis, i.e., the MEKRE93 or Met-Krh1-E93 pathway, was demonstrated to work downstream of JH and ecdysone in insects (Belles and Santos, 2014; Ureña et al., 2014), suggesting that this pathway is also involved in caste regulation in termites (Korb and Belles, 2017; Miura and Maekawa, 2020).

EVOLUTIONARY IMPLICATIONS

One of the most important steps in the evolution of eusociality is the acquisition of reproductive labor division (Wilson, 1971; Lin and Michener, 1972). In termites, especially in basal species with linear caste pathways, immature individuals, that are homologous to "nymphs" in general hemimetabolous insects, engage in colony tasks without participating in reproduction (Korb and Hartfelder, 2008). These immature individuals undergo successive moltings, but they do not completely develop adult-specific characteristics such as compound eyes and wings (Miura et al., 2004; Katoh et al., 2007; Nii et al., 2019). Considering the evolutionary process of termite eusociality, the appearance of immature castes that are engaged in colony tasks (i.e., pseudergates) would be an important step (cf. Nalepa and Bandi, 2000). Before the emergence of such immature castes, bifurcation (i.e., develop or less develop) of imaginal organ development within the colony members is suggested to lead the reproductive division of labor (Bourguignon et al., 2016). Therefore, during the eusocial evolution in termites, it is suggested that the postembryonic development of hemimetabolous insects is partially (i.e., body parts specifically) or completely arrested during the linear adult developmental process (Bourguignon et al., 2016).

Thus, what mechanisms enable neotenic wingless reproductive differentiation? Some studies provided circumstantial evidences that the development of adult-specific characteristics (e.g., reproductive organs, wings, compound eyes, sensillae) is connected and induced under the control of physiological signals (i.e., hormonal signals), leading to alate differentiation (e.g., Miura and Matsumoto, 1996). Therefore, during neotenic differentiation, such alate-specific characteristics could also be expressed. In fact, neotenic reproductives display dark coloration of the cuticles caused by pigmentation and sclerotization during differentiation that resembles alate coloration (e.g., Watson and Abbey, 1985; Hu and Forschler, 2012). Moreover, it was reported that ergatoids possessed wing bud-like structures on their thoraces and slightly developed compound eyes on their heads (e.g., Noirot and Thorne, 1988; Miura and Matsumoto, 1996; da Silva et al., 2019). This phenomenon suggests that the development of these adult-specific characteristics is interlinked, permitting their expression simultaneously with the gonadal development required for the differentiation of functional ergatoids. These links among adult-specific characteristics are assumed to arise from the linkage of gene regulatory networks.

Recently, transcriptomic analysis suggested that Ras-MAPK pathway genes involved in ergatoid differentiation in *Reticulitermes labralis* (Ye et al., 2019). In general, the Ras-MAPK pathway is known to control the signal transduction from plasma membrane to nucleus, regulating downstream genes involved in cell proliferation, differentiation, and cell death (Foster and Malek, 2016). Therefore, specific regulations in such cellular processes may be crucial for ergatoid differentiation. It is unclear whether these gene expression changes also occur during nymphoid differentiation; however, the differences of gene expression patterns between ergatoids and nymphoids may help to understand the genetic networks that regulate body part-specific development.

As mentioned previously, there are several morphological and functional differences between alates and neotenics, and thus, there should be different physiological regulatory mechanisms underlying the differentiation processes leading to the two reproductive types. In both alate and neotenic differentiation, JH and ecdysone pathways might be involved in each molt, although the timing of JH elevation differs between them. Therefore, these gaps might explain the partial differences between alates and neotenics. During neotenic differentiation, only some body parts and organs that are related to reproduction undergo further developmental processes that also occur during alate differentiation (**Figure 2**). In soldier differentiation in termites, such body part-specific morphogenesis, i.e., mandibular enlargement (Koshikawa et al., 2003; Watanabe and Maekawa, 2008; Watanabe et al., 2014; Miura and Maekawa, 2020), is organized by patterning genes providing spatial information, such as Hox genes, which are upregulated under the control of hormonal factors (e.g., Toga et al., 2013; Sugime et al., 2019). Considering these mechanisms, neotenic differentiation should also require similar regulatory mechanisms of gene expression including toolkit genes such as Hox genes and hormonal factors. Obviously, because anterior body parts are dramatically modified during soldier differentiation whereas posterior body parts such as reproductive traits develop during neotenic differentiation, different Hox genes should be responsible for the body-part development in each caste. During the evolutionary processes of the termite lineage, it is suggested that such regulatory mechanisms leading to body part-specific differentiation processes were acquired in the common ancestor, resulting in the appearances of novel castes, such as soldiers and neotenic reproductives. Thus, comparative approaches between termites and sister-group cockroaches, the genus *Cryptocercus*, may uncover the underpinnings of social evolution in termites.

AUTHOR CONTRIBUTIONS

KO, KM, and TM wrote the manuscript. All authors read and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Daniel Aguilera-Olivares, University of Concepción, Chile

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Franck Dedeine, Université de Tours, France

***Correspondence:**
Gillian H. Gile
ggile@asu.edu

†These authors have contributed equally to this work and share first authorship

‡ORCIDs:
Alison Ravenscraft
orcid.org/0000-0003-4974-4804
Petr Stiblík
orcid.org/0000-0001-6141-5603
Jan Šobotník
orcid.org/0000-0002-8581-637X
David Sillam-Dussès
orcid.org/0000-0001-5027-8703
Tiago F. Carrijo
orcid.org/0000-0001-6308-7252
Gillian H. Gile
orcid.org/0000-0003-3955-0942

§Present address:
Francesca De Martini, Life Science Department, Mesa Community College, Mesa, AZ, United States

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Biogeography and Independent Diversification in the Protist Symbiont Community of *Heterotermes tenuis*

Francesca De Martini[1†§], Nicole L. Coots[1†], Daniel E. Jasso-Selles[1], Jordyn Shevat[1], Alison Ravenscraft[2‡], Petr Stiblík[3‡], Jan Šobotník[3,4‡], David Sillam-Dussès[5‡], Rudolf H. Scheffrahn[6], Tiago F. Carrijo[7‡] and Gillian H. Gile[1*‡]

[1] School of Life Sciences, Arizona State University, Tempe, AZ, United States, [2] Department of Biology, The University of Texas at Arlington, Arlington, TX, United States, [3] Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czechia, [4] Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Czechia, [5] Laboratory of Experimental and Comparative Ethology (LEEC), University of Sorbonne Paris Nord, Villetaneuse, France, [6] Fort Lauderdale Research and Education Center, University of Florida, Davie, FL, United States, [7] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, São Bernardo do Campo, Brazil

The eukaryotic microbiome of "lower" termites is highly stable and host-specific. This is due to the mutually obligate nature of the symbiosis and the direct inheritance of protists by proctodeal trophallaxis. However, vertical transmission is occasionally imperfect, resulting in daughter colonies that lack one or more of the expected protist species. This phenomenon could conceivably lead to regional differences in protist community composition within a host species. Here, we have characterized the protist symbiont community of *Heterotermes tenuis* (Hagen) (Blattodea: Rhinotermitidae) from samples spanning South and Central America. Using light microscopy, single cell isolation, and amplicon sequencing, we report eight species-level protist phylotypes belonging to four genera in the phylum Parabasalia. The diversity and distribution of each phylotype's 18S rRNA amplicon sequence variants (ASVs) mostly did not correlate with geographical or host genetic distances according to Mantel tests, consistent with the lack of correlation we observed between host genetic and geographical distances. However, the ASV distances of *Holomastigotoides* Ht3 were significantly correlated with geography while those of *Holomastigotoides* Ht1 were significantly correlated with host phylogeny. These results suggest mechanisms by which termite-associated protist species may diversify independently of each other and of their hosts, shedding light on the coevolutionary dynamics of this important symbiosis.

Keywords: coevolution, microbiome, termite, *Cononympha*, *Pseudotrichonympha*, *Cthulhu*, Rhinotermitidae

INTRODUCTION

The degree of intraspecific variation in the insect gut microbiota varies greatly among lineages. At one extreme, many insects, including butterflies and walking sticks, have such highly variable gut microbial communities that it has been argued they have no specific microbiome (Shelomi et al., 2013; Hammer et al., 2019; Ravenscraft et al., 2019). This high variability likely stems from molting,

which disrupts the gut microbiota, and from the lack of a mechanism for vertical transmission of microbes. At the other extreme, in social insects, intimate interactions among nestmates create a route by which the microbiome can be transmitted and stably maintained (Engel and Moran, 2013; Sanders et al., 2014). As a result, many social insects host a stable and consistent core microbiome at both the intra-colony and intra-species levels (e.g., corbiculate bees, Kwong et al., 2017).

In termites, the social behavior of proctodeal trophallaxis (anal feeding) provides a mechanism for both vertical inheritance of gut microbes and for re-establishment of the microbiota after each molt (Brune and Dietrich, 2015). This behavior emerged in the ancestor of termites and their sister lineage *Cryptocercus* and likely enabled the transition to wood feeding by trapping cellulolytic protists and their nitrogen-fixing endosymbionts in an increasingly specialized digestive symbiosis (Nalepa, 2015). As a result, termites exhibit relatively stable and largely co-diversifying gut microbial communities (Bourguignon et al., 2018).

The eukaryotic component of "lower" termite microbiomes (all families except Termitidae) consists of highly specialized wood-feeding flagellates (protists) that cannot live outside their host. This means that unlike prokaryotic microbiota, the protist communities do not include a transient, environmentally acquired component. Because of this strict vertical inheritance, protist communities are strongly influenced by termite phylogeny and are not expected to have a biogeographical influence independent of their hosts (Tai et al., 2015; Taerum et al., 2018). However, there are two documented evolutionary mechanisms consistent with direct termite-to-termite transmission that could conceivably introduce a biogeographical component to protist distribution within a single host species. These are (1) transmission of protist species from one host species to another, and (2) lineage-specific losses of symbiont species.

Horizontal symbiont transfer (HST) from one termite lineage to another is theoretically possible, according to experiments on laboratory colonies (Light and Sanford, 1928; Dropkin, 1946). It may have taken place between *Hodotermopsis* (Archotermopsidae) and the ancestor of *Reticulitermes* (Rhinotermitidae) (Kitade, 2004; Tai et al., 2015). This would explain the distinct protist community found in *Reticulitermes* as compared to its rhinotermitid relatives (Kitade, 2004). Another ancient HST may have occurred in the ancestor of *Serritermes* and *Glossotermes* (Serritermitidae), again explaining why the symbionts of these host genera differ from those of their rhinotermitid relatives (Radek et al., 2018). To date these are the only two documented inferences of HST in termites. However, if HST is more common than currently appreciated, it could lead to regional differences in protist communities across a host species' range, especially when different parts of that range overlap with different termite species.

Lineage-specific loss of protist symbionts is much more common than HST. The absence of one or two of the expected protist species from a termite colony has been documented by both morphological and molecular methods (Kitade and Matsumoto, 1993; Kitade et al., 2012, 2013; Taerum et al., 2018; Michaud et al., 2020). Such losses are more common for some host species than others, and for some protist species than others (Kitade and Matsumoto, 1993; Kitade et al., 2013; Michaud et al., 2020). Protists are inherited biparentally: both king and queen contribute their symbionts to the nascent colony's microbial community (Shimada et al., 2013; Brossette et al., 2019). Host outcrossing can therefore allow complementation of these deficient communities, helping to maintain a consistent protist community across the host species' range (Michaud et al., 2020). However, if gene flow between host populations becomes restricted, the opportunity to re-acquire a protist species by host inter-population mating would cease, and regional differences in protist communities could emerge.

If these mechanisms can, in fact, lead to a geographical influence on protist distribution, such an influence should be more readily detected in a broadly distributed host. *Heterotermes tenuis* (Hagen) (Blattodea: Rhinotermitidae) occupies a vast geographical range from as far north as southern Mexico and the West Indies down to northern Argentina, and from Ecuador to eastern Brazil (Constantino, 1998; Krishna et al., 2013; Carrijo et al., 2020). It is one of the most common termite species in both dry and forested areas of South America (Mathews, 1977; Davies et al., 2003; Ackerman et al., 2009). Like other *Heterotermes* species, it forages underground and consumes wood in contact with soil (Mathews, 1977). It has been reported to cause damage to buildings and crops, including sugarcane and eucalyptus plantations (Constantino, 2002; Batista-Pereira et al., 2004; Haifig et al., 2008). *Heterotermes tenuis* exhibits both morphological and molecular diversity, suggesting that it may be considered a species complex rather than a singular species (Constantino, 2000; Carrijo, 2013).

The symbiotic protist community of *H. tenuis* is incompletely described. Early morphological descriptions reported one species of *Pseudotrichonympha*, one species of *Cononympha*, and 1-5 species of *Holomastigotoides* (Mackinnon, 1926, 1927; de Mello, 1954). These three protist genera, all belonging to the phylum Parabasalia, are typical of *Heterotermes* and *Coptotermes* hosts and are restricted to the Rhinotermitidae (Yamin, 1979; Kitade, 2004; Jasso-Selles et al., 2017; Jasso-Selles et al., 2020). More recently, molecular investigations have documented two distinct phylotypes of *Pseudotrichonympha* (Saldarriaga et al., 2011), three distinct phylotypes of *Holomastigotoides* (Gile et al., 2018), and a phylotype related to *Cthulhu macrofasciculumque* from *Prorhinotermes simplex* (James et al., 2013). No molecular data from *Cononympha* from *H. tenuis* have been published to date.

In this study, we first revisited the hindgut protist community of *H. tenuis* in order to clarify its composition. Using a combination of light microscopy, 18S rRNA sequences from single isolated cells, and high-throughput amplicon sequencing of *H. tenuis* colonies collected across its range, we identified eight distinct species-level clades, belonging to four genera, which we refer to as phylotypes. We next examined the diversity and distribution of 18S rRNA amplicon sequence variants for each of the protist phylotypes in order to determine whether symbiotic protists might exhibit biogeographical patterns independent from those of their host.

MATERIALS AND METHODS

Termite Collections and Barcoding

Live and RNA later-preserved termites were collected in Ecuador in 2016 under permit No 015 – 2016 – IC – FAU – FLO – DPAO - PNY, in French Guiana in 2019 under permit TREL1820249A/108, and in Panama in 2018 under permit SC/A-24-17. Ethanol-preserved specimens were provided by collections at the University of Florida and the Museu de Zoologia da USP (MZUSP), Brazil. Full accession and collection information for each specimen is provided in **Supplementary Table 1**.

Molecular barcodes for termite specimens were generated by amplifying and sequencing the mitochondrial large subunit 16S ribosomal RNA gene (mt16S), using the primers LRN 5′-CGC CTG TTT ATC AAA AAC AT-3′ and LRJ 5′-TTA CGC TGT TAT CCC TAA-3′ (Simon et al., 1994; Kambhampati and Smith, 1995). Template DNA was extracted from the excised hindgut of live workers or from the macerated abdomen of ethanol- or RNAlater-preserved workers using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The PCR cycle included a 3 min denaturation at 95°C followed by 30 cycles of 95°C for 30 s, 46°C for 30 s, and 72°C for 60 s, and a final 10 min extension at 72°C. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol and sequenced directly on both strands on an Applied Biosystems 3730 capillary sequencer.

Live Protist Isolation and Characterization

Live termite hindguts were removed and their contents suspended in Ringer's solution (8.5 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.1 g $NaHCO_3$ per L, HiMedia Laboratories). Individual *Pseudotrichonympha*, *Holomastigotoides*, *Cononympha*, and *Cthulhu* cells were isolated by drawn-glass micropipette on an AxioVert inverted microscope and photographed with an Axiocam 105 color camera (Zeiss). Additional micrographs were taken on an AxioImager upright compound light microscope with an AxioCam 503 monochrome camera (Zeiss).

Each isolated cell was washed twice in fresh Ringer's solution and transferred into a 0.5 ml tube for DNA extraction using the MasterPure DNA purification kit (Epicentre Biotechnologies) following the manufacturer's protocol, except that purified DNA was resuspended in 5 μl of deionized, ELGA-purified water. The 18S rRNA gene was then amplified using a nested PCR approach. Primer pairs SpiroF1 5′-ATA CTT GGT CGA TCC TGC CAA GG-3′ and SpiroR1 5′-TGA TCC AAC GGC AGG TTC MCC TAC-3′ (Taerum et al., 2019) were used for the for the initial (outer) reaction and GGF 5′-CTT CGG TCA TAG ATT AAG CCA TGC-3′ and GGR 5′-CCT TGT TAC GAC TTC TCC TTC CTC-3′ (Gile et al., 2011) for the second (inner) reaction. PCR cycles for both primer pairs included a 3 min denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and a final 10 min extension at 72°C. Resulting PCR products were separated by gel electrophoresis and visualized with GelGreen stain (Biotium). Bands of the expected size (∼1500 base pairs) were excised from a 1% agarose gel, purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific), cloned using the TOPO TA cloning kit for sequencing (Invitrogen), and sequenced on both strands using the ABI 3730XL capillary sequencer.

Phylogenetic Analyses

We successfully amplified and sequenced the 18S rRNA gene from six individual *Pseudotrichonympha* cells, 10 *Holomastigotoides* cells, three *Cononympha* cells, and one pool of eight individually isolated *Cthulhu* cells. Between two and eight clones were sequenced from each cell, trimmed of vector, and assembled using Geneious R9 (Kearse et al., 2012). For preliminary analyses, all 116 new sequences were aligned with previously published Parabasalia 18S rRNA gene sequences using MAFFT v. 7.222 (Katoh and Standley, 2013). Ambiguously aligned sites were identified by eye and removed in AliView 1.17.1 (Larsson, 2014). A maximum likelihood (ML) phylogeny was computed using RAxML v. 8.0 (Stamatakis, 2014).

For the final parabasalian 18S rRNA phylogenetic analyses, we selected a subset of representative sequences from each protist phylotype in order to reduce redundancy. These sequences were submitted to GenBank under accessions MW353606-MW353628. New and previously published sequences from Honigbergiellida (*Cthulhu* and outgroup), Spirotrichonymphida (*Holomastigotoides* and *Cononympha*), and Trichonymphida (*Pseudotrichonympha* and outgroup) were aligned separately in order to maximize the number of alignable sites in each matrix. The final, manually trimmed, matrix sizes were: Honigbergiellida 18 taxa, 1389 sites; Spirotrichonymphea 31 taxa, 1471 sites; Trichonymphida (family Teranymphidae only) 31 taxa, 1404 sites. Bayesian and ML phylogenetic analyses were performed for each matrix using MrBayes v. 3.2.6 (Ronquist et al., 2012) and RAxML v. 8.0 (Stamatakis, 2014), respectively, under the GTR-Γ model, with support for each node assessed by 1,000 bootstrap replicates (ML) and posterior probability (Bayes). For the Bayesian analyses, two independent chains, sampled once every 100 generations, were run until they converged (the average standard deviation of partition frequency values between the chains dropped below 0.01). Convergence was reached after 20,000 generations for Honigbergiellida, 240,000 generations for Spirotrichonymphida, and 110,000 generations for Teranymphidae. The first 25% of trees were discarded as burn-in and majority rule consensus trees were computed from the remaining 300, 4600, and 1650 trees, respectively. We also computed ML trees from each of these data sets with all amplicon sequence variants (ASVs) from each taxon in order to visualize the phylogenetic diversity of the ASVs (see below for details).

For the host mt16S analyses, new and previously published sequences from *Heterotermes* species were aligned with MAFFT v. 7.222 (Katoh and Standley, 2013), which yielded a clean alignment of 67 taxa by 404 sites. This matrix was subjected to ML and Bayesian phylogenetic analyses under the GTR-Γ model of sequence evolution as above. New termite mt16S barcode sequences were submitted to GenBank under accession numbers MW345686-MW345724 (**Supplementary Table 1**).

Hindgut Community 18S rRNA Amplicon Sequencing

DNA was extracted from the whole gut contents of live termites or the macerated abdomens of termites preserved in ethanol or RNAlater (Thermo Fisher Scientific, Waltham, MA, United States) using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States). Each DNA template was acquired from a single worker individual. Amplicon libraries were generated using a two-step PCR protocol with dual index sequencing (Kozich et al., 2013). The first PCR used gene-specific primers with Illumina adaptor sequences at their 5′ ends: nexF-ParaV45F 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3′ and nexF-ParaV45R 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3′ (Jasso-Selles et al., 2017). The second PCR attached indexing barcodes to both ends of each amplicon (Hamady et al., 2008). PCR programs included an initial 3 min denaturation at 95°C, then 35 cycles of 95°C for 30 s, 48°C (1st step) or 50°C (2nd step) for 30 s, and 72°C for 50 s, followed by a 10 min final extension at 72°C. All PCR reactions used EconoTaq 2x Master Mix (Lucigen) with 2.5 μl of 2 μM stock forward and reverse primers and 2.5 μl of undiluted template DNA in a 30 μl reaction. PCR products were purified using Ampure (Beckman Coulter) magnetic beads in combination with the Beckman Biomek NXp robot and quantified using Qubit broad range dsDNA fluorescent dye (Invitrogen) on a Biotek HT1 Plate Reader. After quantification, samples were pooled at approximately equivalent concentrations and sequenced on the Illumina MiSeq platform. This study includes samples run in 2017 using 2 × 300 paired-end chemistry and samples run in 2019 using 2 × 250 paired-end chemistry.

Amplicon sequence analyses were performed using QIIME 2 2020.2 (Bolyen et al., 2019). Casava 1.8 paired-end demultiplexed fastq files were imported via QIIME tools import. The demultiplexed reads were trimmed, merged, and denoised separately for each sequencing run using DADA2 (Callahan et al., 2016). The 2 × 250 samples did not require trimming. Both sequencing runs were merged into a single dataset after denoising. Samples consisting of fewer than 500 reads for the Parabasalia taxa of interest were discarded. Taxonomy was assigned using a naïve Bayes classifier implemented in QIIME 2 (Bokulich et al., 2018) trained on a manually curated Parabasalia 18S rRNA reference file of 1075 published and unpublished sequences, including all 116 18S rRNA clones from single cells sequenced in this study. Raw demultiplexed fastq files were submitted to NCBI SRA under bioproject PRJNA683896.

ASV Diversity and Distribution Analyses

Mean and maximum pairwise distances between amplicon sequence variants (ASVs) assigned to each protist phylotype and linear regressions of ASV diversity and uniqueness vs. protist prevalence were computed in R version 4.0.1 (R Core Team, 2020). Distributions of ASVs across sample locations were determined by sorting ASVs into individual datasets for each protist phylotype and for each geographical coordinate using QIIME 2 2020.2 (Bolyen et al., 2019). For Mantel tests, all genetic distance matrices (for protist 18S rRNA ASVs and host mt16S rRNA sequences) used uncorrected pairwise ("p") distances with no model of evolution. In cases where multiple unique ASVs were present at the same coordinate, the ASV with the greatest read depth was selected to represent that coordinate in uncorrected pairwise distance matrices for each protist phylotype. Euclidean distance was used as the distance measure for the geographic distance matrices. All full and partial Mantel tests were conducted in R version 4.0.1, using packages vegan (Oksanen et al., 2019) and ape (Paradis and Schliep, 2019), with significance level determined from 999 permutations in each test.

RESULTS

Morphology of *H. tenuis* Hindgut Protist Symbionts

Using light microscopy, we observed the protists inhabiting the guts of live termites collected in Ecuador, Panama, and French Guiana. We identified four genera of protists, all belonging to the phylum Parabasalia: *Pseudotrichonympha*, *Cthulhu*, *Cononympha*, and *Holomastigotoides* (**Figure 1**). Each of these genera had been previously reported to inhabit the gut of *H. tenuis*, though *Cononympha* was previously known as *Microspironympha* or *Spirotrichonympha* (Mackinnon, 1926, 1927; de Mello, 1954; Saldarriaga et al., 2011; James et al., 2013). We did not observe any additional genera beyond those previously reported to occur in *H. tenuis*.

Pseudotrichonympha (Trichonymphida) is a large, hypermastigote, wood-feeding protist. It has an anterior cell portion called a rostrum and a posterior portion called the cell body or post-rostral area. The rostrum has a smooth apical cap and an internal tube-like structure. Except for the apical cap, the entire cell is covered with flagella of three different lengths; short flagella on the rostrum, long flagella forming a fringe around the cell at the base of the rostrum, and medium-length flagella covering the rest of the cell (Grassi and Foà, 1911; Grassi, 1917; Saldarriaga et al., 2011). We observed two *Pseudotrichonympha* morphotypes in *H. tenuis*, one with a raised rim around the margin of its apical cap and one without (**Figures 1A,B**). Whether this characteristic represents a stable, heritable trait or a variable or artifactual one remains unknown. Previously published electron micrographs of *Pseudotrichonympha* from *H. tenuis* also showed a raised rim around the apical cap while light micrographs from the same study lacked this feature (Saldarriaga et al., 2011).

Cthulhu (Honigbergiellida) is a very small protist defined by its bundle of roughly 20 anterior flagella. This feature differentiates it from its relatives *Cthylla* and *Hexamastix*, which each have five anterior flagella (James et al., 2013). The number of flagella borne by the *H. tenuis* symbiont is difficult to capture in still micrographs of live specimens (**Figure 1C**), but video microscopy demonstrates that the number is more than 10 (**Supplementary Video 1**).

Cononympha (Spirotrichonymphida) is similar to *Holomastigotoides* in having spiral bands of flagella, but these come together at the cell apex to form a spiral staircase-like

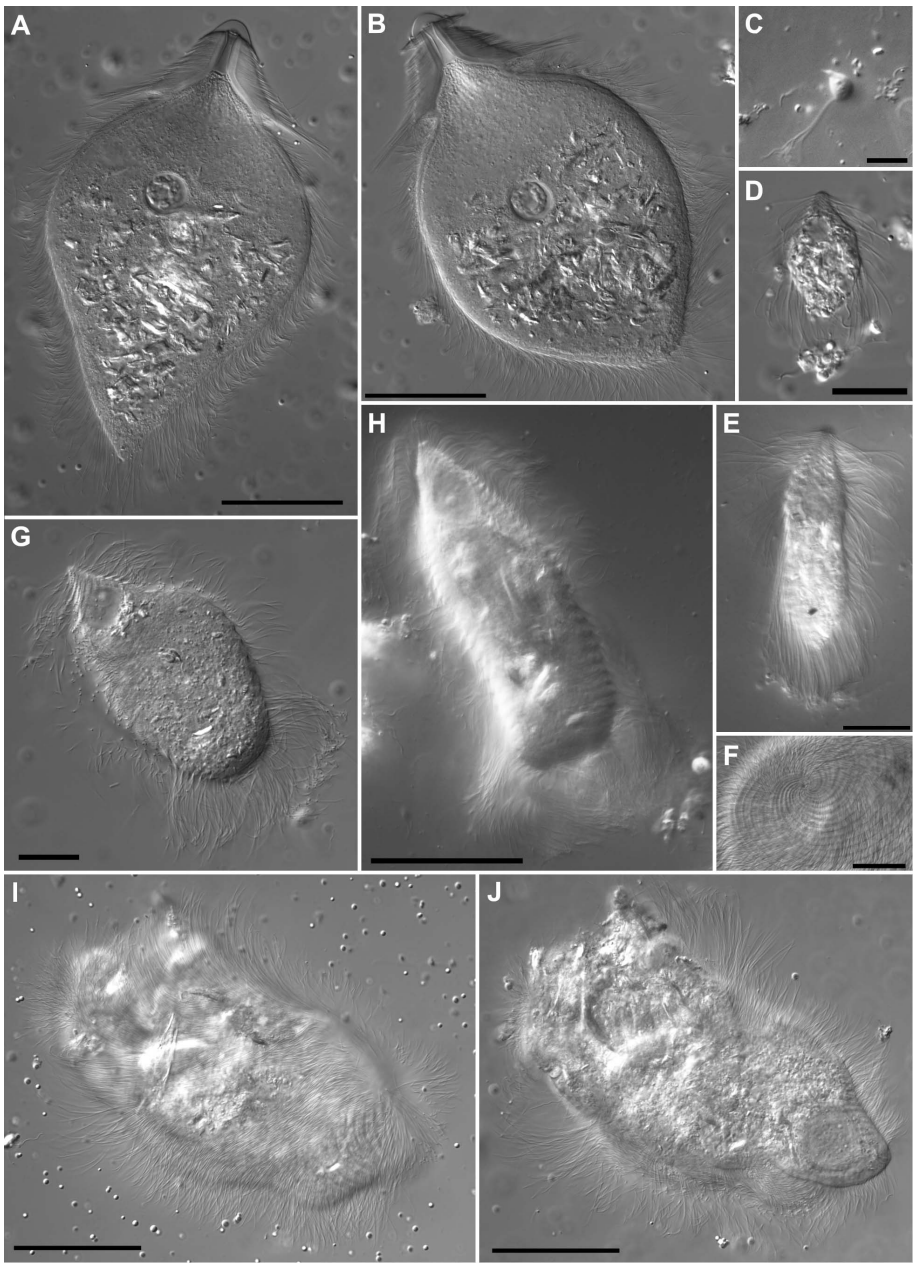

FIGURE 1 | Differential interference contrast light micrographs of *Heterotermes tenuis* parabasalian hindgut symbionts. **(A,B)** *Pseudotrichonympha* cells (Trichonymphida) are characterized by a prominent rostral tube with apical cap or nose cone. Flagella of three distinct length classes cover the cell body, short flagella on the rostral area, long flagella form a fringe around the base of the rostral area, and medium length flagella cover the remainder of the cell body. Both cells have ingested many wood fragments (refractile material throughout cell body) and both are shown slightly flattened by the cover slip. **(A)** Smooth nose cone morphotype. **(B)** Morphotype with marginal ridge on nose cone. **(C)** *Cthulhu* cells (Honigbergiellida) are small and bear several apical flagella. **(D,E)** *Cononympha* cells (Spirotrichonymphida) with characteristic apical pseudorostrum in which flagellar bands form a spiral staircase-like structure. **(D)** Smaller morphotype with central to anterior nucleus location and detritus adhering to the posterior flagella. **(E)** Larger morphotype with more elongate shape and centrally located nucleus. **(F–J)** *Holomastigotoides* (Spirotrichonymphida). **(F)** Origin of spiraling flagellar bands at a cell apex. **(G)** Acuminate apex morphotype (type 3) with prominent anterior nucleus and posterior ingested wood particles. **(H)** Bottle-shape morphotype with anterior nucleus, ingested wood particles, and relatively long flagella. **(I,J)** Large amorphous morphotype cell in two optical sections. **(I)** Glancing surface section with spiral flagellar bands visible. **(J).** Deeper optical section with large anterior nucleus visible. Scale bars: **(A,B,H,I,J)** 50 μm; **(D,E,F,G)** 20 μm; **(C)** 10 μm.

structure called a pseudorostrum with an internal axial tube-like structure called a columella (Koidzumi, 1921; Jasso-Selles et al., 2017). We observed two distinct *Cononympha* morphotypes inhabiting *H. tenuis* (**Figures 1D,E**). One type had rounded cells measuring less than 30 μm in length with a nucleus positioned near the anterior of the cell (**Figure 1D**), while the other type had

larger, more elongated cells that measured 35–65 μm in length and a more centrally positioned nucleus (**Figure 1E**).

Holomastigotoides (Spirotrichonymphida) is also a wood-feeding, highly flagellated protist, but bears helical bands of flagella that originate at the cell apex and encircle the cell nearly to its posterior pole. Its nucleus is positioned anteriorly (Grassi and Foà, 1911; Grassi, 1917; Brugerolle and Lee, 2000). Within this morphological category, we observed a range of cell sizes and shapes, suggesting multiple *Holomastigotoides* species in *H. tenuis* (**Figures 1F–J**). One morphotype was characterized by very large cells, measuring 130–180 μm in length, with a flexible, irregular outline (**Figures 1I,J**). Other cells were smaller with an acutely pointed apex (**Figure 1G**) or an apex that tapered gradually to a point (**Figure 1H**) or a more rounded apex (not shown).

Molecular Phylogenetic Characterization of Individually Isolated Protist Cells

For molecular characterization of these symbionts, we isolated single protist cells and amplified, cloned, and sequenced their 18S rRNA genes. Cells were selected to represent the full range of morphological variability within each genus as much as possible. From 20 single cells, we successfully sequenced 116 clones, which clustered into eight groups of closely related sequences (species-level phylotypes) (**Supplementary Table 2**). We recovered two phylotypes from our isolated *Pseudotrichonympha* cells, one phylotype of *Cthulhu*, two phylotypes of *Cononympha*, and three phylotypes of *Holomastigotoides*, all of which branched with previously characterized congeners, consistent with our morphological identifications. Six of these phylotypes branched closely with previously published sequences from *H. tenuis* symbionts (Noda et al., 2007; Saldarriaga et al., 2011; James et al., 2013; Gile et al., 2018). We labeled our new sequences concordantly, so *Pseudotrichonympha paulistana* follows Saldarriaga et al., 2011, *Pseudotrichonympha* sp. follows Noda et al., 2007, and the three *Holomastigotoides* phylotypes follow previously published clones Ht1, Ht2, and Ht3 (Noda et al., 2007; Saldarriaga et al., 2011; Gile et al., 2018). The single *Cthulhu* phylotype did not require a distinguishing phylotype label. *Cononympha* phylotypes were numbered 1 and 2 arbitrarily but consistently.

In order to maximize the number of alignable sites, we carried out phylogenetic analyses separately for *Pseudotrichonympha* (**Figure 2**), *Cthulhu* (**Figure 3**), and the Spirotrichonymphida genera *Holomastigotoides* and *Cononympha* (**Figure 4**). The position of the two *Pseudotrichonympha* phylotypes was not resolved, though *Pseudotrichonympha* from *Heterotermes* hosts formed a supported clade (**Figure 2**). Our *Cthulhu* sequences were nearly identical to a previously published symbiont clone from *H. tenuis* collected in northern Colombia (James et al., 2013; **Figure 3**). This sequence (JX975351) was amplified from whole gut DNA, not an isolated cell, so it was not attributed to *Cthulhu*, though *Cthulhu* morphotypes were observed in *H. tenuis* in the same study (James et al., 2013). Our new sequence was amplified from a pool of eight *Cthulhu* cells isolated from *H. tenuis* collected in Ecuador (**Figure 3**). For *Cononympha*, the two phylotypes formed a clade with *Cononympha aurea* from *Heterotermes aureus*, the only *Cononympha* sequences so far available from any *Heterotermes* host (Jasso-Selles et al., 2017; **Figure 4**). Finally, two of the three *Holomastigotoides* phylotypes branched together with weak support while the third phylotype branched sister to the *Holomastigotoides* species from *Heterotermes aureus*. In contrast to *Pseudotrichonympha*, the *Holomastigotoides* species from *Heterotermes* hosts did not form a clade (**Figure 4**).

Hindgut 18S rRNA Community Profiling

We also carried out 18S rRNA V4-V5 amplicon sequencing (metabarcoding) to characterize the hindgut parabasalian symbionts of *H. tenuis* sampled across much of its range. We included *H. tenuis* from four countries, French Guiana, Panama, Ecuador, and Brazil, with Brazilian samples coming from 10 states and both forest and savannah habitats (**Figure 5**). In total, we profiled the protist community of 95 individual worker termites from 39 distinct colonies, of which 16 were represented by a single individual (all ethanol-preserved museum specimens) and 21 had 2–4 individuals, while live collections from Ecuador and French Guiana had six and 20 individuals (biological replicates), respectively. In order to link each of these profiles to host genotype, we sequenced the mt16S of at least one individual from each colony. Our maximum likelihood phylogeny of these molecular barcodes recovered three distinct clades with moderate support (**Figure 5**), in agreement with a previous study (Carrijo et al., 2020).

After trimming, merging, and denoising, we assigned taxonomy to our amplicon sequence variants (ASVs) using a custom reference database of full-length Parabasalia 18S rRNA sequences, including all 116 Sanger sequences generated in this study. All Parabasalia ASVs from the 95 samples were assignable to the eight phylotypes we characterized from single cells. Note that ASVs are equivalent to zero-radius OTUs; each has a unique sequence but no longer includes read abundance information. Together the ASVs represent the total sequence diversity present in the 18S rRNA amplicons. The number of ASVs per protist phylotype in our data set ranged from 17 (*Cthulhu*) to 136 (*Holomastigotoides* Ht2), for a total of 557 protist ASVs (**Supplementary Table 3**).

For a closer look at ASV phylogenetic diversity, we aligned all ASVs with the new and previously published 18S rRNA sequences used in the isolated cell analyses above (**Figures 2–4**) and computed phylogenetic trees (**Supplementary Figures 1–4**). All ASVs branched with the phylotypes to which they had been assigned, though they revealed considerably more sequence variability than the fewer single cell clones (**Supplementary Figures 1–4**). Particularly high levels of sequence divergence were exhibited by the two *Cononympha* phylotypes (**Supplementary Figure 1**, **Supplementary Table 3**), but because they lack clearly demarcated subclades, we maintain our interpretation of two phylotypes. In contrast, the ASVs clustering with *Holomastigotoides* Ht1 did form two supported subclades and therefore could alternatively be considered as two phylotypes, possibly representing two distinct species (**Supplementary Figure 2**). In keeping with this possibility, we noted that ASVs from these two subclades are nearly mutually exclusive in distribution, co-occurring in just one out of the nine colonies

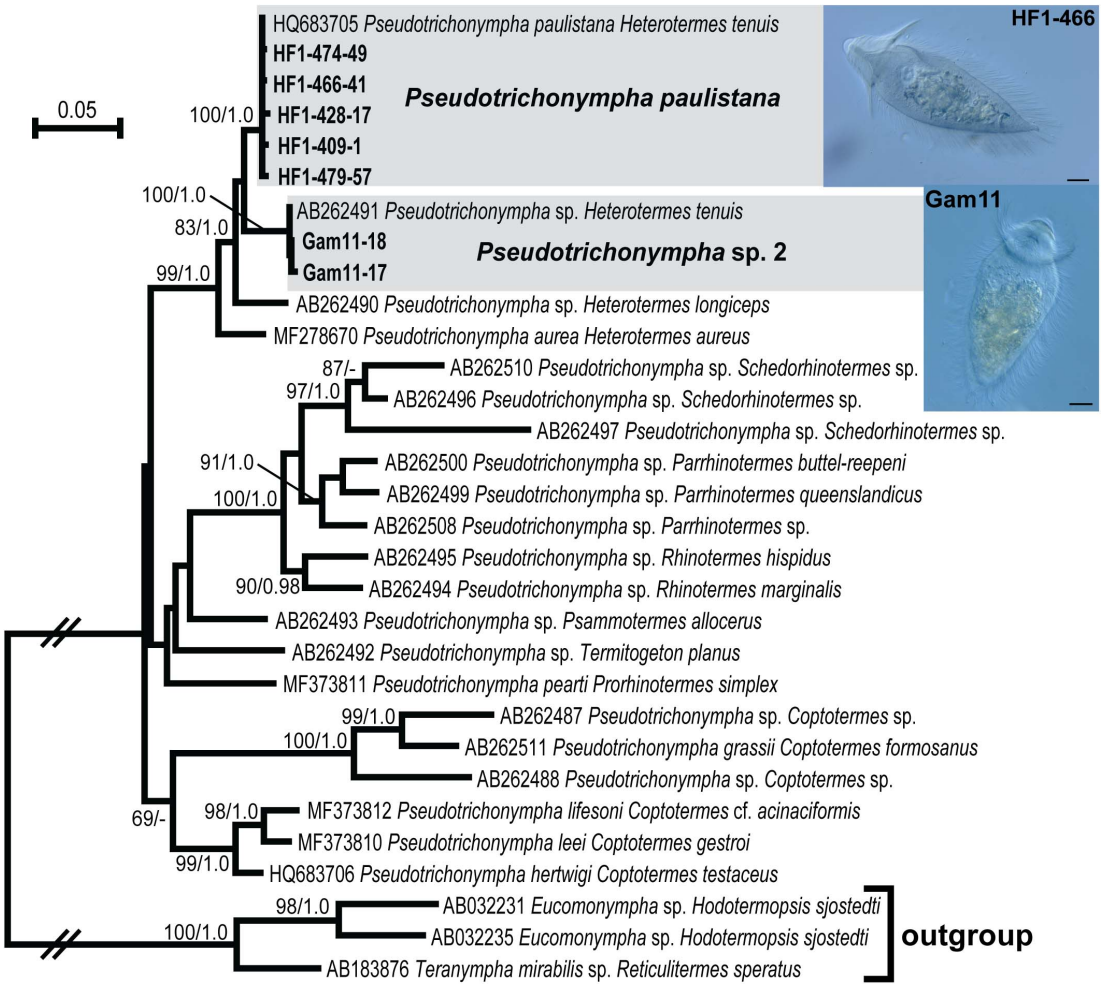

FIGURE 2 | Maximum likelihood phylogeny of 18S ribosomal RNA gene sequences from *Pseudotrichonympha* with outgroup *Eucomonympha* and *Teranympha* (Teranymphidae: Trichonymphida). Protist species-level phylotypes from *H. tenuis* are indicated by gray boxes and new sequences obtained in this study are indicated by bold text. Support at nodes is given out of 100 bootstrap replicates computed by RAxML/Bayesian posterior probabilities if greater than 60/0.95. New sequences from *H. tenuis* symbionts are indicated by bold text. Select cells from which new sequences were derived are shown in micrographs inset at right; sequence names (tip labels) include the cell code. All scale bars: 20 μm.

in which Ht1 was found, and suggesting they might belong to distinct cellular lineages. Because these subclades are very closely related and we did not isolate a representative cell from the additional subclade, we will consider Ht1 as one phylotype in this work. In sum, the ASV diversity across our data set supports eight protist species-level phylotypes as a reasonable accounting of the *H. tenuis* symbiont community. The 18S rRNA sequence variability within each of these protist phylotypes, including Ht1 (**Supplementary Table 3**), is consistent with that previously observed for named termite-associated protist species (Tai et al., 2014; Taerum et al., 2019, 2020; Jasso-Selles et al., 2020).

Distribution of Protist Phylotypes Across *H. tenuis* Colonies

Our sampled colonies contained different combinations of these eight phylotypes, with only two colonies harboring all eight phylotypes (**Supplementary Table 1**). None of the protist phylotypes was restricted to a single host haplotype group or a specific geographical region. In several colonies, we detected only two or three protist phylotypes. However, due to the destructive nature of our sampling, we were limited to just one termite individual from several of our museum collection colonies. This modest level of sampling increases the likelihood that protist species present in these samples' colonies might not be detected by 18S rRNA profiling, whether due to biological variability among nestmates (e.g., in recently molted individuals) or technical limitations (e.g., biased sample degradation or allelic dropout during PCR). We therefore examined the prevalence of each protist phylotype across a reduced set of colonies for which at least two individual termites were sampled.

Holomastigotoides Ht2 was the most prevalent phylotype, present in 22 out of 23 colonies (96%). Next most common were *Pseudotrichonympha paulistana* and *Pseudotrichonympha*

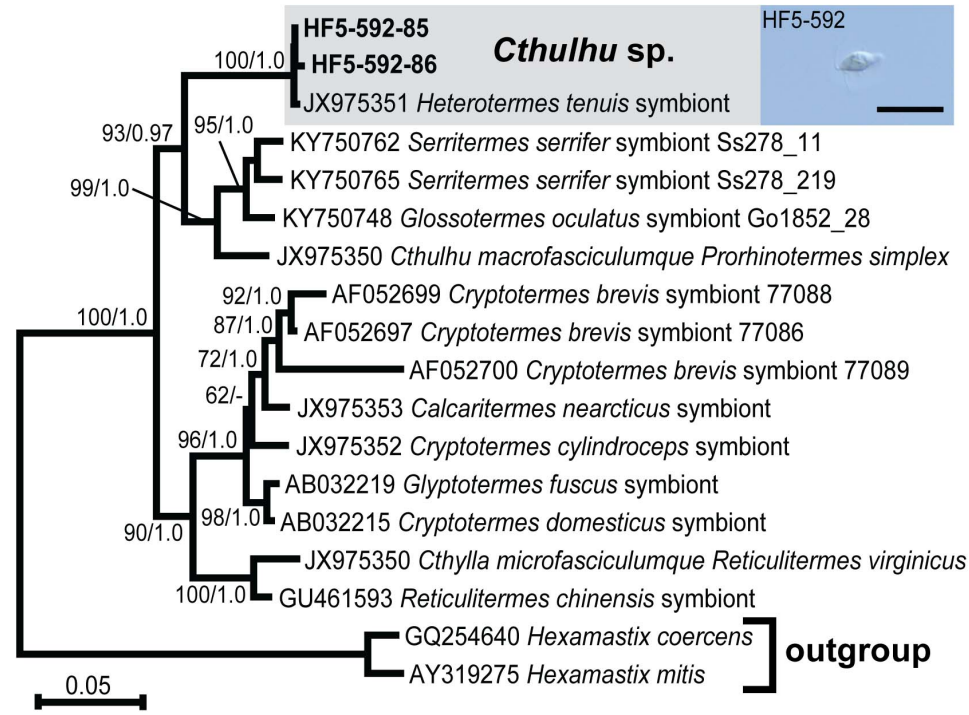

FIGURE 3 | Maximum likelihood phylogeny of 18S ribosomal RNA gene sequences from *Cthulhu* species with *Hexamastix* sequences as outgroup. Protist species-level phylotypes from *H. tenuis* are indicated by gray boxes and new sequences obtained in this study are indicated by bold text. Support at nodes is given out of 100 bootstrap replicates computed by RAxML/Bayesian posterior probabilities if greater than 60/0.95. New sequences from *H. tenuis* symbionts are indicated by bold text. Inset DIC micrograph features one of the eight pooled *Cthulhu* cells that were combined to generate the template for these sequences. Scale bar: 20 μm.

sp., each present in 21 of the 31 colonies (91%). Only one colony harbored neither *Pseudotrichonympha* species: our live colony from Panama that was in visible decline in the lab. While we did isolate a single *Pseudotrichonympha* cell (Gam11) from this colony shortly after collection, by the time we collected gut samples for 18S rRNA profiling no *Pseudotrichonympha* species were apparent by light microscopy. We have also observed *Pseudotrichonympha aurea* disappear from declining lab colonies of *Heterotermes aureus* (Jasso-Selles et al., 2017). The next most prevalent phylotypes were *Cononympha* sp. 1 and *Cononympha* sp. 2, occurring in 17 and 19 out of 23 colonies, respectively (74% and 83%). The phylotypes with the patchiest distributions were *Cthulhu* and *Holomastigotoides* Ht1, present in six and four colonies, respectively (26% and 17%, **Supplementary Table 4**).

Because differences in preservation mode among samples (i.e., ethanol, RNAlater, live) might lead to biased detection of protist phylotypes (Hammer et al., 2015), we also examined protist phylotype prevalence across each sample preservation mode separately (**Supplementary Table 4**). The ethanol-only set showed very similar values to those observed in the 2 + termite subset, with the same ranked order of protist phylotypes by proportion of colonies in which they were detected (i.e., Ht2 > *Pseudotrichonympha* spp. > *Cononympha* sp. 2 > *Cononympha* sp. 1 > Ht3 > *Cthulhu* > Ht1). However, the RNAlater ($n = 7$) and live ($n = 3$) subsets deviated from this trend in some respects. Most notably, both *Cononympha* species were detected in all of the non-ethanol-preserved samples, suggesting that ethanol samples might be biased toward faster degradation of *Cononympha* sequences. *Holomastigotoides* Ht3 was also more prevalent in these sample types than in ethanol, present in six out of seven RNAlater colonies and all three live colonies, while *Cthulhu* was completely absent from RNAlater colonies and present in one out of three live colonies. Without further sampling it is impossible to determine whether these differences reflect technical bias or true biological differences in the sampled colonies. Note that six out of seven RNAlater colonies and one out of three live colonies come from Panama, as opposed to just one out of 29 ethanol-preserved colonies.

The most common number of protist phylotypes recovered across our 2 + termite colonies is six (seen in 9 out of 23 colonies), and these communities generally consist of both *Pseudotrichonympha* species, *Holomastigotoides* Ht2 and Ht3, and both *Cononympha* species. In other words, they most commonly lack *Cthulhu* and *Holomastigotoides* Ht1 (**Supplementary Table 1**). The next most common number of protist phylotypes is five (5 out of 23), and these colonies are most often additionally missing one of the *Cononympha* species or *Holomastigotoides* Ht3. The seven- and eight-protist phylotype colonies (1 and 2 out of 23) add one or both of the rarer *Cthulhu* and *Holomastigotoides* Ht1. From these observations, we qualitatively conclude that the "typical" hindgut protist community of *H. tenuis* consists of *Pseudotrichonympha*

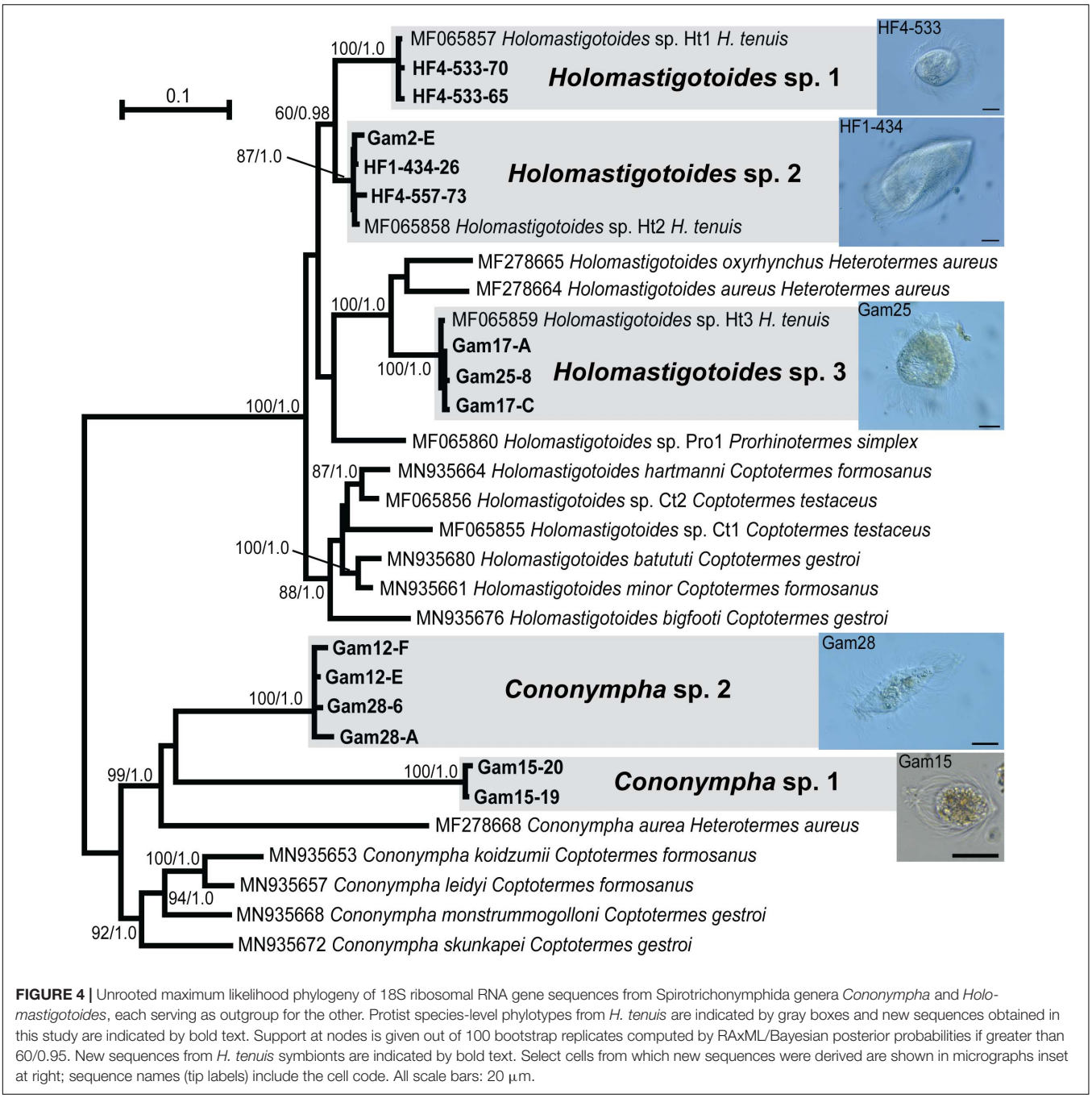

FIGURE 4 | Unrooted maximum likelihood phylogeny of 18S ribosomal RNA gene sequences from Spirotrichonymphida genera *Cononympha* and *Holomastigotoides*, each serving as outgroup for the other. Protist species-level phylotypes from *H. tenuis* are indicated by gray boxes and new sequences obtained in this study are indicated by bold text. Support at nodes is given out of 100 bootstrap replicates computed by RAxML/Bayesian posterior probabilities if greater than 60/0.95. New sequences from *H. tenuis* symbionts are indicated by bold text. Select cells from which new sequences were derived are shown in micrographs inset at right; sequence names (tip labels) include the cell code. All scale bars: 20 μm.

paulistana and/or *Pseudotrichonympha* sp., *Holomastigotoides* Ht2, another of the *H. tenuis*-associated *Holomastigotoides* species, and at least one of the *H. tenuis*-associated *Cononympha* species.

Diversity and Phylogeography of Protist 18S rRNA Sequence Variants

There is considerable diversity among 18S rRNA amplicon sequence variants (ASVs) within each protist phylotype, and the level of diversity itself varies among phylotypes (**Supplementary Figures 1–4**, **Supplementary Table 3**). This is consistent with the intragenomic sequence variability of 18S rRNA genes observed in single cell PCR (e.g., Saldarriaga et al., 2011; Taerum et al., 2018). We recovered between 17 (*Cthulhu*) and 136 (*Holomastigotoides* Ht2) unique ASVs for each protist phylotype across our data set. However, the number of unique ASVs per phylotype correlated strongly with the prevalence of each phylotype across our data set (R^2 = 0.84), suggesting that the observed among-phylotype variability in number of unique ASVs is driven by sampling depth rather than interspecific biological differences (**Supplementary Table 3**). Similarly, the proportion of ASVs that were unique to

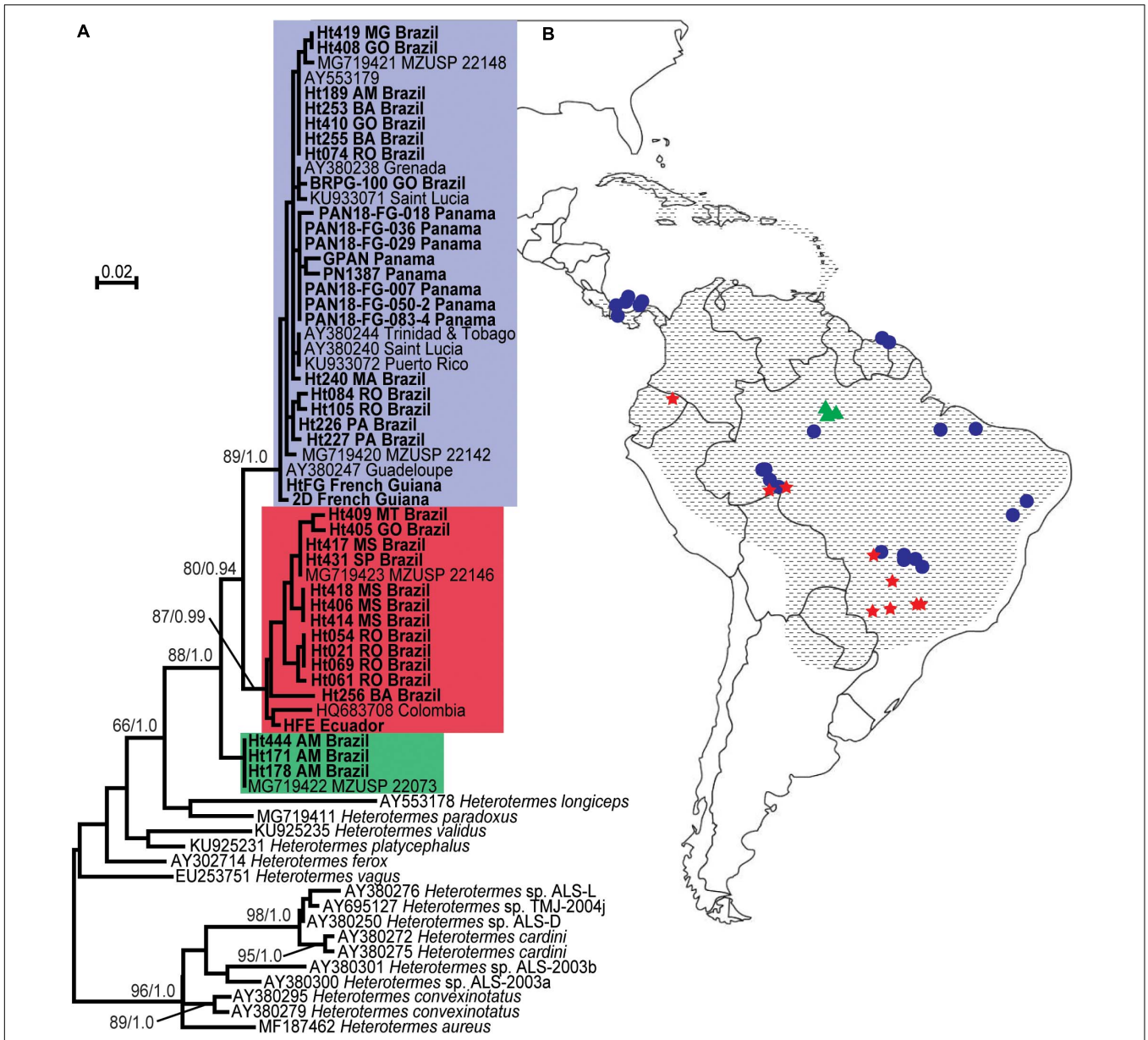

FIGURE 5 | Collection locations and molecular identification of *Heterotermes tenuis* colonies. **(A)** Maximum likelihood phylogeny of *Heterotermes* partial mt16S gene sequences. *Heterotermes tenuis* sequences are indicated by colored boxes (top box is blue, middle is red, bottom is green); new sequences determined in this study are indicated by bold type. Support at nodes is given from 100 bootstrap replicates and Bayesian posterior probabilities where greater than 60/0.9. **(B)** Collection locations of *H. tenuis* colonies. Shapes indicate collection locations of samples included in this study, with color corresponding to the same haplotype group color scheme as in **(A)** (top blue haplotype = circles, middle red haplotype = stars, bottom green haplotype = triangles). Hatching indicates the geographical range of *H. tenuis* (Carrijo et al., 2020).

a single location varied across phylotypes, and these values were also correlated with phylotype prevalence across samples, though weakly ($R^2 = 0.49$, **Supplementary Table 3**).

Looking instead at the sequence diversity among ASVs, the *Cononympha* phylotypes stand out as the most diverse, with mean/maximum uncorrected pairwise distance values of 0.02/0.12 for *Cononympha* sp. 1 and 0.05/0.13 for *Cononympha* sp. 2 (**Supplementary Table 3**). Even *Holomastigotoides* Ht1, which we suspect might reasonably represent two species, has lower ASV diversity, at 0.02/0.04, though still roughly twice as high as *Holomastigotoides* Ht3 (0.01/0.02) and *Holomastigotoides* Ht2 (0.00/0.02) (**Supplementary Table 3**).

We next asked whether the genetic distances among protist ASVs correlate with geographical distances using Mantel tests (**Table 1**). Mantel tests compare two distance matrices (i.e., uncorrected pairwise distances for all ASVs and geographical distances between sample locations) and compute a test statistic from cross products of the two matrices. Significance can

TABLE 1 | Mantel test results (coefficient of correlation, R, and *p*-values).

		Full		Partial	
		Geography	Host phylogeny	Geography (control host phylogeny)	Host phylogeny (control geography)
Holomastigotoides Ht1	r statistic	0.032	0.500	−0.072	0.503
	p value	0.364	0.005	0.660	0.014
Holomastigotoides Ht2	r statistic	0.010	0.022	−0.005	0.020
	p value	0.404	0.360	0.519	0.362
Holomastigotoides Ht3	r statistic	0.211	0.127	0.181	0.063
	p value	0.024	0.110	0.029	0.287
Pseudotrichonympha paulistana	r statistic	0.056	−0.017	0.079	−0.058
	p value	0.210	0.538	0.136	0.773
Pseudotrichonympha sp.	r statistic	−0.091	−0.006	−0.099	0.041
	p value	0.779	0.419	0.859	0.342
Cthulhu	r statistic	0.303	−0.067	0.368	−0.228
	p value	0.205	0.690	0.140	0.886
Cononympha sp. 1	r statistic	−0.023	−0.044	0.002	−0.038
	p value	0.674	0.543	0.470	0.490
Cononympha sp. 2	r statistic	−0.012	−0.135	0.102	−0.168
	p value	0.612	0.940	0.122	0.962
Heterotermes tenuis	r statistic	−0.005			
	p value	0.496			

Green background indicates statistical significance ($p < 0.05$). For full Mantel tests, each protist species' ASV uncorrected pairwise distances were compared to geographical distances and to host mt16S uncorrected pairwise distances (host phylogeny), and H. tenuis mt16S distances were compared to geographical distances. Partial Mantel tests were carried out between protist ASV distances and host mt16S distances, with geographical distances as a control, and between protist ASV distances and geography, with host mt16S distances as a control.

be assessed by comparison to a distribution of test statistics from permutated data (Diniz-Filho et al., 2013; Buttigieg and Ramette, 2014). Note that while biased sample degradation would be expected to impact the inferred presence or absence of a phylotype, it would not be expected to impact the sequence of nucleotides in an amplified fragment. Our Mantel tests therefore include ASVs from all 39 colonies sampled in this study.

For nearly all protist species, there was no correlation between geographical distance and genetic distance of ASVs. Only one species, *Holomastigotoides* Ht3, showed a weakly significant correlation between the genetic distances among its ASVs and the geographical distances between their sample locations (r = 0.211, p = 0.024; **Table 1**). These results indicate an overall lack of geographical pattern in the protist ASV sequence diversity in our data set.

An important caveat to this test is that protist biogeography depends on host biogeography, and host biogeography might be correlated with host phylogeny. We therefore carried out a series of Mantel tests to examine this relationship further. First, we compared host mt16S distances to host geographical distances and found no significant correlation, suggesting that host phylogeny should not be a confounding factor in this case. Next, we computed partial Mantel tests between protist ASV genetic and geographical distances while controlling for host phylogeny, and again found only a weakly significant correlation between *Holomastigotoides* Ht3 genetic and geographical distances (r = 0.181, p = 0.029; **Table 1**).

We also checked for correlation between protist ASV distances and host mt16S distances. Although host phylogeny is known to impact protist phylogeny and community composition in termites (Tai et al., 2015), it is unknown whether this influence manifests or is detectable at the host population level. In both full and partial Mantel tests, there was no detectable correlation between host and protist genetic distances, except for one species: *Holomastigotoides* Ht1, whose genetic distance showed a significant correlation with that of the host genetic distance (full test r = 0.500, p = 0.005; partial test controlling for geography r = 0.503, p = 0.014; **Table 1**).

DISCUSSION

Drivers of Protist Diversification

In this study, we asked whether the considerable intraspecific variation in protist ASVs exhibits any patterns with respect to geography. Because protist communities are reported to be quite consistent across their termite host species, it is perhaps unsurprising that there was no significant correlation between most of the protists' 18S rRNA ASV sequence diversification and their geographical or host phylogenetic distances. This suggests that there is as much ASV diversity within samples and/or between close (geographically or host phylogenetically) samples as there is across more distant samples. However, we did detect a weakly significant correlation between geographical and ASV

pairwise distances of *Holomastigotoides* Ht3 (**Table 1**). This phylotype exhibits considerable shared sequence divergence (i.e., long branches) among subsets of ASVs, though their phylogenetic relationships are not resolved (**Supplementary Figure 2**). The most divergent *Holomastigotoides* Ht3 ASVs are closely related to our single-cell 18S rRNA sequences from Panama while the rest of the ASVs mainly come from Brazilian samples. This appears to be driving the correlation between ASV and geographical distances, which are greatest between Panama and southern and eastern Brazil.

The other significant correlation in our data set was between host mt16S pairwise distances and the pairwise distances of ASVs from *Holomastigotoides* Ht1. This is the protist phylotype for which the ASVs formed two supported, sister subclades in phylogenetic analyses (**Supplementary Figure 2**). As mentioned previously, these ASV subclades had a nearly mutually exclusive distribution across host colonies, making it unlikely that they co-exist in a single genome. While both subclades were found in samples from both the red and blue host haplotype groups (**Figure 5**, **Supplementary Table 1**), they were differentially distributed among the haplotype subclades. In particular, host colonies HFE, Ht061, and Ht069, which branch relatively deeply in the red haplotype group, harbor one of the *Holomastigotoides* Ht1 subclades, while host colonies Ht409, Ht431, and Ht406 in the crown clade of the red haplotype group harbor the other subclade of *Holomastigotoides* Ht1. This differential distribution of ASV subclades within the red host haplotype group appears to be driving the correlation between ASV and host mt16S distances.

Whether or not these correlations reveal incipient speciation in *Holomastigotoides* Ht1 or Ht3, they certainly highlight plausible scenarios by which termite-associated protists might speciate independently of their hosts or of each other (**Figure 6**). Such examples are welcome because the coevolutionary history of termites and their protists is too complex to be explained by co-speciation alone. In the case of *Holomastigotoides* Ht3, whose ASV genetic distances correlate with geographical distances, a divergent subset of ASVs is found almost exclusively in samples from Panama, on the margin of its mainland range. This pattern is reminiscent of peripatric speciation, in which a small marginal population becomes isolated from the parent population and diverges by genetic drift (Mayr, 1954). Although the host genetic distances do not show this pattern, *Holomastigotoides* Ht3 in Panama would be isolated from its conspecifics if they were missing from contiguous host populations toward the mainland. As our data suggest, the lack of *Holomastigotoides* Ht3 in certain host colonies is not only plausible but common.

Holomastigotoides Ht1 is the only *H. tenuis* symbiont whose ASV genetic distances are significantly correlated with the mt16S genetic distances of its host. While co-phylogeny at the genus level has been demonstrated between *Pseudotrichonympha* and its rhinotermitid hosts (Noda et al., 2007), incomplete protist 18S rRNA lineage sorting would be expected to obscure co-diversification in recently diverged host lineages (Maddison, 1997). Indeed, there is no detectable protist diversification in symbionts of recently diverged *Zootermopsis* species (Taerum et al., 2018), and evidence of incomplete lineage sorting has been reported from *Teranympha* symbionts of *Reticulitermes* (Noda et al., 2018). So why is host/symbiont co-diversification detectable in *Holomastigotoides* Ht1? We suspect that the cause is the patchy distribution of this protist phylotype. Because *Holomastigotoides* Ht1 is more commonly absent than present among our sampled *H. tenuis* colonies, a host lineage lacking *Holomastigotoides* Ht1 is unlikely to gain it by mating with a different host lineage, and daughter colonies are unlikely to inherit *Holomastigotoides* Ht1 from both parents, unless both parents belong to the same host lineage. In this way, protist populations could become isolated even while their hosts maintain gene flow. Protist rarity would therefore strengthen the congruence between maternally inherited mt16S distances and biparentally inherited protist ASVs. Another consequence of protist rarity could be the pruning

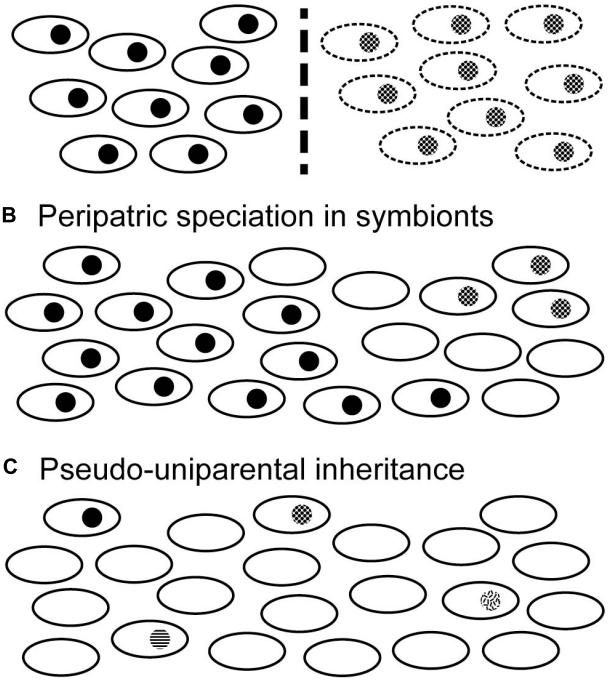

FIGURE 6 | Scenarios of evolutionary diversification in termite-specific protists. Open black ovals represent termite colonies while filled circles indicate one of their symbiotic protist species. Note that termite-specific protists are biparentally inherited and do not persist in the environment outside their hosts. **(A)** Co-diversification of host and symbiont species. If host populations become separated by some barrier to gene flow (dashed vertical line), symbiont populations will also be separated. Over time protists and hosts can co-speciate. **(B)** If certain host colonies lose the symbiont species (empty ovals), they can form a barrier isolating two populations of symbionts. Symbiont populations can then diverge from one another in the absence of host diversification. This might occur more readily on the margins of a host species' range, in a process reminiscent of peripatric speciation. Note that other symbiont species occupying the same host species could remain unaffected. **(C)** If a symbiont species becomes rare due to loss from many host colonies, its inheritance becomes nearly uniparental: any host colony harboring that symbiont species would have inherited it from just one parent (i.e., king or queen), unless both parents came from the same colony. In this pseudo-uniparental inheritance scenario, symbiont genetic diversification is more likely to be congruent with that of the uniparentally inherited mitochondrial genome.

of ASV diversity whenever a protist species fails to be transmitted to a daughter colony. This would further accelerate the ASV lineage sorting process.

Variability of Protist Community Composition

From early morphology-based observations it became a paradigm that symbiont communities are highly consistent across populations of a termite species with only occasional exceptions where a colony might be lacking one of the expected protist species (Kirby, 1937, 1949). This assumption was first directly tested in Japanese *Reticulitermes* species, in which colonies missing protist species were found to be common (Kitade and Matsumoto, 1993). For example, 10 out of 41 *Reticulitermes speratus* nests were found to be missing one or two of the 15 identified protist morphospecies (Kitade and Matsumoto, 1993). A similar prevalence of missing protists was seen in *Hodotermopsis sjostedti*, in which 13 out of 34 nests were missing one or two protist morphospecies (Kitade et al., 2012). These relatively high-variability termite species stand in contrast with *Coptotermes formosanus*, in which all 11 nests investigated contained all three of the recognized morphospecies, though an unidentified small trichomonad flagellate was also present in three of the colonies examined (Kitade et al., 2013). In our study, 21 out of 23 *H. tenuis* colonies were missing at least one of the eight protist species, 20 were missing two or more, and 11 were missing at least three species. By these metrics, the *H. tenuis* protist community has far higher inter-colony variability than any other reported to date. In *H. tenuis*, colonies missing protist species are the norm rather than the exception.

An important caveat to this comparison is the difference in methods. First, our combined morphological and molecular approach likely detects more species than a purely morphological approach, providing more opportunity to detect missing species. For example, the protist community of *C. formosanus* was long considered to consist of three species, *Pseudotrichonympha grassii*, *Holomastigotoides hartmanni*, and *Cononympha (Spirotrichonympha) leidyi* (Koidzumi, 1921), but molecular studies have detected an additional *Holomastigotoides* and an additional *Cononympha* species (Jasso-Selles et al., 2020; Nishimura et al., 2020). Thus, although the morphology-based study of *C. formosanus* found all three of the traditional morphospecies in all 11 colonies examined, the absence of a *Holomastigotoides* and/or *Cononympha* species could have gone unnoticed (Kitade et al., 2013). Next, molecular approaches have different biases. Amplicon sequencing may fail to detect some protist species, particularly those present in low abundance (Michaud et al., 2020). On the other hand, index hopping may result in protist species being erroneously detected in samples where they are absent (van der Valk et al., 2020). By considering only the colonies for which we sampled at least two termite individuals, and by employing dual indexing in our experimental design, we aimed to mitigate both of these potential biases, but we still likely detected more inter-colony variability than a morphological study alone would have.

CONCLUSION

Here, we have characterized the *H. tenuis* symbiont community as consisting of eight protist phylotypes. By combining 18S rRNA data from single isolated cells with high-throughput amplicon sequencing, we were able to confidently delimit these phylotypes and investigate their distribution across much of the geographical range of *H. tenuis*. None of the protist phylotypes was restricted to a single host haplotype group or a specific geographical region. Instead, we found that the protist symbiont community of *H. tenuis* is highly variable, with only two colonies harboring all eight phylotypes, and the vast majority of colonies missing at least two phylotypes. This result should be interpreted with caution due to limitations in our level of sampling, but it is so extreme that even a heavy bias, once corrected, would leave *H. tenuis* with the title of most variable protist community. We also investigated each phylotype's genetic diversity across host colonies and found an overall lack of correlation with geographical or host phylogenetic distances, unsurprisingly for samples from a single host species. However, the two exceptions highlighted theoretical mechanisms of protist speciation that could work within the context of a diverse and widespread host species such as *H. tenuis*. These mechanisms bring a new perspective to the complex patterns of coevolution between termites and their protist symbionts.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the manuscript/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DJ-S and GG performed microscopy. DJ-S performed single cell isolation. FM, NC, DJ-S, JS, and AR performed molecular work. FM, NC, and GG performed data analyses. PS, JŠ, DS-D, RS, and TC performed termite collection, maintenance, and preservation. GG performed study conception and manuscript writing. FM, NC, DJ-S, JS, AR, PS, JŠ, DS-D, RS, TC, and GG performed manuscript editing and improvement. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.640625/full#supplementary-material

Supplementary Figure 1 | Unrooted maximum likelihood phylogenetic tree of *Cononympha* 18S rRNA gene sequences from isolated cells and from amplicon profiling of termite gut contents. Nodes are intentionally compressed to show backbone structure. Isolated cell sequences determined in this study are nearly full length (∼1500 bp) and are indicated by red font. ASV sequences from amplicon profiling comprise only the V4–V5 regions of the 18S rRNA gene (∼450 bp) and are indicated by qiime2-generated sequence IDs in black text.

Supplementary Figure 2 | Unrooted maximum likelihood phylogenetic tree of *Holomastigotoides* 18S rRNA gene sequences from isolated cells and from amplicon profiling of termite gut contents. Nodes are intentionally compressed to show backbone structure. Isolated cell sequences determined in this study are nearly full length (∼1500 bp) and are indicated by red font. ASV sequences from amplicon profiling comprise only the V4–V5 regions of the 18S rRNA gene (∼450 bp) and are indicated by qiime2-generated sequence IDs in black text.

Supplementary Figure 3 | Unrooted maximum likelihood phylogenetic tree of *Cthulhu* 18S rRNA gene sequences from isolated cells and from amplicon profiling of termite gut contents. Isolated cell sequences determined in this study are nearly full length (∼1500 bp) and are indicated by red font. ASV sequences from amplicon profiling comprise only the V4–V5 regions of the 18S rRNA gene (∼450 bp) and are indicated by qiime2-generated sequence IDs in black text.

Supplementary Figure 4 | Unrooted maximum likelihood phylogenetic tree of *Pseudotrichonympha* 18S rRNA gene sequences from isolated cells and from amplicon profiling of termite gut contents. Nodes are intentionally compressed to show backbone structure. Isolated cell sequences determined in this study are nearly full length (∼1500 bp) and are indicated by red font. ASV sequences from amplicon profiling comprise only the V4–V5 regions of the 18S rRNA gene (∼450 bp) and are indicated by qiime2-generated sequence IDs in black text.

Supplementary Table 1 | Termite collection data, number of individuals assayed per collection, and protist taxonomy assignment of 18S rRNA ASV reads.

Supplementary Table 2 | Isolated cell codes, localities, length measurements, and number of clones sequenced.

Supplementary Table 3 | Diversity and distribution metrics of ASVs for each protist phylotype.

Supplementary Table 4 | Protist phylotype prevalence across samples.

Supplementary Video 1 | Live *Cthulhu* cell in motion displaying > 10 flagella in an anterior bundle. Scale bar 10 μm.

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Assessing the Australian Termite Diversity Anomaly: How Habitat and Rainfall Affect Termite Assemblages

Rebecca A. Clement[1,2], Habacuc Flores-Moreno[1], Lucas A. Cernusak[3], Alexander W. Cheesman[3,4], Abbey R. Yatsko[1], Steven D. Allison[5,6], Paul Eggleton[7†] and Amy E. Zanne[1†]*

[1] Department of Biological Sciences, George Washington University, Washington, DC, United States, [2] Computational Biology Institute, The Milken Institute School of Public Health, The George Washington University, Washington, DC, United States, [3] College of Science and Engineering, James Cook University, Cairns, QLD, Australia, [4] College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom, [5] Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA, United States, [6] Department of Earth System Science, University of California, Irvine, Irvine, CA, United States, [7] Life Sciences Department, The Natural History Museum, London, United Kingdom

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***Correspondence:**
Rebecca A. Clement
rebeclem@gmail.com

† These authors have contributed equally to this work and share senior authorship

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Termites are important ecosystem engineers in tropical habitats, with different feeding groups able to decompose wood, grass, litter, and soil organic matter. In most tropical regions, termite abundance and species diversity are assumed to increase with rainfall, with highest levels found in rainforests. However, in the Australian tropics, this pattern is thought to be reversed, with lower species richness and termite abundance found in rainforest than drier habitats. The potential mechanisms underlying this pattern remain unclear. We compared termite assemblages (abundance, activity, diversity, and feeding group composition) across five sites along a precipitation gradient (ranging from ∼800 to 4,000 mm annual rainfall), spanning dry and wet savanna habitats, wet sclerophyll, and lowland and upland rainforests in tropical North Queensland. Moving from dry to wet habitats, we observed dramatic decreases in termite abundance in both mounds and dead wood occupancy, with greater abundance and activity at savanna sites (low precipitation) compared with rainforest or sclerophyll sites (high precipitation). We also observed a turnover in termite species and feeding group diversity across sites that were close together, but in different habitats. Termite species and feeding group richness were highest in savanna sites, with 13 termite species from wood-, litter-, grass-, dung-, and soil-feeding groups, while only five termite species were encountered in rainforest and wet sclerophyll sites—all wood feeders. These results suggest that the Australian termite diversity anomaly may be partly driven by how specific feeding groups colonized habitats across Australia. Consequently, termites in Australian rainforests may be less important in ecosystem processes, such as carbon and nutrient cycling during decomposition, compared with termites in other tropical rainforests.

Keywords: Isoptera, community assembly, ecosystem engineers, Blattodea, termite community assembly, carbon cycle, Australian tropical forest, savanna

INTRODUCTION

Termites play an important but under-recognized role in the functioning of tropical ecosystems (Bonachela et al., 2015; Ashton et al., 2019; Elizalde et al., 2020). Though best known as pests, fewer than 10% of the 3,000 termite species are categorized as such (Rouland-Lefèvre, 2011), while the majority are integral in carbon and nutrient cycling, breaking down wood, soil, grass and leaf litter. These activities contribute a significant amount of methane (CH_4) to the atmosphere, estimated at 1–3% of the global CH_4 budget (Nauer et al., 2018). In addition to CH_4 release, their nitrogen-fixing activities and redistribution of organic matter, combined with soil movement and compaction through nest-building, provide hotspots of nutrients that support significant biological activity, especially in nutrient deficient or drought affected areas (De Oliveira-Filho, 1992; Jouquet et al., 2011; Ashton et al., 2019). Therefore, understanding termite distributions is key to understanding biogeochemical cycling during decomposition (Sugimoto et al., 2000), especially in the tropics.

In addition to irregular distributions over local space, termite biomass and abundance vary globally (Eggleton et al., 1996). Estimates have been made that termites comprise nearly 10% of animal biomass in the tropics, where they are most diverse and abundant, and are responsible for >55% of decomposition (Bignell, 2006; Jones and Eggleton, 2010; Griffiths et al., 2019). In tropical ecosystems, termite diversity, abundance and biomass increase with rainfall with the greatest values in lowland tropical rainforests (Eggleton, 2000; Bignell, 2006; Davies et al., 2015). For example, in some tropical African rainforests, termites make up 80% of insect biomass, with biomass as high as 300 kg ha^{-1} and richness as high as 67 species ha^{-1} (Sugimoto et al., 2000; Davies et al., 2003; Dahlsjö et al., 2014). Estimates for savanna or grassland habitats are <200 kg ha^{-1} termite biomass with sometimes fewer than eight species ha^{-1} (Sugimoto et al., 2000; Davies et al., 2015). Although patterns of termite species richness and abundance are affected by many local environmental factors as well as vegetation type, elevation, and latitude, in general, all metrics of termite prevalence in the tropics increase with increasing rainfall.

In contrast, Australia does not appear to follow the patterns observed in other tropical regions, as Australian termite abundance and diversity are thought to be higher in dry tropical ecosystems compared with rainforests, a pattern defined here as the Australian termite diversity anomaly (part of the global termite functional diversity anomaly) (Eggleton, 2000; Davies et al., 2003; Dahlsjö et al., 2014). This anomaly may be due to taxonomic disparities: in Australia, there is a marked deficiency of two of the most abundant and speciose termite feeding groups worldwide—the soil feeders and fungus farmers (Krishna et al., 2013). Whereas, in South/Central America, Africa, and Southeast Asia, soil-feeding and/or fungus-farming termites comprise a large portion of the total termite diversity (Davies et al., 2003), in Australia, these feeding groups are rare (soil feeders) or absent (fungus farmers). Their absence could explain why, despite being sampled extensively, Australian rainforests are very termite species-poor in comparison with other tropical rainforests (Calaby and Gay, 1959; Watson and Gay, 1991). Due to the lack of particular termite feeding groups, there is reason to believe that precipitation plays a different role in shaping termite assemblages in Australia as compared with other tropical regions. Preliminary evidence suggests that although termite activity is impacted by rainfall, it may be in a manner counter to other tropical regions (Cheesman et al., 2017). While we have qualitative descriptions of this anomaly, to date we lack quantitative assessments of how termites change with shifts in rainfall.

In this study, we quantified these responses across a wide rainfall spectrum at a local scale (<100 km) to explore which aspects of termite communities represent the termite diversity anomaly. We surveyed termite assemblages in tropical North Queensland measuring differences in termite abundance, activity, taxonomic richness and feeding group diversity across different habitats and rainfall levels. Under the termite diversity anomaly, we predicted that compared with rainforest, drier savanna sites would have greater termite (i) abundance, (ii) activity in wood pieces, and (iii) species and feeding group density. We explore the changes in termite assemblages across contrasting habitat types and examine the potential causes and consequences of the Australian termite diversity anomaly. We compare our findings with other published studies to contextualize how Australian termites respond to changes in rainfall when compared with other tropical regions.

MATERIALS AND METHODS

Study Sites

We sampled termites from five sites across a 100 km transect in North Queensland, Australia, with annual rainfall ranging from 800 to 4,260 mm: (1) Pennyweight—dry savanna (Sav1), (2) Station Creek—open Eucalyptus woodland/savanna (Sav2), (3) Mt. Lewis—wet sclerophyll forest (Scl1), (4) Mt. Lewis—upland rainforest (Rft1), and (5) Daintree Rainforest Observatory–lowland rainforest (Rft2) (**Figure 1** and **Table 1**). These sites are connected within continuous forest cover; at increasing distance from the coast, rainfall decreases, and habitats shift. All sites are located on the traditional homeland of the Kuku-Yalangi people. The first four of these sites are situated on Australian Wildlife Conservancy's Brooklyn Sanctuary[1], and the Daintree Rainforest Observatory[2] is part of James Cook University. Three of the sites (Sav2, Scl1, and Rft1) change habitat type within 5 km of each other. All sites experience a distinct wet and dry season, with 77% of rainfall occurring between November and April (Cheesman et al., 2017). Because of this seasonality, we conducted termite sampling in both July 2019 (dry season), and December of either 2018 or 2019 (wet season). Three of the sites (Sav1, Sav2, and Scl1) had been burned 6 months to a year prior to surveys, and both of the savanna sites (Sav1 and Sav2) experience low-density feral cattle grazing. Rainfall was estimated using the 20-year trends (2000–2020) of annual rainfall of the closest grid point using SILO LongPaddock gridded data (Jeffrey et al., 2001).

[1]https://www.australianwildlife.org/where-we-work/brooklyn/

[2]https://www.jcu.edu.au/daintree

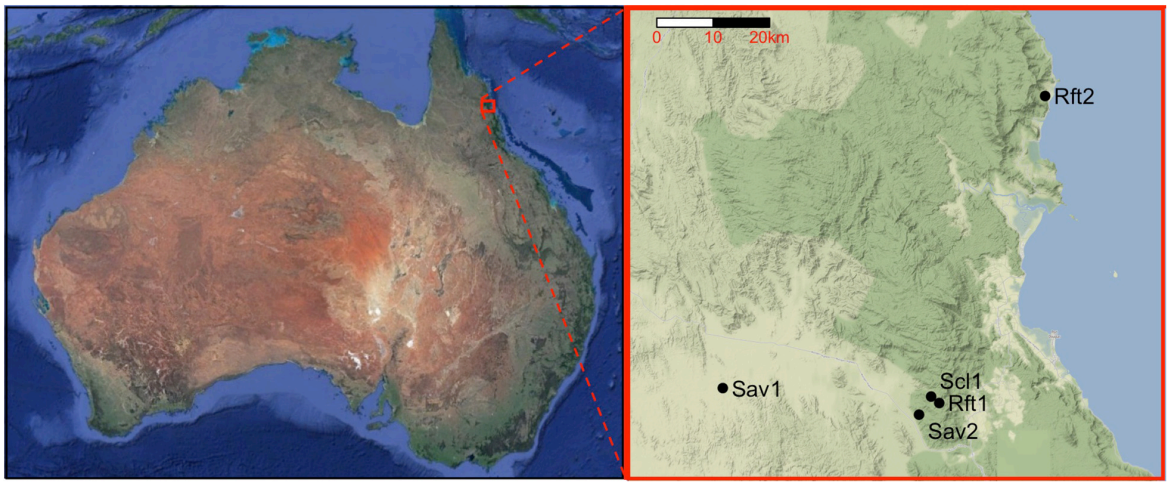

FIGURE 1 | Location of the five sites in the study area in Northern Queensland, Australia: Sav1 (Pennyweight savanna), Sav2 (Station Creek savanna), Scl1 (Mt. Lewis sclerophyll), Rft1 (Mt. Lewis rainforest), and Rft2 (Daintree rainforest). Australia map by Google Earth, earth.google.com/web/. Local map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

TABLE 1 | Habitat type, rainfall, elevation, distance from coast, and localities for sites surveyed in this study.

	Sav1	Sav2	Scl1	Rft1	Rft2
Site name	Pennyweight station	Station Creek	Mt. Lewis sclerophyll	Mt. Lewis rainforest	Daintree rainforest
Habitat type	Dry savanna	Open eucalyptus woodland/savanna	Wet sclerophyll	Rainforest	Rainforest
Rainfall estimate (mm)	800	1,250	1,480	1,500	4,260
Elevation (m)	315	420	935	980	70
Distance from coast (km)	60	24	21	20	2
Coordinates	−16.5746°N 144.9163°E	−16.610°N 145.2400°E	−16.5830°N 145.2620°E	−16.5933°N 145.2743°E	−16.1012°N 145.4444°E

Termite Sampling

Routine termite sampling normally includes four microhabitats: visible mounds, dead wood, soil, and arboreal habitats (Jones et al., 2005; Davies et al., 2021), however, since Australia lacks soil-feeding termites (Eggleton, 2000), soil pits seldom have termites in them (Dawes-Gromadzki, 2005). Additionally, methods for sampling arboreal termites are difficult and dangerous (Jones et al., 2005). To maximize termite encounters per effort, in this study, we targeted termite field surveys in termite mounds and dead wood only (**Table 2** and **Supplementary Table 1**).

Abundance: Termite Mound Sampling

Termites build mounds or nests in various shapes using soil mixed with termite saliva, soil and feces, ultimately constructing shelters for termite colonies and food storage space (Jouquet et al., 2016). As recommended for areas with dominant mound-building termites, we conducted mound surveys following Davies et al. (2021). We set up 50 m × 50 m plots at each of our five sites and divided them into 25 subplots (10 m × 10 m) for more accurate mound sampling and an exhaustive search of mounds in the given area. In each subplot, we mapped and recorded all mounds that were on the ground or low on trees as a measure of termite abundance (**Figure 2**). Each mound was initially measured (maximum height and maximum diameter), subsequently a small portion of mound material was removed to expose the termites. Where possible, we collected two soldiers and five workers from each mound preserved in 96% ethanol. Mound sampling took place in December 2018 (wet season) with the exception of the dry savanna site, Sav1, which was inaccessible

TABLE 2 | Summary of termite encounters, taxonomic richness, and feeding group counts from surveys at Pennyweight dry savanna (Sav1), Station Creek open woodland/savanna (Sav2), Mt. Lewis wet sclerophyll (Scl1), Mt. Lewis rainforest (Rft1), and Daintree rainforest (Rft2).

Site	Sav1	Sav2	Scl1	Rft1	Rft2	Total
Encounters						
All surveys	**50**	**79**	**4**	**1**	**4**	**138**
Mound surveys only	44	68	2	0	1	115
Dead wood surveys only	6	11	2	1	3	23
Richness						
Species from all surveys	**6**	**11**	**2**	**1**	**4**	**19**
Mound surveys only	4	8	1	0	1	11
Dead wood surveys only	3	5	1	1	3	10
Count of genera	4	6	2	1	4	10
Count of families	2	2	1	1	2	3
Count of feeding groups	4	4	1	1	1	5

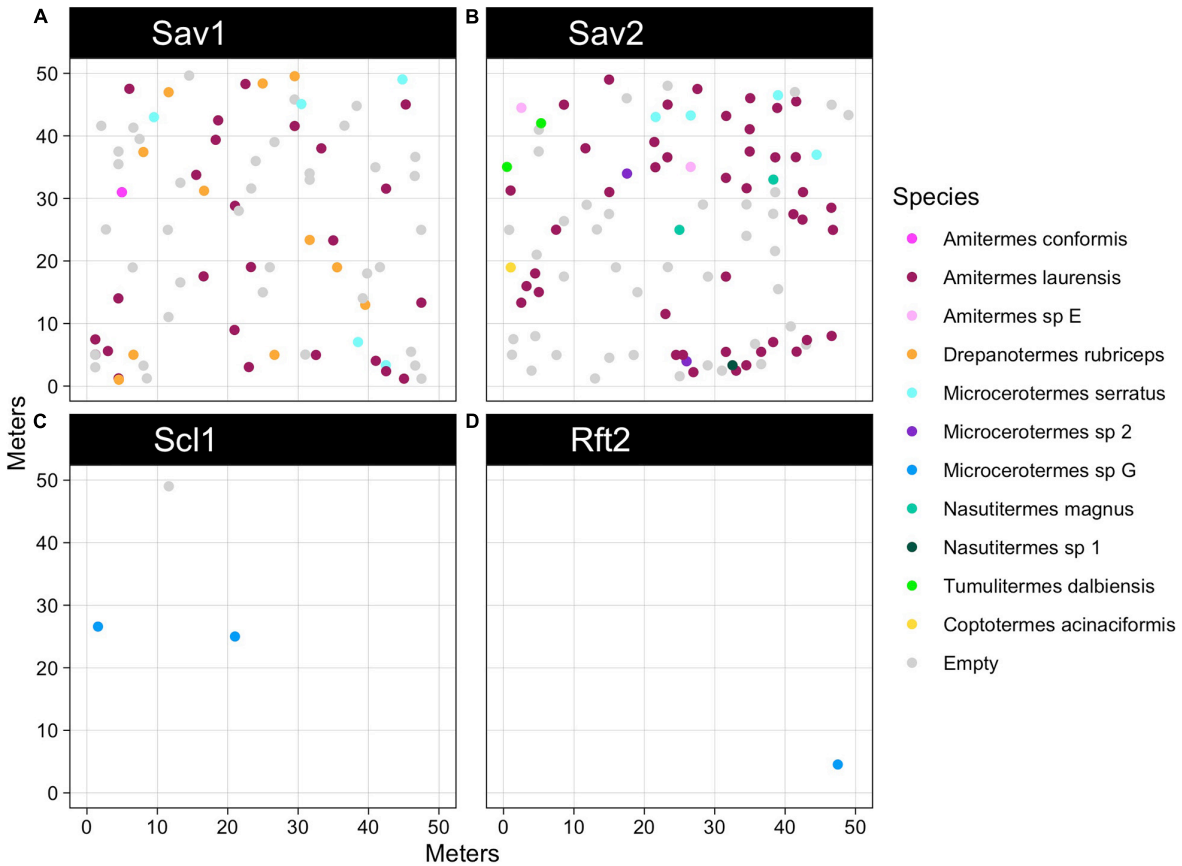

FIGURE 2 | Mound abundance and mapping for each site: **(A)** Pennyweight savanna (Sav1) n = 80 mounds, **(B)** Station Creek savanna (Sav2) n = 98 mounds, **(C)** Mt. Lewis wet sclerophyll (Scl1) n = 3 mounds, **(D)** Daintree rainforest (Rft2) n = 1 mound. Mt. Lewis rainforest (Rft1) is not shown here because there were no mounds in the plot. Termite mounds colored by species, mapped across sites in quarter hectare plots. Gray points indicate empty mounds where no termites could be collected.

at that time, and was sampled in July 2019. Because of the longevity of mounds, we do not expect that this difference in sampling season biased the data. We avoided sampling during the hottest part of the day when termite activity may decline in mounds. We compared the mean and standard deviation of mound abundance between savanna and non-savanna habitat types (wet sclerophyll and rainforest).

Activity: Dead Wood Sampling

Dead wood serves as both food and shelter for many termites (Korb, 2007). In each 50 m × 50 m plot (described above), we used a line intersect method (Warren and Olsen, 1964; Van Wagner, 1968; Kimber and Eggleton, 2018) to assess termite presence and damage in dead wood as a measure of termite activity. We laid out two 50 m transects in a randomly assigned position across the plots in both the wet season (Dec. 2018 or Dec. 2019) and the dry season (July 2019) for a total of four transects per plot. For each piece of dead wood with a diameter >2 cm that intersected a transect, we measured the length and diameter and used a drywall hammer to break the wood open in three places to search for termites. When one or more termites from the same morphospecies were present in a piece of wood, it was considered a single termite encounter. When more than one termite morphospecies was encountered in the same piece of wood, we considered it a separate encounter. For each termite encounter, we collected five workers and two soldiers into a 2 mL tube of 96% ethanol. We also recorded signs of damage on the wood from termites (piping, runways or termite tunnels) and/or fungus (white rot, fungus fruiting bodies or discoloration). We measured dead wood volume using the method described in Kimber and Eggleton (2018). Termite activity in Australia is influenced by seasonality, with the highest levels of termite activity during the transition period from wet to dry (Dawes-Gromadzki and Spain, 2003; Davies et al., 2012); we sampled dead wood at the end of both the wet and dry seasons to capture the maximum amount of termite activity in dead wood.

Termite activity was defined as (a) average number of termite encounters per 50 m transect (**Figure 3**), (b) number of termite encounters per dead wood volume, (c) percent of wood pieces with termites present, and (d) percent of dead wood pieces showing termite damage (**Table 3**). As wood-dwelling microbes, especially fungi, are the main alternate decay agents to termites in these systems, we also compared percentages of wood pieces damaged by fungi across habitat types. To assess

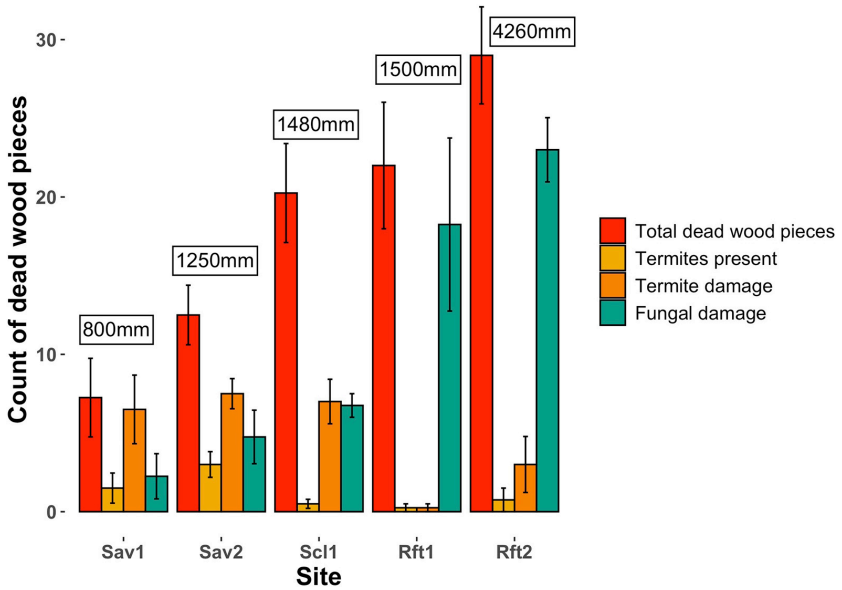

FIGURE 3 | Average count of pieces of dead wood, dead wood with termites present, deadwood with termite damage, and dead wood with fungal damage, encountered across four 50 m transects from savanna (left) to rainforest (right) sites: Sav1 (Pennyweight savanna), Sav2 (Station Creek savanna), Scl1 (Mt. Lewis sclerophyll), Rft1 (Mt. Lewis rainforest), and Rft2 (Daintree rainforest). Error bars show standard error. Measurements in boxes show rainfall at each site.

the relationship between rainfall and habitat type and counts of termite encounters, wood pieces with termite damage, wood pieces with fungal damage per transect and total wood pieces, we built a generalized linear mixed model with habitat and rainfall as fixed predictors and sites as a random effect. We used a negative binomial distribution to account for zero-inflated variables, and a Poisson distribution for count of wood pieces, which was not zero inflated. We also fitted a generalized linear mixed-effects model to each metric jointly with its respective wood count using habitat and rainfall as fixed predictors and sites as a random effect using a binomial distribution. All statistical analyses were performed using R version 3.6.3 (R Core Team, 2020).

In the rainforest and wet sclerophyll sites, we performed additional searching beyond the four dead wood transects because there were very few mounds. We broke open all pieces of dead wood in five 10 × 10 m subplots and recorded the total number of pieces of wood encountered and termite presence/absence. We collected termites found in these wood pieces where present.

Termite Identification

After affirming generic assignment using morphological features of soldiers, we used DNA barcoding for accurate species identification (Davies et al., 2021). For each of the 138 termite collections, we extracted the DNA from one worker using a Qiagen DNeasy Blood and Tissue kit. We amplified the *COII* gene from each termite using A-tleu and Btlys primers (Inward et al., 2007). Samples were sequenced at Eurofins Genomics. Resulting paired sequence contigs were assembled using Geneious and matched with the closest-related species on NCBI using BLAST (Johnson et al., 2008). If sequences had a unique >97% identity with a sequence from a described species on GenBank, they were assigned to that species. An additional 205 *COII* sequences from congeneric and Australian termites were downloaded from GenBank (**Supplementary Table 2**) and aligned with the sequences from this study using MAFFT (Katoh and Standley, 2013). We used IQTree (Nguyen et al., 2015) to reconstruct a maximum likelihood tree of the combined sequences. If a *COII* sequence from this study fell within a described species clade on the *COII* tree, we classified it as the given species. Otherwise, we labeled each cluster of our samples as sp, sp 1, sp 2, etc. A few of these clusters matched at high (>98%) sequence similarity with prospective species from a recent genomic study (Bourguignon et al., 2017), and we assigned them accordingly for consistency (*Amitermes* sp. E, *Microcerotermes* sp. G, *Microcerotermes* sp. I). Generated termite *COII* sequences have been deposited in GenBank under accessions MW772943-MW773117 (**Supplementary Table 1**).

We also used IQTree to generate a maximum likelihood tree of the *COII* sequences unique to this study with automatic model selection (GTR + F + G4), 1000 ultrafast bootstraps and an SH-like approximate likelihood ratio test with 1,000 replicates. Species, habitat, and feeding group were mapped onto the tips of the tree using iTOL (Letunic and Bork, 2019) to visualize taxonomic changes across habitat (**Supplementary Figure 1**).

Termites are on loan from James Cook University and will be lodged at the Natural History Museum, London, United Kingdom.

Taxonomic and Group Diversity

Taxonomic richness was measured at the species, genus, and family level (**Table 2**). Each species was assigned to one of the

following feeding groups: wood-feeder, wood-and-dung feeder, litter-and-dung feeder, litter-and-grass feeder, grass feeder or soil feeder, based on literature from an Australian termite trait database (Cornwell, unpublished). Feeding group richness was measured as the number of feeding groups at each site. We compared termite communities across sites using the Simpson beta-diversity index (**Supplementary Table 3**) and visualized site differences using non-metric multidimensional scaling (nMDS) based on Bray-Curtis dissimilarity index (**Supplementary Figure 2**).

We fitted multivariate generalized linear models to predict the effect of combined and individual environmental variables (i.e., rainfall, elevation, latitude, distance from the coast) on termite species presence and abundance using R package mvabund (Wang et al., 2012). For the species presence models we used a binomial distribution; for the abundance models we used a Poisson distribution. For abundance and presence data we ran separate single predictive models for all species at all sites, assuming different environmental response for different species (GLM-LASSO, Brown et al., 2014). In these models the species presence or abundance were the response variables, and the environmental variables were predictors. These models apply a lasso penalty which sets non-significant terms to zero [lasso penalty = log(number of sites)].

RESULTS

Through our mound and dead wood surveys, we encountered termites 138 times across our five sites in North Queensland, including specimens from 19 species, ten genera, three families and five feeding groups. The open eucalyptus woodland/savanna site (Sav2) had the highest species richness and number of encounters with 11 species and 79 encounters, followed by the dry savanna (Sav1) with 6 species and 50 encounters. The upland and lowland rainforests (Rft1, Rft2) and the sclerophyll site (Scl1) had fewer encounters and termite species (**Table 2**).

Mound Abundance

We observed a major reduction in termite mound abundance from savanna to forest habitats (**Figure 2**). We found that the mean mound abundance for savanna sites ($M = 359$ mounds ha^{-1}, $SD = 55.2$) was 68 times greater than the mound abundance for non-savanna sites ($M = 5.3$ mounds ha^{-1}, $SD = 6.1$). In the savannas, we found densities of 320 mounds ha^{-1} (Sav1) and 398 mounds ha^{-1} (Sav2), compared to 12 mounds ha^{-1} in the sclerophyll site (Scl1), zero in upland rainforest (Rft1), and four mounds ha^{-1} in lowland rainforest (Rft2). Most of the mounds sampled in either of the savannas were <1 m tall conical mounds, built by *Amitermes laurensis* (78/112 or 68% of occupied mounds), but termites from 11 species overall were represented in mounds across sites including *Amitermes conformis*, *Amitermes* sp. E, *Drepanotermes rubriceps*, *Microcerotermes serratus*, *Microcerotermes* sp. 2, *Microcerotermes* sp. G, *Nasutitermes magnus* and *Nasutitermes* sp. 1, *Tumulitermes dalbiensis*, and *Coptotermes acinaciformis* (**Table 4**). Of the savanna mounds in Sav1 ($n = 80$) and Sav2 ($n = 98$), only 52.5% ($n = 44$) and 69.4% ($n = 68$), respectively, were occupied by termites. In Scl1, only two of the three mounds were occupied.

Termite Dead Wood Activity

We encountered the most pieces ($M = 29$) and highest volume ($M = 192.3$) of dead wood from transects in our lowland rainforest site Rft2. There was a positive effect ($p < 0.05$) of rainfall on pieces of wood per transect with fewer pieces of wood per transect in savanna habitats ($p < 0.05$). However, despite having fewer pieces of wood, we found both a higher number of termites in dead wood per transect ($p < 0.05$) and a higher proportion of termite encounters per wood count ($p < 0.05$) in savanna habitats (**Table 3**). Rainfall had a positive effect ($p < 0.05$) on the number of pieces of wood damaged by termites, but only after accounting for the negative effect of rainforest habitat on termite damage ($p < 0.05$). For either of the rainforest sites Rft1 and Rft2, only one of the four transects at each site resulted in termite encounters despite there being 18–36 wood pieces per transect. In savannas, the majority (60–90%) of pieces of dead wood were damaged by termites compared with 34% of wood pieces in the sclerophyll site, and 1 and 10% of the pieces of wood in the two rainforest sites, respectively (**Table 3** and **Figure 3**). Even though we encountered more dead wood and there was more primary productivity in rainforest and sclerophyll transects, wood in savannas had more frequent termite dead wood damage and termites were more abundant. In contrast, fungal damage was higher in the rainforest compared to savannas ($p < 0.05$). Most (80%) of rainforest dead wood showed signs of fungal damage compared to only 30–40% of savanna and sclerophyll dead wood pieces that were damaged by fungi.

Seasonality only affected termite activity in dead wood in rainforest sites. In rainforests Rft1 and Rft2, we only found

TABLE 3 | Termite encounters, percent occupancy or damage by termites or fungus, and items/volume of wood per transect from driest to wettest sites: Pennyweight dry savanna (Sav1), Station Creek open woodland/savanna (Sav2), Mt. Lewis wet sclerophyll (Scl1), Mt. Lewis rainforest (Rft1), and Daintree rainforest (Rft2).

Site	Sav1	Sav2	Scl1	Rft1	Rft2
Average number of termite encounters/transect	1.5	3	0.5	0.25	0.75
Termite encounters per volume dead wood (m^3/ha)	1.02	0.61	0.0054	0.0085	0.00098
Percent dead wood pieces occupied by termites	21%	24%	2%	1%	3%
Percent wood pieces damaged by termites	90%	60%	35%	1.1%	10%
Percent wood pieces damaged by fungus	31%	38%	33%	83%	80%
Average pieces of wood/50 m transect	7.25	12.5	20.25	22	29
Volume dead wood (m^3/ha)	1.47	4.93	92.4	29.3	769.2

TABLE 4 | Termite species found in five sites in North Queensland [Pennyweight dry savanna (Sav1), Station Creek open woodland/savanna (Sav2), Mt. Lewis wet sclerophyll (Scl1), Mt. Lewis rainforest (Rft1), and Daintree rainforest (Rft2)] with feeding groups and sampling method: Mound, dead wood, or both.

	Savanna		Sclerophyll	Rainforest				
Species	**Sav1**	**Sav2**	**Scl1**	**Rft1**	**Rft2**	**Total**	**Feeding group**	**Sampling method**
Lower termites								
Kalotermitidae								
Neotermes sp.	–	–	–	1 (+2)	–	**1**	Wood	Dead wood
Rhinotermitidae								
Coptotermes acinaciformis	–	1	–	–	–	**1**	Wood	Mound
Coptotermes dreghorni	–	–	–	–	1	**1**	Wood	Dead wood
Heterotermes sp.	1	1	–	–	(+2)	**2**	Wood, dung	Dead wood
Parrhinotermes sp.	–	–	–	–	1 (+4)	**1**	Wood	Dead wood
Schedorhinotermes sp.*	–	–	(+3)	–	(+1)		Wood, dung	Dead wood
Higher termites (Termitidae)								
Amitermes group								
Amitermes conformis	1	–	–	–	–	**1**	Litter, dung	Mound
Amitermes laurensis	27 (+3)	51	–	–	–	**78**	Litter, dung	Mound
Amitermes sp. E	–	3	–	–	(+2)	**3**	Wood, dung	Mound
Drepanotermes rubriceps	12 (+1)	(+2)	–	–	–	**12**	Grass, litter	Mound
Microcerotermes serratus	8	6	–	–	–	**14**	Wood, dung	Both
Microcerotermes sp. 1	1	3	–	–	–	**4**	Wood	Dead wood
Microcerotermes sp. 2	–	6	–	–	–	**6**	Wood	Both
Microcerotermes sp. G	–	–	2 (+2)	–	1 (+4)	**3**	Wood	Mound
Microcerotermes sp. I	–	2	–	–	–	**2**	Wood	Dead wood
Nasutitermes group								
*Nasutitermes graveolus**	–	(+1)	–	–	–		Wood, litter	Mound
Nasutitermes magnus	–	2	–	–	–	**2**	Grass	Mound
Nasutitermes sp. 1	–	1	–	–	–	**1**	Grass	Mound
Nasutitermes sp. 2	–	–	2 (+4)	–	–	**2**	Wood	Dead wood
*Nasutitermes walkeri**	–	–	–	–	(+7)		Wood	Dead wood
Tumulitermes dalbiensis	–	3	–	–	–	**3**	Litter, dung	Dead wood
Termes group								
*Macrognathotermes errator**	–	(+2)	–	–	–		Soil	Mound
Paracapritermes sp.	–	–	–	–	1 (+2)	**1**	Wood	Dead wood
Survey encounters	**50**	**79**	**4**	**1**	**4**	**138**		
Species richness	6	11	2	1	4	19		

*Numbers in parentheses (+) and species with asterisks * were found in additional searching (not as part of either of the main surveys) but reported here to highlight all of the diversity encountered. The bold species designate which groups the species are in.*

termites present or termite damage in dead wood transects at the end of the wet season. In the savanna and sclerophyll sites, seasonality was not related to termite activity. Ten species of termites from 23 total encounters were obtained from dead wood surveys across the five sites.

From additional searching of dead wood (non-survey), there were only four termite encounters in 144 pieces of dead wood in lowland rainforest Rft2 (3%), two termite encounters in 215 pieces of dead wood in upland rainforest Rft1 (1%) and eight encounters in 138 pieces of dead wood in the sclerophyll Scl1 (6%).

Termite Taxonomic Richness

We collected 19 termite species through mound and dead wood surveys, and an additional four species through non-survey searching. This total includes close to half of the known species diversity of the surrounding areas (50–65 species) (Watson and Abbey, 1993; Abensperg-Traun and Steven, 1997)[3]. While our surveys did not capture all termite diversity in the area (for example, we did not recover *Mastotermes darwiniensis* in these surveys though it was sighted in Sav1 on a previous trip), we sampled a similar proportion to what Jones and Eggleton (2000) estimate for typical termite surveys in Borneo, Malaysia and Cameroon (31–36% of termite species).

Of the 19 species collected from our mound and dead wood surveys, 14 were higher termites; nine were in the Amitermes group, four were from the Nasutitermes group, and one was from the Termes group. Five were lower termites—four from the family Rhinotermitidae and one from the family Kalotermitidae (**Table 4**). Surveys from all sites resulted in 10 genera of termites,

[3]GBIF.org

with the highest generic diversity at Sav2 with 6 genera, and 4 at both Sav1 and Rft2.

The open eucalyptus woodland/savanna site (Sav2) had the most species at any site with 11 total species, with the second highest species richness at the dry savanna (Sav1, 6 species, **Table 2**). Although we only encountered termites four times in surveys in the Daintree rainforest (Rft2), each encounter was from a different species for four total species. The Sclerophyll site (Scl1) had only two species, and the site with the fewest species was the upland rainforest (Rft1), which had a single individual, *Neotermes* sp. Only one species of *Neotermes* is recorded in Australia, *Neotermes insularis*, but because the match with the *N. insularis* on GenBank was less than 90%, we expect this is a new species, at least for the continent of Australia.

Additional (non-survey) searching (**Table 4** in parentheses) recovered four additional species for a total of 23 species throughout all 5 sites, reinforcing that our surveys uncovered a majority, but not all of the species present in these sites. Additional termite species collected were *Amitermes* sp. E, *Heterotermes* sp., *Nasutitermes walkeri*, and *Schedorhinotermes* sp. from Rft2, *Schedorhinotermes* sp. from Scl1, and *Drepanotermes rubriceps*, *Macrognathotermes errator*, and *Nasutitermes graveolus* from Sav2.

Out of the 183 *COII* sequences from our samples, only 114 (<65%) matched to a described species on GenBank with more than 97% identity, suggesting the need for more barcode sequencing and associated termite taxonomic work in this region.

Taxonomic Turnover Across Sites and Habitat Types

Although the dry savanna (Sav1) and open woodland/savanna (Sav2) shared four species and the wet sclerophyll (Scl1) and lowland rainforest (Rft2) shared one species, there was a complete turnover in species among the sites of different habitat types that were within 5 km of each other (Sav2, Scl1, and Rft1) (**Figure 1**, **Supplementary Figure 2**, and **Supplementary Table 3**). Four species were shared between savanna sites: *Microcerotermes serratus*, *Microcerotermes* sp. 1, *Heterotermes* sp., and *Amitermes laurensis*. One species, *Microcerotermes* sp. *G* was shared between Rft2 and Scl1, and is a close sister group to *Microcerotermes serratus*, shared between Sav1 and Sav2.

We observed a compositional difference in the termites across the habitat types. Of the five rainforest species encountered through surveys, three of them were lower termites. Two of the thirteen savanna species were lower termites (**Table 4** and **Supplementary Figure 1**). Species of genera *Microcerotermes*, *Nasutitermes*, and *Coptotermes* were found across savanna and rainforest sites.

With the additional non-survey collections included, Rft2 shares two species with Sav2 and two species with Scl1, and Sav2 and Sav1 share five species.

Termite Diversity and Abundance in Response to Environmental Variables

We found evidence for a significant effect of environmental variables on termite species presence ($p = 0.005$). Both habitat type ($p = 0.009$) and elevation ($p = 0.005$) are significantly associated with termite local species diversity. Rainfall ($p = 0.008$), habitat type ($p = 0.001$) and distance from the coast ($p = 0.007$) are significantly associated with termite species turnover across communities, but the effect of rainfall and distance from coast on local species richness is statistically unclear ($p > 0.05$). There was also no clear effect of latitude on local species diversity or species turnover ($p > 0.05$).

Our GLM-LASSO model predicts that within our sites, species differ in their response to environmental variables in both presence and abundance (**Supplementary Figures 3A,B**).

Feeding Group Richness

Of the 138 termite encounters from this study, 22 were wood-feeders, 19 were wood-and-dung feeders, 3 were grass feeders, 12 were grass-and-litter feeders, and 82 were litter-and-dung feeders (**Table 4**). The savanna habitats had all five feeding groups. In the rainforest and sclerophyll sites, we found only wood-feeders. None of the 19 termite species encountered through surveys in this study were soil feeders or fungus-farming termites. However, one species of soil-feeding termite, *Macrognathotermes errator*, was encountered in additional non-survey collections in Sav2.

DISCUSSION

Through this study, we quantified differences in termite abundance, activity, taxonomic richness, and feeding group diversity across habitat types and rainfall levels in North Queensland, Australia as a documentation of the termite diversity anomaly. Our results confirm quantitatively and more comprehensively the earlier findings that in Australia, termite mound abundance, dead wood activity, and species and feeding group richness is greater in savanna and limited in rainforest sites, contrasting with patterns seen in many other tropical regions (Bignell, 2006). Additionally, we found a complete turnover of observed species across habitat types despite sites being within only a few kilometers of each other. Finally, we found differences in feeding group distribution with rainforests supporting only wood-feeding termites, while savannas had wood, litter, dung, grass and soil feeders. Below, we contextualize the results of our study as we examine the relationship between termite diversity and rainfall globally and inspect the patterns that comprise the Australian anomaly. We discuss potential mechanisms for the Australian termite diversity anomaly and how these patterns in turn affect decomposition in the Australian tropics. The Australian termite diversity anomaly combined with local environmental factors affect termite distributions at a local and continent-wide scale in at least four aspects.

Patterns of Termite Assemblages in Australia

Australian Termite Species Richness Is Not Shaped by Rainfall

Similar to earlier work showing a positive correlation between generic richness and net primary productivity across three continents (Eggleton et al., 1994), our analysis of termite species

richness patterns from surveys with methods similar to ours shows that globally, there is a significant positive correlation between termite species richness and rainfall (**Figure 4** and **Supplementary Table 4**). However, our surveys in tropical Australia (**Figure 4**, circled triangles) show that while rainfall can have a positive effect on incidences of termite damage in wood within a habitat type, overall it is not associated with local termite diversity. The overall pattern of increased termite species richness with rainfall is driven by large, significant relationships seen in surveys in Africa and Central/South America, while in Australia, Madagascar, and Southeast Asia, these two variables are unrelated. This suggests that the termite diversity anomaly could apply to other continents, and perhaps there is a range of responses across continents of how tightly termite diversity is tied to rainfall. The anomaly is most extreme in Australia; sites in other tropical rainforests had 10–20 times the species richness of the wettest rainforest site from our study (Constantino, 1992; Eggleton et al., 1995; Gathorne-Hardy et al., 2001), and across habitat types, Australian termite species richness is lower than on other continents. This indicates that Australian termite richness is not shaped by rainfall in the same way it is on other continents, or at least not to the same degree. Further investigation is needed to gauge to what extent the anomaly shapes relationships between termite species and rainfall on other continents.

At a local scale, other environmental factors influenced species richness in our sites more than rainfall. Both habitat type and elevation contributed significantly to local species diversity. Savanna sites had higher species richness than any of the other sites. The two sites with the lowest species diversity (Scl1 and Rft1) were 500–900 m higher elevation than any of the other sites; termite species richness on other continents is known to drop with even a 100 m increase in elevation (Gathorne-Hardy et al., 2001). Disturbance may have also played a role in species richness at our sites. For example, we encountered fewer than half as many species in dry savanna Sav1 as open woodland/savanna Sav2, which differed only 450 mm in rainfall, but Sav1 had more frequent cattle visitation and fire frequency. Termite species richness tends to be lower in heavily grazed areas (Holt et al., 1996), and we noticed several termite mounds had been toppled, potentially by cows. Although latitude is a good predictor of termite richness on other continents, Australia is known to differ in this regard (Abensperg-Traun and Steven, 1997), and our analysis also showed no significant effect of latitude on either alpha or beta species diversity. Our sites however, showed very little variation in latitude. Additionally, distance from the coast and rainfall contributed significantly to beta diversity. Our predictions show that species respond differentially to environmental variables implying that some species respond strongly to rainfall, but most of the species in our sites are better predicted by elevation, habitat type and disturbance.

Mound Abundance and Termite Activity in Dead Wood Are Greatest in Savanna Sites

Termite activity and mound abundance levels from our sites were within the known range of other savanna sites in North Queensland and Northern Territory (Holt et al., 1996; Dawes-Gromadzki and Spain, 2003; Dawes-Gromadzki, 2008). Termite mound densities were much higher in savannas than forested areas, with *Amitermes laurensis* responsible for much of this mound-building activity. This litter-and-dung-feeding termite

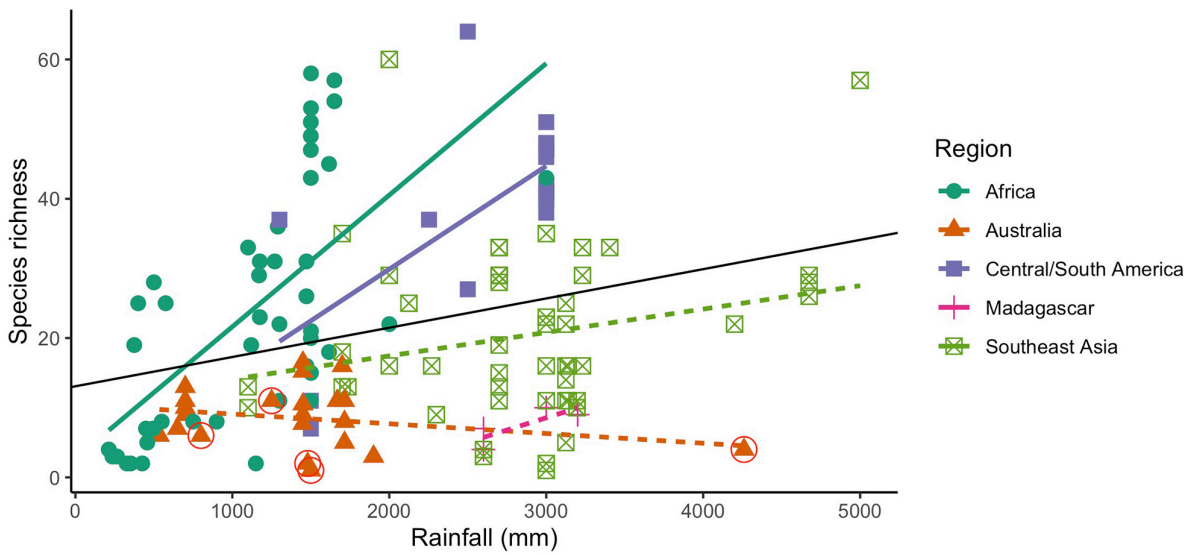

FIGURE 4 | Relationship between termite species richness and rainfall from 143 tropical sites from 31 studies of surveys in 5 different regions (**Supplementary Table 4**) (Sands, 1965; Mathews, 1977; Wood, 1977; Abe and Matsumoto, 1979; Collins, 1980; Buxton, 1981; Wood et al., 1982; Braithwaite et al., 1988; Constantino, 1992; Holt et al., 1996; Gathorne-Hardy et al., 2001; Davies et al., 2003; Dawes-Gromadzki and Spain, 2003; Dawes-Gromadzki, 2005, 2008; Jamali et al., 2011; Davies et al., 2015; Houston et al., 2015). Lines indicate best linear model fit: Africa (slope: 0.019, R^2_{adj}: 0.396, $p < 0.001$), Central/South America (slope: 0.015, R^2_{adj}: 0.413, p: 0.002), Southeast Asia (slope: 0.0036, R^2_{adj}: 0.0311, p: 0.11), Madagascar (slope: 0.0070, R^2_{adj}: 0.46, p: 0.20), Australia (slope: −0.0014, R^2_{adj}: −0.0186, p: 0.28). Black line shows linear regression for all sites and rainfalls (slope: 0.0042, R^2_{adj}: 0.0751, $p < 0.001$). Dashed lines are not significant. Circles indicate sites from the current study.

builds the smallest and most fragile of the termite mounds we encountered. The high frequency of *A. laurensis* mounds could be due to a lower building cost than mounds that are sturdier and denser, or multiple mounds may be from the same termite colony. With an almost endless amount of grass and its corresponding litter in these savannas, mound-building species like *A. laurensis*, *N. magnus*, and *D. rubriceps* have become dominant, feeding on these substrates. Although there is also litter in rainforests, it may be that the type of litter prohibits these species from inhabiting rainforest habitats as well. Mound building may also be contingent on the makeup of the soil. In savannas in Australia, there is a negative relationship between soil fertility and the number of termite mounds (Ratcliffe et al., 1952; Goodland, 1965), which suggests that Australian mound-building termites have adapted to poor soil conditions.

It is remarkable that there are few termites in dead wood in the rainforest sites, especially in comparison with rainforests in other tropical regions. In rainforest dead wood surveys in Malaysia, termites are typically found in ∼20% of wood pieces (Kimber and Eggleton, 2018), but we found termites in fewer than 3% of wood pieces in North Queensland rainforests. Additionally, in our rainforest sites, we found that over 80% of pieces of dead wood had been damaged by wood-dwelling fungi, compared with less than 40% of dead wood in savanna sites. These results suggest that dead wood decomposition in Australian rainforests is especially dependent on fungal decomposers (Cheesman et al., 2017). It is unclear whether any of this pattern is driven by direct competitive interactions between termites and fungi. Viana-Junior et al. (2018) suggest that wood-inhabiting fungi are potential facilitators of fungi, but other studies (Um et al., 2013) say the opposite. Further testing through exclusion experiments is needed to determine whether fungi and termites regulate one another's access to dead wood, and if Australian fungi contribute to the termite diversity anomaly.

High Turnover of Species Across Habitat Types

The high level of species turnover across different habitat types that are very close together (Sav2, Scl1, Rft2, **Figure 1**, **Supplementary Figure 2**, and **Supplementary Table 3**) suggests that habitat type is an important determinant of termite assemblage composition in this region of Australia. This habitat-related pattern may be a byproduct of how termite species sorted as they arrived to and radiated across Australia. Many Australian termites descended from the dry-adapted termites that first diversified in Australia (Arab et al., 2017), and there may not have been time for these lineages to invade rainforests. The rainforest termites may be from other lineages that drifted over at a different time. If these contrasting habitats were filled by lineages adapted to contrasting habitats, the shift between rainforest and savanna would form a strong barrier to dispersal for the termites.

Feeding Groups Are More Diverse in Savannas Than Rainforests

Our fourth key observation about the termite diversity anomaly is that habitat had strong consequences for the diversity of feeding groups. The rainforest termites in this study were limited not only in species richness and total abundance, but also in feeding groups. All of the termites found in our rainforest surveys were wood feeders, despite the presence of abundant litter, as well as some grass and dung. This finding is consistent with other Australian monsoonal rainforests in Darwin where only wood feeders were detected (Dawes-Gromadzki, 2005) and could be connected with the limited number of higher termites in rainforest sites, as most lower termites are wood feeders. The savanna termites were from a diversity of feeding groups, mainly dominated by litter-and-dung or grass-and-litter feeders, but also characterized by wood feeders, grass feeders, wood-and-litter, wood-and-dung, and soil feeders from both higher and lower termites. Similar to our rainforest finding for wood that despite numerous wood pieces present, few termites were found, we also noted high amounts of rainforest litter, but there were no litter-feeding termites present. This result suggests either that Australian termites are not targeting rainforest leaf litter or that amount of substrate is not the limiting factor for termites in rainforests.

Potential Mechanisms of the Australian Termite Diversity Anomaly

Without true soil-feeding termites or fungus farming termites, the termite communities in Australia are quite different from those on other continents. Australian termite communities lack three major clades of higher termites—Apicotermitinae, which are soil-feeding termites in the Neotropics and Afrotropics; Cubitermitinae, which are common soil-feeding termites in African tropics; and Macrotermitinae, which are fungus-farming termites in Africa and Southeast Asia (Braithwaite et al., 1988; Davies et al., 2003). The Nutrient-Poverty/Intense-Fire theory claims that most of the anomalous features of Australian flora and fauna are evolutionary consequences of adapting to low nutrient availability and frequent fire (Orians and Milewski, 2007). Australian soils are poor in P, Zn, I, Co, Mg, and Se, and do not receive nutrients in aerosols because of Australia's distance from other continents (Braithwaite, 1990; Orians and Milewski, 2007). To explain the lack of fungus-growing termites, the Megacatalyst Theory (Milewski and Diamond, 2000), states that these species are unable to live in Australia because they are dependent on I, Co, and Se in soils for their growth and rapid reproduction. Additionally, studies from Malayan rainforests show a positive relationship between termite richness and soil nutrient content (Salick and Tho, 1984), suggesting that termites, especially those that are feeding on soil, would be unable to survive in Australia's nutrient-poor soil. Of note, Braithwaite et al. (1988) found that in Darwin, Australia, termite richness was greatest in areas with poorer-quality soils, which may indicate that the Australian termites are particularly adapted for nutrient-poor soil conditions, albeit not as soil feeders.

It seems likely that at the core of the Australian termite diversity anomaly is a selection process influenced by the biogeographical history of termites in Australia. The first termites likely evolved at the end of the breakup of Pangea and at the beginning of the breakup of Gondwana ∼140 Ma (Bourguignon et al., 2014). When Termitidae, or the higher

termites (comprising the bulk of termite diversity worldwide), evolved in Africa ∼50 Ma, rainforests were important for their diversification and cladogenesis (Eggleton, 2000). However, when closed canopy forests first evolved in other tropical regions, Australia was still at a very southern latitude, meaning the termites that evolved in these rainforests may have been unable to raft to or survive in Australia (Davies et al., 2003). Australia has been colonized by higher termites at least five times over the past 20 M years, many of these dispersal events happening when the arid biome was expanding in Australia, which made it possible for dry-adapted higher termites to diversify and take over ecosystems (Eggleton, 2000; Bourguignon et al., 2017). Most of these dispersals have happened through rafting across the ocean on pieces of wood (Bourguignon et al., 2016). Soil feeders and fungus farmers, which make up >50% of all termite species worldwide, do not produce supplementary reproductives that can raft in wood. Thus, Australia's termite assemblage is made up mostly of nutrient-poor or dry adapted higher termites (Abensperg-Traun and Steven, 1997), which are mostly wood, grass, litter, and dung feeders.

Effect of Australian Termite Diversity Anomaly on Carbon and Nutrients

Only by gaining better quantitative estimates of termite assemblages can scientists uncover the impact of termites on ecosystem processes and predict how they shape carbon and nutrient turnover. These findings have important implications for modeling the breakdown of carbon and nutrients in tropical systems. As termites tunnel and build mounds, they increase soil bioturbation and nutrient levels, the effects of which can last for decades (Beaudrot et al., 2011). Termites differ from fungi in the speed at which they break down wood and the pathways by which they release carbon from wood (e.g., termites release both CO_2 and CH_4 as compared with fungi that just release CO_2). In some tropical rainforests, termites are responsible for 58–64% of decomposition (Griffiths et al., 2019), but our observations suggest that a lack of termite activity in Australian rainforests could slow down dead wood turnover as compared with other tropical rainforests in the world.

Knowledge of the diversity, abundance, activity, and feeding group composition of termites is critical in modeling carbon cycling and storage. For example, fungus farming termites have the capacity to break down lignin. Without lignin decomposition, termites will instead translocate lignin from termite decayed wood into nests, mounds, and soils. Therefore, feeding groups, or in this case the lack of certain feeding groups, has implications for the way that carbon is stored or released into the surrounding environment. The Australian termite diversity anomaly is an important key to understanding the varying effects of termite assemblages on ecosystem services and carbon cycling around the globe.

Based on our study, greater quantification of termite responses to shifts in rainfall is warranted in other parts of the world. As we document biogeographic shifts in relationships between termites and environmental gradients, these differences need to be incorporated into how we model carbon and nutrient cycling. Our work suggests that such cycling is likely very different in Australia than in other tropical regions, with termites playing a smaller role as decomposers in rainforests in Australia compared with tropical rainforests in Africa and South America (Cheesman et al., 2017). As relationships between rainfall/habitat type and termite assemblages in Australia and elsewhere are clarified, we can better understand how termites are shaping ecosystems globally and incorporate them more accurately into our models of carbon turnover.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

RC, AZ, SA, and PE conceived of the project design. RC, AZ, PE, HF-M, LC, AC, and AY carried out the experiment. RC performed the analysis with help from HF-M, AY, and AZ. RC took lead in writing the manuscript. PE provided the conceptual background. AZ supervised the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.657444/full#supplementary-material

Supplementary Figure 1 | Maximum likelihood tree of termite COII genes from termites in this study with sites and feeding groups.

Supplementary Figure 2 | Non-metric multidimensional scaling (NMDS) analysis representing similarity of termite species across sites.

Supplementary Figure 3 | Heat map from predictive model assuming different environmental response for different species (GLM-LASSO, Brown et al., 2014) showing association between **(A)** species presence/absence and **(B)** species abundance as responses and standardized environmental variables as predictors.

Supplementary Table 1 | Termite samples collected, their sites, collection method, and GenBank accession numbers.

Supplementary Table 2 | Accession numbers from 205 termite COII sequences from GenBank used for tree-building and termite species identification.

Supplementary Table 3 | Dissimilarity matrix of termite species across sites based on Simpson beta-diversity index.

Supplementary Table 4 | Compiled sources of termite surveys with region, site name, average annual rainfall, habitat type, and number of species (N).

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Complementary Contribution of Fungi and Bacteria to Lignocellulose Digestion in the Food Stored by a Neotropical Higher Termite

*Edimar A. Moreira[1†], Gabriela F. Persinoti[2†], Letícia R. Menezes[1], Douglas A. A. Paixão[2], Thabata M. Alvarez[3], João P. L. Franco Cairo[4†], Fabio M. Squina[5], Ana Maria Costa-Leonardo[6], Andre Rodrigues[7], David Sillam-Dussès[8] and Alberto Arab[1]**

[1] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC (UFABC), Santo André, Brazil, [2] Laboratório Nacional de Biorrenováveis (LNBR), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, Brazil, [3] Graduate Programme in Industrial Biotechnology, Universidade Positivo, Curitiba, Brazil, [4] Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, Brazil, [5] Programa em Processos Tecnológicos e Ambientais, Universidade de Sorocaba (UNISO), Sorocaba, Brazil, [6] Departamento de Biologia Geral e Aplicada, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, Brazil, [7] Center for the Study of Social Insects, São Paulo State University (UNESP), Rio Claro, Brazil, [8] Laboratory of Experimental and Comparative Ethology UR 4443, University Sorbonne Paris Nord, Villetaneuse, France

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George Tsiamis,
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Reviewed by:
Gaku Tokuda,
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Purdue University, United States

***Correspondence:**
Alberto Arab
albertoarab@gmail.com

[†] These authors have contributed equally to this work

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Lignocellulose digestion in termites is achieved through the functional synergy between gut symbionts and host enzymes. However, some species have evolved additional associations with nest microorganisms that collaborate in the decomposition of plant biomass. In a previous study, we determined that plant material packed with feces inside the nests of *Cornitermes cumulans* (Syntermitinae) harbors a distinct microbial assemblage. These food nodules also showed a high hemicellulolytic activity, possibly acting as an external place for complementary lignocellulose digestion. In this study, we used a combination of ITS sequence analysis, metagenomics, and metatranscriptomics to investigate the presence and differential expression of genes coding for carbohydrate-active enzymes (CAZy) in the food nodules and the gut of workers and soldiers. Our results confirm that food nodules express a distinct set of CAZy genes suggesting that stored plant material is initially decomposed by enzymes that target the lignin and complex polysaccharides from fungi and bacteria before the passage through the gut, where it is further targeted by a complementary set of cellulases, xylanases, and esterases produced by the gut microbiota and the termite host. We also showed that the expression of CAZy transcripts associated to endoglucanases and xylanases was higher in the gut of termites than in the food nodules. An additional finding in this study was the presence of fungi in the termite gut that expressed CAZy genes. This study highlights the importance of externalization of digestion by nest microbes and provides new evidence of complementary digestion in the context of higher termite evolution.

Keywords: Isoptera, nest microrganisms, carbohydrate active enzymes, saprotrophs, food storage, nutrition, Blattodea, Syntermitinae

INTRODUCTION

Termites (Blattodea: Isoptera) are eusocial cockroaches comprising over 3,000 species widespread in tropical and subtropical regions. These insects play an important role in ecosystems through the decomposition of plant lignocellulose (Brune, 2014; Mikaelyan et al., 2015), having a positive impact in nutrient cycling and soil-water dynamics through the construction of their nests and tunnel networks in soil (Neupane et al., 2015; Siebers et al., 2015; Jouquet et al., 2018).

All termites live in nests containing hundreds to thousands of individuals divided in castes (reproductive individuals, workers, and soldiers) that cooperatively conduct the tasks of the colony. Nests protect termites against the environment and predators and offer a place for feeding, reproduction, and nursery (Bignell and Eggleton, 2000). Higher termites (Termitidae) have evolved remarkable nesting strategies including the construction of epigeal mounds which can reach a prominent size in some species (Korb, 2011). Mounds are usually made of micro-aggregates of soil and feces forming porous structures that control the microclimate (Korb, 2003; Singh et al., 2019) and could function as an external reservoir of microorganisms (Fall et al., 2007; Manjula et al., 2014) or even like a fermenting chamber (Korb, 2011; Schmidt et al., 2014). Some authors have hypothesized that externalization of digestion by nest microorganisms, as seen in the subfamilies Macrotermitinae and Sphaerotermitinae (Garnier-Sillam et al., 1989; Aanen et al., 2002), was the first step in the evolution of Termitidae and might have driven the loss of gut protozoans and the acquisition of specialized lignocellulolytic bacteria resulting in higher efficiency in plant decomposition (Brune, 2014; Chouvenc et al., 2021).

Cornitermes cumulans (Kollar, 1932) (Termitidae: Syntermitinae) is a mound-building termite in pastures and savannas of central South America and in some areas its nest density is exceptionally high compared to other termite species (Coles De Negret and Redford, 1982; Buschini, 2006). The hard epigeal mounds are made externally of soil with an internal core of soft carton made from fecal material and soil particles (Redford, 1984). Although feeding *in situ* has been reported (Coles De Negret and Redford, 1982), *C. cumulans* is a harvester termite, cutting and transporting grass and litter into its mound. The plant material is then stored in nodules made with feces and saliva (**Figure 1A**) and subsequently consumed by workers (Lima and Costa-Leonardo, 2007). Food nodules showed a high hemicellulolytic activity possibly mediated by bacteria and fungi. It is possible that the pre-processing of plant material by the action of microbes in the food nodules before gut passage acts as a complementation of biomass digestion in *C. cumulans*. However, only partial taxonomic information for fungal strains was reported because less than 10% of reads were assigned to order level at the time (Menezes et al., 2018).

In this study, we assessed the taxa and guilds that differentiate fungal communities associated to the food nodules of *C. cumulans* by using deep taxonomic and guild assignment of datasets generated through internal transcribed spacer (ITS) sequencing. To investigate the potential complementation of lignocellulose digestion among the termite host, the gut symbionts and the microbiota of food nodules, we then performed metagenomic and metatranscriptomic analyses on this tri-partite system to assess: (i) the repertoire and functional contribution of lignocellulolytic enzymes; (ii) how these enzymes are linked taxonomically to the termite holobiont (host, gut, and nest microbiota); and (iii) the differences of enzyme functionality across sterile castes.

MATERIALS AND METHODS

ITS Datasets, Sequence Analysis and Guild Assignment

Internal transcribed spacer reads were obtained from raw Illumina datasets of three (3) *C. cumulans* colonies (CC1, CC2, and CC3) deposited in the European Nucleotide Archive (ENA) with accession no. PRJEB17080 (Menezes et al., 2018). Libraries were processed using the UPARSE pipeline (Edgar, 2013). Paired end reads were first merged using fastq_mergepairs from USEARCH package version 8.1.1803. Reads with a minimum overlap of 50 bp and a maximum expected error of 0.5 were mapped to fungal ITS2 region using ITSx software (Bengtsson-Palme et al., 2013). Reads were further compared to ITS2 UCHIME reference dataset to filter chimera sequences using chimera UCHIME (Edgar et al., 2011), also implemented in USEARCH package. The filtered reads were then subjected to clustering into OTUs (operational taxonomic units) at 97% of sequence similarity according to UPARSE-OUT algorithm. The OTU table was generated by mapping the reads from each sample back to the OTUs. Taxonomic assignment was performed using *sintax* command as implemented in USEARCH version 10.0.240 software using RDP Warcup training set v2 (Deshpande et al., 2016) database. Fungal genera with *sintax cutoff* > 0.7 were considered to discriminate taxa that significantly differentiate fungal communities. Relative abundances were calculated as the number of reads per taxon. OTUs were further classified into ecological guilds using FUNGuild, only considering assignments that were either "highly probable" or "probable" (Nguyen et al., 2016). Fungi were then assigned to saprotrophs (undefined saprotrophs and dung saprotrophs), symbiotrophs (endophytic and lichenized fungi) and plant pathogens. We also used publicly available fungal datasets generated through Illumina sequencing of the ITS2 region, that included samples of: (1) soil from pastures located nearby our sampling sites, which commonly contain *C. cumulans* mounds (de Oliveira et al., 2020); (2) the carton nest core of the sympatric non-food-storing termite *Procornitermes araujoi* (Syntermitinae) which builds mounds with outer walls made of thin layer of soil with an internal layer lined with fecal material (**Supplementary Figure 1**; Moreira et al., 2018); (3) different species of bark beetles (Rassati et al., 2019; Seibold et al., 2019); and (4) nests of a leaf-cutting bee (Rothman et al., 2019).

Diversity and Community Structure Analyses

We used R version 3.4.4 (R Core Team, 2018) to conduct analyses using different software packages. Alpha-diversity

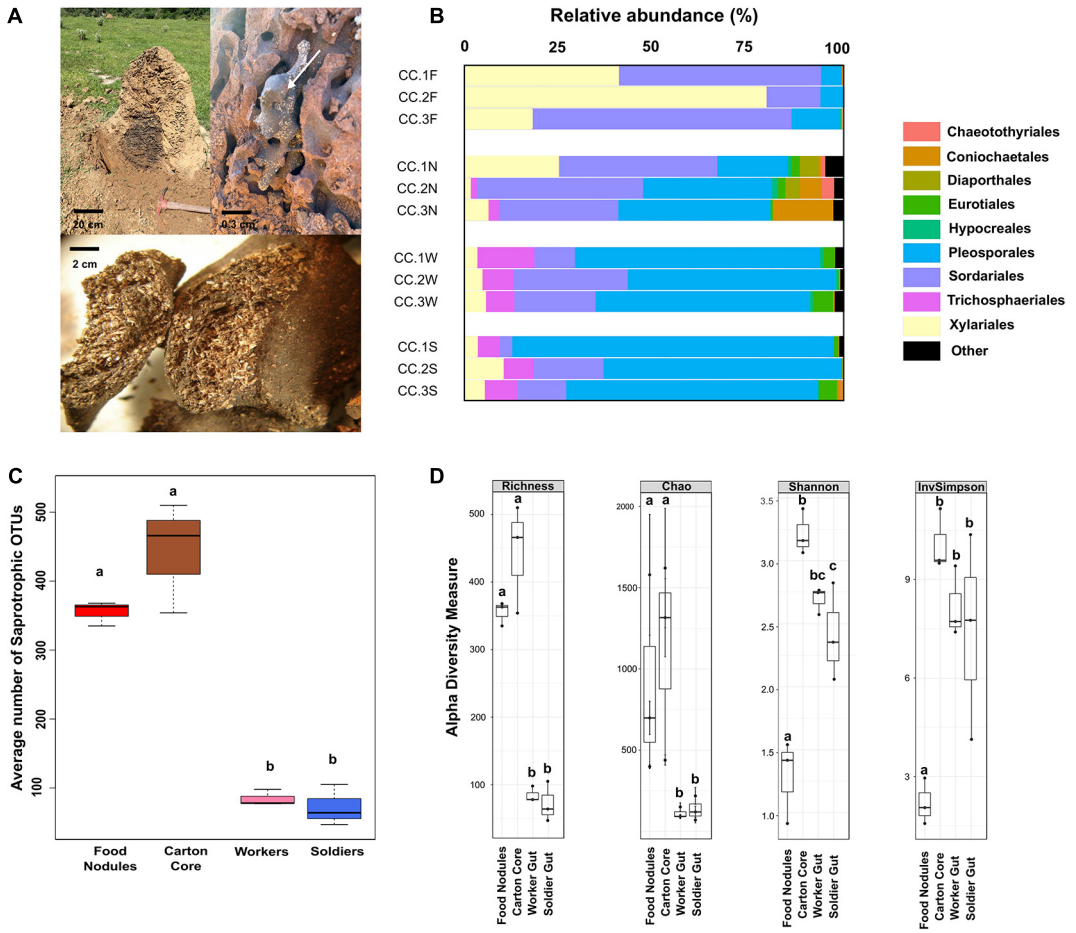

FIGURE 1 | (A) Nest of *Cornitermes cumulans* showing the central carton core with a food nodule (arrow) and a zoomed picture of the plant material inside a food nodule. **(B)** Proportion of reads assigned to different fungal orders in gut and nest substrates for each sampled colony. **(C)** Average number of saprotrophic fungal OTUs associated to each one of the samples. **(D)** Alpha-diversity indices estimated for fungal ITS amplicons. Graphics report the observed operational taxonomic units (OTUs) richness, Chao1 estimator of richness, and Shannon's and Simpson's indices. Boxplots report median, upper and lower quartiles, and maximum and minimum values. Different letters denote significant differences (p-values < 0.05 in GLMM). Samples codes: CC1 = colony 1, CC2 = colony 2, and CC3 = colony 3. F, food nodules; N, carton nest core; W, worker gut; S, soldier gut.

estimates were calculated using the function *plot_richness* in the phyloseq package (McMurdie and Holmes, 2013). Rarefaction was assessed using vegan package (Oksanen et al., 2018). Generalized linear mixed models (GLMM) were performed to check for overall significant differences of α-diversity estimates and the total number of OTUs among samples. The type of substrate was included as fixed effects, while the colony number was included as random effect. Multiple comparisons were conducted by general linear hypotheses using the *glht*-function in the multcomp package (Hothorn et al., 2008). A redundancy analysis (RDA) (999 permutations) of Hellinger-transformed abundance data using the vegan package (Oksanen et al., 2018) was used to evaluate whether nest and gut substrates differ in fungal composition at the OTU level. The *anova* function from the package vegan were used to test the significance of RDA models and axes, respectively. We used Calypso web-server (Version 8.58), an online platform (Zakrzewski et al., 2017), to construct taxonomic heatmaps and the RDA of fungal metanalysis. The transmission of fungi between nest substrates and the gut of workers and soldiers was evaluated by analyzing the number of intersecting OTUs using the UpSetR package (Conway et al., 2017). We used linear discriminant analysis (LDA) effect size (LEfSe) to detect taxa that significantly differentiate fungal communities (Segata et al., 2011). To assess LEFSe, we used Galaxy web application, an online platform for the evaluation of multiple microbial community composition data (Afgan et al., 2016). The LDA score threshold was 4.0. Relative abundances less than 0.01% of the total reads were omitted from further analysis. We then identified variation in taxa using GLMM. To identify OTUs that were significantly different among samples, we used DESeq2 package (Love et al., 2014). Statistical significance was defined as $p \leq 0.05$ in both statistical methods. Only OTUs identified by both LEfSe and DESeq2 were discussed. Plots were constructed with the packages ggplot2 (Wickham, 2016) and ggord (Beck, 2017).

DNA Extraction and Metagenomic Sequencing

The whole gut of 200 workers and soldiers and 50 mg of nest substrates (the carton nest core and food nodules) from colony CC1 were extracted in 2 ml tubes containing 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate (SDS). Gut samples were obtained by anesthetizing termites on ice for 5 min and dissected with fine forceps. All the samples were kept at −20°C until DNA extraction and purification using a bead-beating protocol (Yu and Morrison, 2004). DNA integrity was assessed by agarose gel electrophoresis (1.0 w/v) and the quantification was performed using NanoDrop spectrophotometer by measuring the absorbance at 260 nm. For metagenomic sequencing purposes, a library was constructed, using the Nextera library preparation kit (Illumina), according to the manufacturer's instructions. The prepared library was validated and quantified using the Agilent bioanalyzer 2100 system with a 12,000 DNA assay kit (Agilent) and Kapa Biosystems next-generation sequencing library qPCR kit (Kapa Biosystems), respectively. Sequencing was performed at LNBR NGS facility (CNPEM – Campinas, São Paulo, Brazil) using an Illumina HiSeq 2500 platform and applying the paired-end protocol (2 × 100-bp paired ends).

Metatranscriptome Library Preparation and Sequencing

Total RNA (10 μg) was extracted from 200 termite guts and 50 mg of the nest substrates of colony CC1 using Trizol reagent protocol (Invitrogen). Trizol/Chloroform step was performed twice. The total RNA was purified using the RNeasy Plant Mini Kit (Qiagen) following manufacturer's instructions. The quality of RNA was verified using RNAnano chip Bioanalyzer 2100 (Agilent). Good quality RNAs (RIN > 8.0) were submitted to rRNA depletion using the RiboZero rRNA Removal Kit under manufacturer instruction with a slight modification: at depletion step, we used a blend of rRNA removal solution from RiboZero Gold and RiboZero Bacteria kits (1:1) aiming to deplete both prokaryotic and eukaryotic rRNAs. Finally, the depleted RNAs were purified using Ampure XP beads, following the manufacturer's instructions, and kept at −80°C until library preparation. A total of 50 ng of depleted RNA were used for library preparation using the TruSeq Stranded Total RNA Sample Preparation kit (Illumina), following the manufacturer's instructions. Quality control, quantification, and sequencing were performed as described before.

Metagenome and Metatranscriptome Data Analysis

Metagenome (MG) and metatranscriptome (MT) raw reads were quality checked using FastQC[1] and quality-filtered using Trimmomatic v.0.36 (Bolger et al., 2014). MT reads were also inspected using SortMeRNA to remove rRNA reads, and then both MG and MT reads were taxonomically classified using Kaiju (Menzel et al., 2016). Next, the whole set of MG trimmed reads were *de novo* co-assembled using IDBA_UD (version 1.1.1) (Peng et al., 2012). Gene prediction and annotation of was performed using Prokka v.1.11 with the meta parameter (Seemann, 2014) and carbohydrate-active enzymes (CAZy) annotation was performed using dbCAN database (Zhang et al., 2018). Taxonomy of predicted protein coding genes were inferred according the easy-taxonomy function of MMseqs2 suite (Steinegger and Söding, 2017) to identify putative bacterial, fungal, and termite-host genes by LCA method. Both, MG and MT reads were mapped to the complete set of predicted genes recovered from the MG *de novo* assembly using Kallisto v. 0.46.1 (Bray et al., 2016) to estimate genes and transcripts abundance. Normalized expression of protein coding genes was estimated with the Transcripts Per Kilobase Million (TPM) method and used to analyze the abundance of expression of the predicted fungal, bacterial, and host putative CAZy genes. Homology to peptide pattern (Hotpep) was used to infer CAZy function (Busk et al., 2017).

[1]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

RESULTS

Which Fungal Taxa Are Associated to the Gut and the Nest of *Cornitermes cumulans*?

We detected 968 fungal OTUs (2,163,723 sequences from the ITS region) associated to the gut and the nests of *C. cumulans*. Of these, 579 OTUs (1,370,000 sequences, 63.3% of the total) were annotated to the food nodules, 774 OTUs (739,691 sequences, 34.2%) to the carton nest core and 197 OTUs (54,032 sequences, 2.5%) to the gut of workers and soldiers. Deep taxonomic analyses using the Warcup database successfully assigned 88 and 84% of fungal reads at the order and family level, respectively. Overall, nest and gut samples yielded the phyla Ascomycota (99.9% of sequence reads) and Basidiomycota, representing 13 classes, 42 orders, 98 families, and 212 genera (**Supplementary Tables 1, 2**). Rarefaction curves indicated adequate sampling of fungi for a valid comparison among fungal communities (**Supplementary Figure 2**). The average number of OTUs was significantly higher in nest samples, the majority classified as undefined saprotrophs (on average 76.5% of sequence reads) and symbiotrophs (endophytes 4.8% and lichenized 2.5%) of the orders Pleosporales, Sordariales, and Xylariales (Ascomycota) using FUNGuild (**Figures 1B,C** and **Supplementary Figure 3**).

Fungal Composition Differs Between Gut and Nest Substrates

Observed richness was significantly lower in gut assemblages (**Figure 1D**). On the other hand, food nodules and carton core samples of *C. cumulans* nests did not differ in richness and shared approximately 99% of their sequence reads (293 OTUs), but estimated diversity was significantly lower in food nodules. Constrained ordination showed that community composition of food nodules formed a distinct cluster separated from carton nest core (F = 2.84, df = 1, p = 0.002) and gut samples (F = 6.61, df = 1, p = 0.001) (**Figures 2A,B**) and

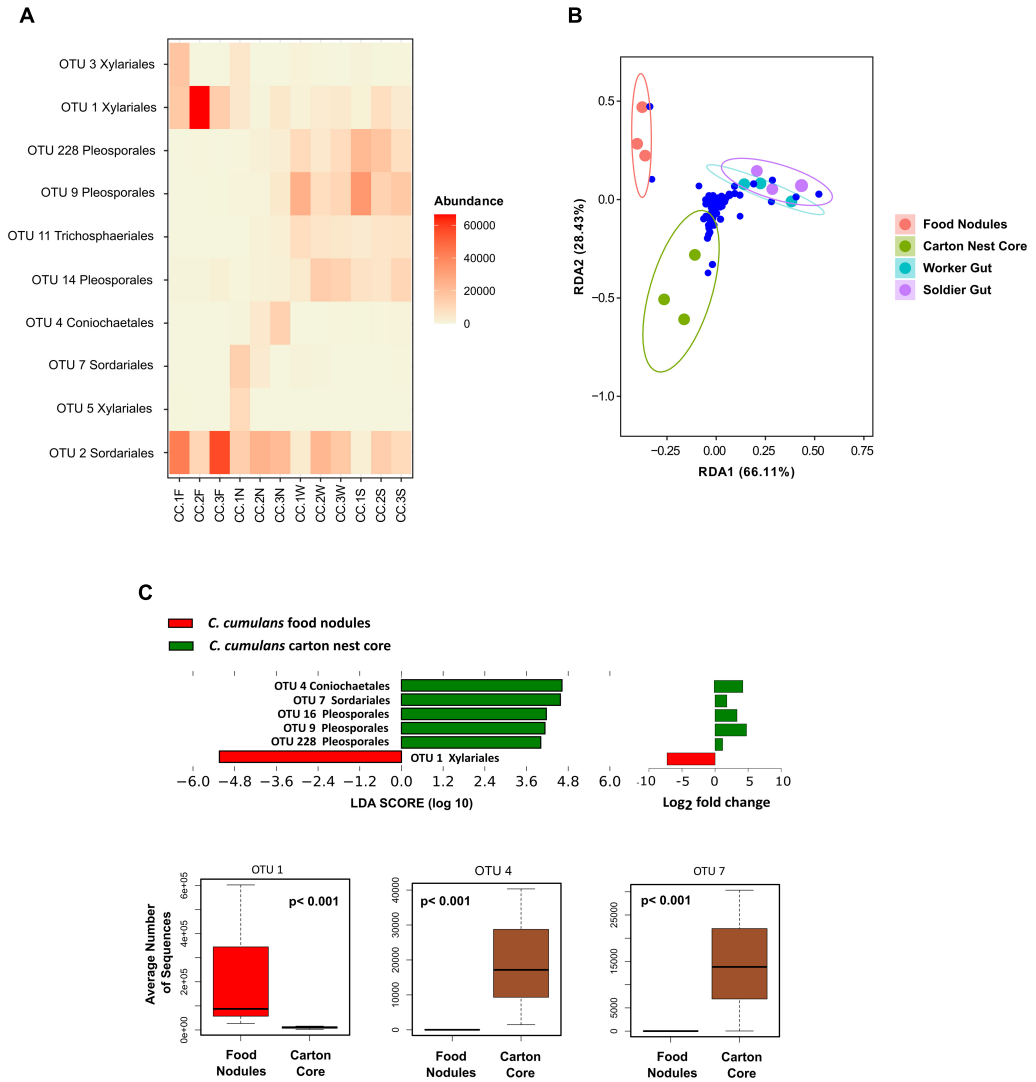

FIGURE 2 | (A) Comparison of the relative abundances (>1%) of fungal OTUs of *C. cumulans*. **(B)** Ordination plots showing fungal communities of gut and nest samples after redundancy analysis of Hellinger transformed abundance of OTUs (blue dots) with colony number (colored circles) as conditional variable. RDA1 Nest Substrates; variance = 0.140, *p*-value = 0.001, and RDA1 Termite Gut; variance = 0.06, *p*-value = 0.002, permutations tests (n = 999). **(C)** Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe, score > 4) and DESeq2 ($p \leq 0.05$) analyses to identify the fungal OTUs responsible for the differences between nest substrates. Boxplots report median, upper and lower quartiles, and maximum and minimum values of the relative abundance of taxa identified by both DESeq2 and LEfSe analyses. A *p*-value < 0.05 was considered significant in GLMM analyses. See **Figure 1** for samples codes.

was characterized by the abundance of saprotrophic OTUs of Sordariales and Xylariales (**Supplementary Tables 3**, **4**). We then applied LEfSe and DESeq2 in order to determine the variation in the composition of fungal communities and both analyses consistently showed that the saprotroph OTU 1 of Xylariaceae was strongly associated to food nodules (LDA = 5.24; p = 0.04; DESeq2: $\log_2$ fold change = −4.32, p = 0.002), whereas the saprotroph OTU 7 of Sordariales (LDA = 4.57; p = 0.04; DESeq2: $\log_2$ fold change = 1.15, p < 0.001) and the endophyte OTU 4 (Coniochaetales: *Lecythophora*) (LDA = 4.62; p = 0.04; DESeq2: $\log_2$ fold change = 3.26, p < 0.001) were the most prominent fungal species found in the carton nest core (**Figure 2C**). No differences were observed among colonies. All the results were confirmed by GLMM (**Supplementary Table 5**).

Fungal Transmission Between Nest Substrates and Termites

The gut mycobiota of workers and soldiers was represented by 11 classes, 26 orders, 52 families, 90 genera, and 197 fungal OTUs (**Supplementary Table 1**) with the prevalence of the genus *Phoma* (Pleosporales) (**Supplementary Tables 2**, **3**). Diversity and composition of fungal gut assemblages were not influenced by the type of caste (RDA, F = 0.84; df = 1; p = 0.540) (**Figure 2B**) and no features with significant differences were

found between workers and soldiers. However, we detected a high percentage (between 89 and 99%) of transmission of fungal OTUs between nest substrates and the gut of workers and soldiers. The majority of shared OTUs belongs to Sordariales and Pleosporales. Additionally, workers and soldiers shared between 97.5 and 98.4% of their sequence reads (**Supplementary Figure 4**).

Termites Nest Exhibits Different Fungal Assemblages

Analyses of the variation in community structure showed that the composition of Ascomycota fungi differed significantly among termite nests (*C. cumulans* and *P. araujoi*), soil samples and substrates associated with two phylogenetically distant insects (RDA, $F = 15.47$; df = 5; $p = 0.001$) (**Supplementary Figure 5A**). The nest substrates of termites formed a cluster, clearly separated from other fungal assemblages. The more prominent genera associated to termite nest substrates, like *Daldinia* (Xylareaceae) and *Lecythophora* (Coniochaetaceae), were found in lower abundances in other assemblages. However, *Thielavia* and *Chaetomium* (Chaetomiaceae) were similarly abundant in both soil and termite nests. We also found that fungal communities associated to bark beetles and bee nests formed separated clusters with their prominent genera present in lower abundances in termite nests (**Supplementary Figure 5B**).

Bacteria Dominated Microbial Assemblages in Gut and Nest Substrates

The microbial assemblages from colony CC1 were further investigated by metagenomics (MG) and metatranscriptomics (MT) sequencing. Taxonomy analyses of the sequences showed a higher abundance of bacteria over fungi in both nest and gut samples (**Figures 3A,B**). Actinobacteria and Proteobacteria were dominant in the nest substrates, whereas Firmicutes and Spirochetes were prevalent in the termite gut, confirming our previous results with 16S rRNA sequencing (Menezes et al., 2018). Ascomycota was present in both nest and gut samples. However, a fungus of genus *Neocallimastix* (Chytridiomycota: Neocallimastigales) was only found in the gut of workers

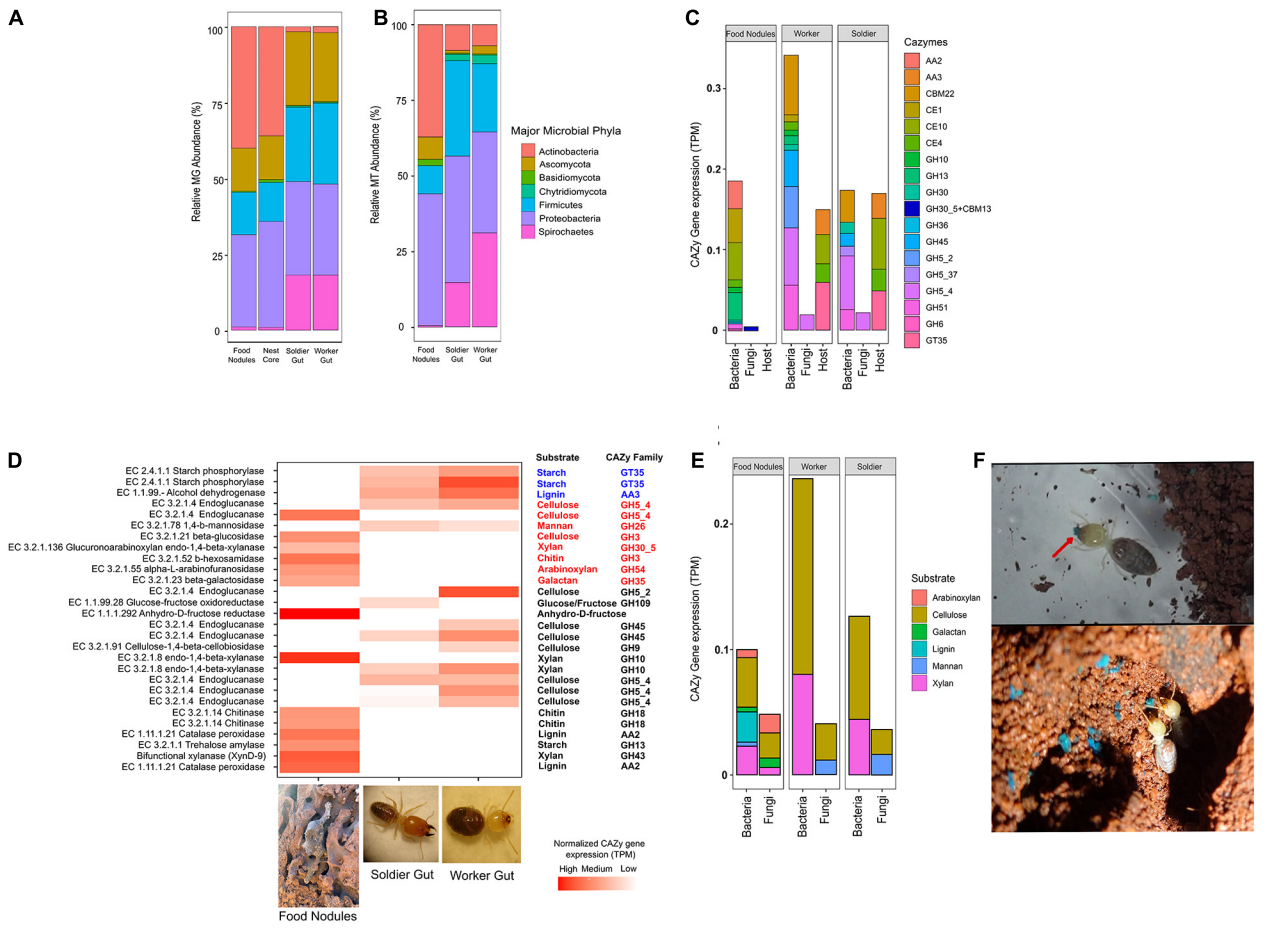

FIGURE 3 | (A) Relative metagenomic (MG) and **(B)** metatranscriptomic (MT) abundance assigned to major phyla in *C. cumulans*. **(C)** Contribution of the termite holobiont (gut, nest microbiota and host) to CAZy expression. **(D)** Complementary CAZy gene expression and lignocellulolytic activity in food nodules and gut of workers and soldiers. The heatmap shows normalized expressions of protein coding genes of major CAZy families colored according to taxonomy assignment (Blue: termite host; Red: fungi; Black: bacteria). **(E)** CAZy gene expressions predicted for lignocellulosic substrates in food nodules and gut of workers and soldiers. **(F)** Detail of the food storage behavior of *C. cumulans* workers on the nest walls using blue-marked sugar-cane (red arrow).

and soldiers. *Bacillus* (Firmicutes), *Treponema* (Spirochetes), *Streptomyces* (Actinobacteria), and *Aspergillus* (Ascomycota) were the most abundant microbial genera in the samples (**Supplementary Tables 6, 7** and **Supplementary Figure 6A**). We did not obtain RNA from nest core samples; however, the MG showed a similar profile between the carton nest and food nodules (**Figure 3A**). The MT sequences from fungi were less abundant (**Figure 3B**), suggesting a lower metabolic activity from fungi over bacteria. Although our data was derived from a single colony (CC1), the fungal taxa responsible for the expression of CAZy transcripts showed similar abundances among the three colonies used in ITS sequencing, for food nodules (H = 0.74; df = 2; p = 0.689; Kruskal–Wallis test) and the gut of workers and soldiers (H = 1.11; df = 5; p = 0.953) (**Supplementary Table 2**).

Expression of Cellulases and Hemicellulases in the Food Nodules

The MG assembly of food nodules yielded 124,543 bacterial and 3,926 fungal genes. Of these, 213 were assigned to bacterial phyla and 86 to fungal CAZy-coding genes. The majority of transcripts (95%) corresponded to the glycoside hydrolases (GHs) and glycosyl transferases (GTs) (**Figure 3C**) and more than 60% of CAZy transcripts were specific to the food nodules (**Supplementary Figures 6B,C**). By using peptide-based annotation, we predicted that the expression of CAZy genes is high for cellulases and hemicellulases. Consequently, the lignocellulosic activity reported previously in these food storage structures (Menezes et al., 2018) is associated to the expression of bacterial and fungal endoglucanases (EC 3.2.1.4), a fungal beta-glucosidases (EC 3.2.1.21), a bacterial xylanase (EC 3.2.1.80), a fungal xylanase (EC 3.2.1.136), and fungal galactosidase (EC 3.2.1.23), and these enzymes were taxonomically assigned to the phyla Proteobacteria and Ascomycota (**Supplementary Figure 6A**). We also obtained transcripts associated to a catalase-peroxidases (EC 1.11.1.21) and an amylase (EC 3.2.1.1) which were linked to Actinobacteria, and chitinases of bacterial (EC 3.2.1.14) and fungal (EC 3.2.1.52) origin (**Supplementary Tables 6, 7** and **Figures 3D,E**).

Gut Symbiotic and Host Contribution to Lignocellulose Digestion

The gut microbiota of *C. cumulans* yielded 88,454 bacterial and fungal 3,370 genes. Of these, 121 were assigned to bacterial and 34 to fungal CAZy. GH5_2 and GH5_4 genes are the highest expressed GH families associated to the gut microbiome. Additionally, the non-catalytic carbohydrate-binding module gene (CBM22) is also abundant. Although the functional lignocellulolytic profile overlapped partially with food nodules (**Figure 3D**), it was linked to different microbial taxa. We also detected a high expression of transcripts associated with endoglucanase (EC 3.2.1.4), xylanase (EC 3.2.1.8), and endomannanase (EC 3.1.2.78) which were linked to Firmicutes, Chytridiomycota and Ascomycota, respectively (**Supplementary Figure 6A**). On the other hand, we identified 2,262 genes in the termite *C. cumulans*, but only 21 CAZy gene transcripts. A starch phosphorylase (EC 2.4.1.1), an alcohol-2-dehydrogenase (EC 1.1.99.-) and the carbohydrate esterases (CE4 and CE10) were predicted in the MG assembly (**Supplementary Table 8** and **Figure 3D**). The total CAZy expression levels associated to cellulose and xylan degradation were higher in the gut of termites, and nearly equally expressed in workers and soldiers (**Figure 3E**).

DISCUSSION

Termites rely on a mutualistic association with gut symbionts to decompose the plant biomass; however, the digestive efficiency is achieved by the functional complementation with host endogenous lignocellulases (Tokuda and Watanabe, 2007; Scharf, 2008; Scharf et al., 2011). Moreover, termites also interact with the surrounding soil microbiota and at least two extant lineages (Macrotermitinae and Sphaerotermitinae) engaged in an external association with nest microorganisms, where each component of the termite holobiont has a collaborative role in the decomposition of lignocellulose (Poulsen et al., 2014; Chouvenc et al., 2021).

In this study, we present evidences of complementary digestion by external nest microorganisms in a neotropical grass-harvester termite *C. cumulans* (Syntermitinae) that store plant material inside its nests (**Figure 3F**). Our findings revealed that plant biomass (previously cut and packed in the food nodules) is initially decomposed by enzymes that target the lignin and complex polysaccharides as cellulose, xylan, arabinoxylan, and starch. We hypothesized that pre-processing of the plant material reduces the recalcitrance of the lignocellulose before gut passage. Since lignin breakdown by peroxidases needs O_2 (Breznak and Brune, 1994), food nodules are a suitable environment for this process. Peroxidases and dehydrogenases of bacterial origin are known to participate in lignin degradation (Pelmont et al., 1989; Tamboli et al., 2011; Chauhan, 2020). The catalase-peroxidase (EC 1.11.1.21) assigned to Actinobacteria in this study was able to oxidase lignin when incubated with lignocellulosic material (de Gonzalo et al., 2016). The final step in the lignocellulose digestion process involves the passage of the pre-processed plant material in the gut of workers where it is again targeted by xylanases, possibly for overcoming any xylan residuals and making the cellulose more accessible to the endoglucanases produced by the gut microbiota. Although we found that endoglucanase transcripts were expressed in the food nodules, our results showed higher expression of cellulases in the gut of termites.

Other CAZy predicted in the gut were the non-catalytic carbohydrate-binding module CBM22 that promotes the association of xylanases and the carbohydrate esterases of *C. cumulans* (CE4 and CE10) (Sermsathanaswadi et al., 2017) that could be acting in the breakdown of hemicelluloses (Franco Cairo et al., 2016). The presence of chitinases was predicted in the food nodules and linked to bacteria of the phylum Firmicutes (EC 3.2.1.14) and to an ascomycete fungus (EC 3.2.1.52). Chitinases could be related to fungi cell-wall growth or the activity of mycolytic bacteria strains (Solanki et al., 2012) found in the food nodules. Chitinase transcripts were not obtained in the gut of *C. cumulans*; however, other higher termites are known to produce chitinases which can improve the digestibility

of fungal substrates and decaying wood (Poulsen et al., 2014; Hu et al., 2019) or increase the permeability of peritrophic matrix in the midgut for the absorption of nutrients.

Although there is functional overlap between the food nodules and gut, CAZy were linked to different taxa. It could be explained by the different conditions that make these places more suitable to different kind of microorganisms. In the food nodules, cellulose and hemicellulose digestion were linked to Proteobacteria and Ascomycota, whereas catalase activity was assigned to Actinobacteria. On the other hand, lignocellulases in the gut of *C. cumulans* were linked to Firmicutes, Ascomycota, and Chytridiomycota. All these microbes dominated the core microbiota and are known to produce cellulases, hemicellulases (Couturier et al., 2016; Grieco et al., 2019; Matsuzawa et al., 2019, 2020) and laccases (Murphy et al., 2021). In fungus-growing insects that exploit plant-derived resources, the association between fungi and bacteria result in a multipartite functional metabolism of lignocellulose (Barcoto et al., 2020). The ecological relevance of such microbial interaction in the food nodules of *C. cumulans* needs to be determined.

Because our data was derived from a single colony, we cannot exclude the possibility of intercolonial variation that may affected the expression levels. Indeed, the relative abundances of some fungi orders of food nodules showed variation among the colonies used for ITS sequencing. However, the taxa responsible for CAZy expression showed similar abundances for bacteria (Menezes et al., 2018) and fungi (this study).

Fungi were less abundant than bacteria in all the samples and their CAZy expression levels associated to cellulose and xylan were also lower. Nevertheless, a noteworthy observation in this study was that the termite gut of *C. cumulans* is inhabited by fungi that expressed CAZy gene transcripts. One gene transcript, assigned to GH5_4 family and classified as endoglucanase EC 3.2.1.4 was linked to *Neocallimastix* (Chytridiomycota: Neocallimastigales). This finding suggests this termite possesses a functional mycobiota in its gut that contributes to the lignocellulose digestion. Neocallimastigales comprises anaerobic highly active lignocellulolytic fungi of the gastrointestinal tract of herbivores, which has also been reported in termites (Lee et al., 2015; da Silva et al., 2017).

Most of the fungi associated to the nests of *C. cumulans* (Syntermitinae) corresponded to saprotrophic Ascomycota, but other guilds within this same fungal phylum are also present in this microenvironment. Like termites, saprotrophic fungi are important plant-litter decomposers in terrestrial ecosystems using lignocellulolytic enzymes (Crowther et al., 2012) and their incidence inside termite nests could be explained by the addition of feces to the internal walls (Brauman, 2000), creating an appropriate environment for the survival and reproduction of these microorganisms (Holt, 1998; Vesala et al., 2019). Lichenized and endophytic fungi were also detected in the nest substrates but in lower abundances. The presence of these fungi has been reported in dead plant material probably consuming organic compounds released by saprotrophic microbes acting on the decomposition of the plant matter (Ottosson et al., 2015), and the mechanisms by which they colonize termite nests are unknown, but they were possibly transported from foraging parties to the mounds by workers. We found a distinct fungal composition of food nodules in comparison to the carton nest core, characterized by the abundance of saprotrophic Xylariales and Sordariales. Members of these fungal orders are known to colonize several substrates, including leaf litter, vertebrate dung, and the interior of termite nests (Okane et al., 2008; Guedegbe et al., 2009). In fungus-growing termites, Xylariales appear to be opportunistic saprotrophs of the stored plant material and fungus combs either due to the failure of microbe suppression mechanisms or after the nests have been abandoned by the termites (Mueller and Gerardo, 2002; Visser et al., 2011).

Approximately 90% of fungal reads were shared between nest fecal material and the gut of *C. cumulans*. One possible explanation for this is that termites consume the fecal material of their nests, independently of the food storing behavior. Carton feeding has been reported in other termites (Brauman et al., 2000), but the precise function of this behavior is not completely understood. Fungal colonization of lignocellulosic substrates is known to enrich its nitrogen content (Jacobson et al., 2015) and because most wood- and litter-feeding termites have a diet poor in nitrogen (Tayasu et al., 1994), consumption of nest carton could also help increase nitrogen intake by the termites. An alternative hypothesis is that nest fungi were originally gut symbionts deposited by the workers together with their feces and saliva on the nest walls. Since we found small differences in the number and nature of fungi of gut samples between colonies within each termite species, it is possible that the fungus is part of the natural gut symbionts and could function in their nests as a supplementary reserve of lignocellulolytic microbiota for these termites (Nalepa et al., 2001; Fall et al., 2004). The large number of shared microbes and CAZy transcripts between sterile castes is consistent with the transmission of microorganisms among nestmates. Soldiers, larvae, and reproductive castes cannot feed themselves and depend on workers for nutrition through trophallaxis (Machida et al., 2001).

In wood-feeding termites, fungi are known to play facultative interactions by attracting individuals to the food source or by improving colony development; however, the effect of fungi may also depend on the termite species (Birkemoe et al., 2018). For example, it is known that the wood decayed by the brown rot fungus *Gloeophyllum trabeum* attracts the termites *Reticulitermes virginicus*, *R. flavipes* (Rhinotermitidae), and *Nasutitermes columbicus* (Termitidae) (Esenther et al., 1961; Smythe et al., 1967) because of the presence of a particular alcohol in this decayed wood, (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (Matsumura et al., 1968, 1969), known to be the only compound or one of the compounds of the trail pheromones of these termite species (Sillam-Dussès, 2010). Besides the potential benefits of nest fungi, we cannot discard the possibility of fungal entomopathogens or commensals to be present in the termite nest. Some representatives of Hypocreales found in our samples could be potential entomopathogens (Araújo and Hughes, 2016), but they were found in low frequency because they may be suppressed by the abundant Actinobacteria (Chouvenc et al., 2018) or by termite gland secretions (Rosengaus et al., 2004) or these fungi are just dormant in this environment (Hajek, 2017).

In this work we improved ITS sequence analyses to bring a more comprehensive understanding of the fungal communities associated to the nest, gut, and food nodules stored by *C. cumulan*s and we bring additional metagenomic and metatranscriptomic analyses of the microbial communities associated with this termite species. Taken together with our previous analyses of the 16S rRNA bacterial communities and the differential lignocellulolytic activity in food nodules (Menezes et al., 2018), we have provided important new evidence suggesting complementary digestion of the plant material inside food storage structures before the passage through the gut of a Neotropical higher termite species. We have shown that fungi are also CAZy contributors in the termite holobiont. This could explain why the survival of this termite increased significantly when workers were fed with food nodules compared to other plant feedstocks (Janei et al., 2020). From the perspective of termite evolution, this study brings new information about the multicomponent nature of the holobiont in higher termites. The complementary digestive strategy of *C. cumulans* may resemble the one linking the fungus-growing termites and their mutualistic fungus *Termitomyces* (Poulsen et al., 2014). The association with nest microorganisms is considered as an evolutionary novelty in higher termites that originated millions of years ago in two sister lineages (Macrotermitinae and Sphaerotermitinae) after transition of gut symbionts in the ancestor of Termitidae (Bucek et al., 2019). However, our findings in *C. cumulans* supports the hypotheses that externalization of the digestion was an ancestral condition of higher termites that evolved through the incorporation of soil lignocellulolytic microbes into the fecal nest material (Chouvenc et al., 2021). Consequently, this association led to the loss of gut flagellates and the acquisition of novel bacterial gut symbionts before the diversification of Termitidae. Thus, we could speculate that ectosymbiosis with nest microorganisms is a plesiomorphic characteristic of higher termites. The externalization of digestion is also present in phylogenetically related species as *Syntermes dirus* (unpublished results) (Syntermitinae) and *Velocitermes heteropterus* (Nasutitermitinae) (Menezes et al., 2018), emphasizing the need to further investigate the ecological relevance of nest microbiota in Termitidae.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the ENA accession no. PRJEB17080, https://www.ebi.ac.uk/ena/browser/view/PRJEB17080.

AUTHOR CONTRIBUTIONS

AA, GP, AC-L, and FS designed the study. EM, LM, TA, and DP performed the experiments. AA and GP analyzed data. AA, GP, JC, AR, and DS-D contributed to new analyses and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.632590/full#supplementary-material

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High Exploration Behavior of Termite Propagules Can Enhance Invasiveness

*Louis Pailler[1], Arthur Matte[1], Alexis Groseiller[1], Pierre-André Eyer[2], Fanny Ruhland[1] and Christophe Lucas[1]**

[1] *Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS – University of Tours, Tours, France,* [2] *Department of Entomology, Texas A&M University, College Station, TX, United States*

Social life is usually associated with enhanced propagule pressure, which increases the chance of introducing several individuals during a single introduction event. Social insects are therefore among the most successful invasive species, benefiting from rapid establishment and increased foundation success in new habitats. In termites, propagule pressure may also be increased by the development of reproductive individuals from a small group of foraging workers. This suggests that enhanced exploration activity may increase propagule pressure through an elevated chance of transporting isolated groups of foragers. Here, we analyzed the exploration behavior of three termite species of the *Reticulitermes* genus, comparing the invasive species *Reticulitermes flavipes* (testing both native and introduced populations) to the native species *Reticulitermes grassei* and *Reticulitermes lucifugus*. Different features representative of the exploration capacity were measured during 48 h, including: the number of tunnels, the length of tunnels, the number of foragers, and the interindividual distance of foragers in a straight line or through tunnels. Our results show that compared to the native *Reticulitermes* species, *R. flavipes* foragers from both populations dug more tunnels with a longer total length, and individuals were more spatially dispersed and covered a larger exploration zone. These findings suggest that the enhanced exploration ability of *R. flavipes* may have played a role in its invasion success, by increasing its propagule pressure through a higher chance of human-mediated transport. In addition, the absence of differences between the native and introduced populations of *R. flavipes* suggests that the exploration behaviors facilitating the worldwide invasion of this species originated in its native range.

Keywords: exploration behaviors, propagule pressure, termites, social insects, invasive species

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****Correspondence:***
Christophe Lucas
christophe.lucas@univ-tours.fr

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INTRODUCTION

Biological invasions are a serious global environmental threat (Walther et al., 2009) and have economic repercussions due to their impairment of ecosystem services and destruction of human infrastructure (Scanes, 2018). The spread of an invasive species occurs in three stages: introduction of propagules (i.e., small group of individuals), establishment, and proliferation (Allendorf and Lundquist, 2003). An increased propagule pressure, which corresponds to larger propagule

sizes (number of individuals involved in an invasion event) and higher propagule numbers (number of release events), impacts these three stages and is therefore a key element of species invasiveness (Britton and Gozlan, 2013). Specific life history traits (longevity, fecundity, or number of generations) can increase invasiveness by not only accelerating the development of introduced colonies (Lockwood et al., 2005; Fahrner and Aukema, 2018), but also by enhancing the success of dispersal events.

Social insects are among the most successful species at invading new habitats, with 57% of invasive insects being social (Bertelsmeier, 2021). The invasive success of Hymenoptera (ants, bees, and wasps) and Isoptera (termites) is primarily attributed to their social life, whereby the cooperation of many individuals may buffer stressful environmental conditions in their novel environment(s). Furthermore, social life may increase propagule pressure by increasing the probability of introducing several individuals during a single introduction event (Duncan et al., 2014). In addition to social life, invaders can also exhibit specific life history traits that increase their propagule pressure and thus enhance their invasiveness (Chapman and Bourke, 2001; Hanna et al., 2013; Evans, 2021; Eyer and Vargo, 2021). For example, the presence of numerous queens per colony (i.e., polygyny) and colony foundation by fission increase propagule pressure by increasing the foundation success of new colonies (Yang et al., 2012), which results in lower dispersal costs (Rust and Su, 2012; Hanna et al., 2013). In subterranean termites, several biological factors are known to promote invasiveness. First, these termite species nest in wood or soil, which are globally traded materials. This feature increases the number of propagules dispersed, as it favors their chance of being introduced through unintended transport. The invasive success of many termite species is also enhanced by the development of neotenic reproductives from nymphs or workers, which can transform a small group of workers into a viable propagule (Eyer and Vargo, 2021). Both of these features increase their propagule pressure, as every transported wood/soil material containing a colony fragment may represent an invasive threat (Evans et al., 2013). The development of numerous reproductives in mature colonies also augments the reproductive capacity of the colony (Perdereau et al., 2015). In subterranean termites, colonies are often composed of multiple satellite nests and feeding sites connected by underground tunnels. The large number of workers per colony increases their capacity to exploit food through tunneling [sometimes over 100 m (Dronnet et al., 2005)]. In addition to representing a significant threat to human infrastructure (Patel et al., 2020; Shults et al., 2021), high foraging activity in hidden environments may enhance propagule pressure through an elevated chance of accidentally transporting isolated groups of workers. Notably, the ability of an invasive species to associate itself with human activity and survive transport may also favor the successful establishment of its propagules (Su, 2013). Moreover, higher exploratory behavior may facilitate discovery of favorable environment allowing to increase survival success. Interestingly, a variation in foraging strategy is often present between different species of the same genus (Mizumoto et al., 2020), sometimes even between colonies of the same species (Mizumoto and Matsuura, 2013). These variations in foraging strategy may therefore differentially influence invasion success. For example, in drywood termites of the *Cryptotermes* genus, invasion proficiency is associated with the construction of longer tunnels and a foraging preference for small pieces of wood, which increases human-assisted dispersion (note that the one-piece genus *Cryptotermes* could not be directly compared to the multiple-piece genus *Reticulitermes*). Understanding the mechanisms driving the exploration behaviors of different termite species is therefore essential to better control their spread and reduce the associated economic costs.

Among subterranean termites, the genus *Reticulitermes* is one of the most costly pests, inflicting heavy damage upon wooden structures worldwide (Vargo and Husseneder, 2009; Evans et al., 2013; Buczkowski and Bertelsmeier, 2017). *Reticulitermes flavipes* (Kollar, 1837) is well established in France, after it was introduced from the eastern United States during the 18th century with the expansion of trade shipment (Vargo and Husseneder, 2009; Evans et al., 2013; Buczkowski and Bertelsmeier, 2017). Interestingly, differences in colony structure have been observed between native and introduced populations of this species. In the native populations, most colonies are headed by a couple of primary reproductives, whereas introduced colonies are several orders of magnitude larger and composed of hundreds of neotenic reproductives (Vargo and Husseneder, 2009; Baudouin et al., 2017). In its introduced range, *R. flavipes* is present in several urban areas, as well as in pine forests along the Atlantic coast. Concerning *Reticulitermes grassei* (Clément, 1978) and *Reticulitermes lucifugus* (Rossi, 1792), they are both considered as native in Europe. Interestingly, most *R. flavipes* populations occur in sympatry with *R. grassei* (Baudouin et al., 2018; Perdereau et al., 2019). In these populations, *R. flavipes* is dominant and outcompetes *R. grassei*, particularly in urban areas (Perdereau et al., 2011), which could also enhance dispersion by increasing chance of human mediated transport. Overall, the differences in ecological dominance between the different species, together with drastic changes in colony structure and colony size between the native and invasive ranges of *R. flavipes*, suggest that these species and populations potentially exhibit strong divergences in their exploration behaviors after propagule introductions.

In this study, we aimed at determining whether invasiveness is influenced by a shift in exploration behavior in the *Reticulitermes* genus after introduction of a propagule in a new environment. Using three species of this genus (*R. flavipes*, *R. grassei*, and *R. lucifugus*), we determined whether small groups of workers display differences in their exploration behaviors. These small groups were composed of thirty workers – the initial number of individuals required to form a viable propagule (Pichon et al., 2007). Due to the previously observed variations between species within this genus, we hypothesized that different species of *Reticulitermes* exhibit differences in their exploration behaviors. For the invasive species *R. flavipes,* we also compared these behaviors between native and introduced populations. We hypothesized that the changes in social organization observed between native and invasive populations may underlie differences in exploration behaviors between the two populations. Finally, we predicted that the two populations of *R. flavipes*

possess higher exploration efficiency compared to the two native species (*R. grassei* and *R. lucifugus*), whereby high foraging activity potentially drives invasive success *via* enhanced propagule pressure.

MATERIALS AND METHODS

Study Species and Laboratory Conditions

Fifty-three colonies of three different species of the *Reticulitermes* genus were collected in the field. Two populations of *R. flavipes* were collected in 2019, including sixteen colonies from Oléron (Charente-Maritime, France) for the invasive French population (called *R. flavipes FR*), and thirteen colonies from Lake Bryan (TX, United States) for the native American population (called *R. flavipes US*). Fourteen colonies of *R. grassei* were collected in 2019 from Oléron (Charente-Maritime, France), and ten colonies of *R. lucifugus* were collected in 2020 from Sainte-Maxime (Var, France). For each species, all colonies were sampled at least 300 m apart to ensure that distinct colonies were collected (Perdereau et al., 2010). Colonies were maintained under standard lab conditions (26 ± 1°C and >95% RH) within black individual plastic boxes (Starpack) containing ultrapure paper (47 mm diameters; Whatman, grade 42 Ashless), moistened sand, and pine wood sawdust (Lucas et al., 2018).

Experimental Design

Behavioral observations were performed using a glass sheet design (Brossette et al., 2017). A hole (15 mm diameter) was drilled in the center of one of the two glass sheets to allow the introduction of termites into the exploration area. A diamond drill bit was used to drill the hole in the center to prevent the glass from cracking. The two glass sheets (220 mm × 220 mm) were separated by two spacers (100 mm × 10 mm × 1.4 mm) located on each side, to obtain a 200 mm × 200 mm exploration area. Sand was introduced between the two glass sheets and moistened with Milli-Q water 24 h before the introduction of individuals, and no food was added. One hour before observations, 30 workers were randomly selected per colony and placed in plastic boxes (50 mm diameter; Starpack Cat#04913) containing moistened pure cellulose paper (47 mm diameter; Whatman, GE Healthcare). Individuals were sorted under CO_2 then placed in a 1.5 ml tube to facilitate their introduction into the arena. The glass hole was plugged with plexiglass and covered with a glass blade to prevent escape. Measurements were carried out for 48 h using cameras (Basler acA1300 – 60gc) driven by the software Labview (v16.0). Cameras were fixed to a rail above the arenas. Pictures of the arena were taken at three observation times: 6, 24, and 48 h. For each species and population, five factors were investigated: the number of tunnels (NT), the total length of the tunnels, the number of foragers (NF) (individuals dispersed away from the introduction area), and the shortest distances between individuals (distance in a straight line and distance through tunnels). Data were recorded and analyzed blindly regarding the treatments (Penn and Frommen, 2010).

Spatial Data Analyses

The cartography of tunnels was analyzed using QGIS (v3.10.2). Pictures of the two-glass systems for each time were implemented as raster files. The RGF93/Lambert93 EPSG:2154 coordinate system was derived to scale the pictures (Bech et al., 2017). The spatial distribution was analyzed, using a point shape (on the neck) to spot each forager (individual dispersed away from the introduction area), as well as to calculate the shortest distance between foragers. Tunnels were counted and drawn to obtain an overall tunnel layout, thereby allowing the total length of the tunnels to be summed. Distances between foragers through tunnels were calculated using the previous tunnel layouts. Note that termites could block unused tunnels; however, in our analyses, such tunnels were not counted anymore. Therefore, the total length of the tunnels could sometimes decrease over time.

Statistical Analyses

The number and the length of tunnels, the interindividual distances (in a straight line and through tunnels) and the number of foragers were analyzed using linear mixed models (LMM). To fit with a normal distribution of residuals and homoscedasticity, all variables were log (+1)-transformed except the number of foragers. Two-way ANOVAs were performed for each variable and the explanatory factors were the observation time and the species. Colony IDs and observation times were used as random factors. The models were first tested with interactions between variables, which were removed when not significant ($p > 0.05$). To analyze significance across the different species for each observation time (if applicable) we performed Tukey's HSD all-pairwise comparisons tests.

Edge effects of the arena were tested by comparing colonies with tunnels hitting the edges to colonies without tunnels hitting the edges. Edge effects were calculated for the five variables and for each species/population. We performed either a Student's *t*-test or Wilcoxon test depending on normality of the data. Data which did not fit a normal distribution were the NT for *R. flavipes FR* and *R. lucifugus*, as well as the NF for *R. lucifugus*.

At the end of the experiments, termites were extracted to count the total number of alive individuals. No mortality effect was found (all $p > 0.20$); with a mortality range between 0 and 5 individuals per colony (1.2 deads on average). Over the 53 tested colonies, 19 colonies showed no mortality and only 2 colonies showed 5 deads.

To infer the relationships between the five variables, we conducted a Principal Component Analysis (PCA) for each observation time. These analyses provided five orthogonal principal components (PCs), out of which we retained the first two PCs (total variances explained >83.9; **Table 1**). We extracted loadings of each variable on the two PCs (**Table 1**) to analyze its contribution to the overall PCA, and therefore to determine its influence in driving the difference between species. We applied Mardia's principle, which states that a PC is loaded by a variable when the value of the loading is higher than 0.8 (Mardia et al., 1979). We then extracted projection values of colonies on the two PCs of the PCA. These values were used as explanatory variables in three linear models (one for each observation time), in which

TABLE 1 | Loadings of each variable on the two principal components (PC1 and PC2) for each observation time (6, 24, and 48 h).

	6 h		24 h		48 h	
	PC1	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Number of tunnels (NT)	**0.951**	−0.184	**0.833**	−0.426	0.587	0.781
Tunnels length (TL)	**0.978**	−0.057	**0.948**	−0.105	**0.917**	0.116
Interindividual distance – straight lines (DL)	**0.976**	−0.084	**0.958**	0.057	**0.945**	−0.217
Interindividual distance – through tunnels (DT)	**0.978**	−0.089	**0.965**	−0.002	**0.939**	−0.174
Number of foragers (NF)	**0.892**	0.449	**0.816**	0.491	0.671	−0.291
Eigenvalues	4.563	0.254	4.108	0.437	3.418	0.795
Variance explained (%)	91.3	5.1	82.2	8.8	68.2	15.7
Cumulative variance explained (%)	91.3	96.4	82.2	91	68.2	83.9

Values showing significant differences between species are in bold. Eigenvalues and explained variances are also represented.

the species was entered as fixed factor. We conducted one-way ANOVAs to compare PC values across species (one for each PC). When applicable, pairwise comparisons between species were tested using Tukey's post-hoc tests.

All analyses and graphs were performed using the *lme4* (Bates et al., 2015), *car* (Fox et al., 2013), *emmeans* (Lenth, 2020), *FactoMineR* (Lê et al., 2008), *sciplot* (Morales, 2017), and *factoextra* (Kassambara and Mundt, 2020) packages in R v3.6.1.[1]

RESULTS

The five variables showed the same pattern (**Figure 1**). The number of tunnels (ANOVA, $F_{6,53} = 25.49$, $p < 0.001$) and the total length of the tunnels (ANOVA, $F_{6,53} = 70.05$, $p < 0.0001$), the interindividual distances in a straight line (ANOVA, $F_{6,53} = 117.43$, $p < 0.0001$) and through tunnels (ANOVA, $F_{6,53} = 45.26$, $p < 0.0001$), and the number of foragers (ANOVA, $F_{6,53} = 28.05$, $p < 0.0001$) were dependent on the interaction between the species and the observation time. No difference between species/populations were observed at 48 h, regardless of the variable considered (Tukey's multiple comparisons test, all $p > 0.0794$).

The number of tunnels at 6 h was higher in the two populations of *R. flavipes* than in *R. grassei* and *R. lucifugus* (Tukey's multiple comparisons test, all $p < 0.024$; **Figure 1A**). But at 24 h, *R. flavipes FR* constructed more tunnels than only *R. lucifugus* (Tukey's multiple comparisons test, $p = 0.004$). The total length of the tunnels at 6 h for *R. flavipes FR* and *R. flavipes US* was higher than *R. grassei* and *R. lucifugus* (Tukey's multiple comparisons test, all $p < 0.0001$; **Figure 1B**). But at 24 h, tunnels of *R. flavipes FR* and *R. flavipes US* were longer than only *R. lucifugus* (Tukey's multiple comparisons test, all $p < 0.042$). Both interindividual distances were higher for *R. flavipes FR* and *R. flavipes US* compared to *R. grassei* and *R. lucifugus*, 6 h after introduction (in a straight line, Tukey's multiple comparisons test, all $p < 0.0001$; **Figure 1C** and through tunnels, Tukey's multiple comparisons test, all $p < 0.01$; **Figure 1D**). However, no significant differences were observed between species/populations after 24 or 48 h. At 6 and 24 h (**Figure 1E**), the number of foragers was higher for *R. flavipes FR* compared to *R. grassei* (Tukey's multiple comparisons test, both $p < 0.001$) and *R. lucifugus* (Tukey's multiple comparisons test, both $p < 0.038$). The number of foragers for *R. flavipes US* was significantly higher compared to *R. grassei* (Tukey's multiple comparisons test, both $p < 0.023$), but not compared to *R. lucifugus* (Tukey's multiple comparisons test, both $p > 0.067$) at 6 or 24 h. No edge effect was found between species for the five variables (all $p > 0.067$). Nevertheless, it must be noted that colonies were rarely observed with tunnels hitting edges, as only four colonies of *R. flavipes FR*, one colony of *R. flavipes US* and three colonies of *R. lucifugus* made it to the edge (and none for *R. grassei*). Overall, these results revealed a strong difference in exploration strategies between the two populations of *R. flavipes* and the two other *Reticulitermes* species at 6 h, and that this difference subsequently faded over time (**Figure 2**). Additionally, no difference was ever observed between the native and invasive populations of *R. flavipes*, and no difference was observed between the two non-invasive species, *R. grassei* and *R. lucifugus*.

The differences in exploration behavior between species at 6 h were highlighted on the PCA, which segregated the two populations of *R. flavipes* from the two other species. This segregation mostly results from a difference between species on the first component (PC1) (**Figure 3A**; ANOVA, $F_{3,53} = 12.8$, $p < 0.01$), which explained 91.29% of the variation observed. Consistent with our results above, significant differences were observed between *R. flavipes* and the two non-invasive species, while no difference was observed between the native and invasive populations of *R. flavipes*, and no difference was observed between *R. grassei* and *R. lucifugus*. At 6 h, PC1 was positively loaded by all five variables (**Table 1**), which were all correlated together, thereby reflecting the importance of all variables in explaining the differences between species (**Figure 3B** and **Table 1**; all the loadings >0.816). However, no difference between species was observed at 6 h on PC2 (**Figure 3A**; ANOVA, $F_{3,53} = 1.27$, $p = 0.29$) and no variable was loaded on PC2; but it is important to note that this component only slightly explained the observed variance (5.09%).

At 24 h, although the PCA only slightly segregated the different species studied, PC1 was able to discriminate the two populations of *R. flavipes* from *R. lucifugus* (**Figure 3C**, Tukey's multiple comparisons test, both $p < 0.03$), as well as *R. flavipes FR* and *R. grassei* (**Figure 3C**; Tukey's multiple comparisons

[1]www.r-project.org

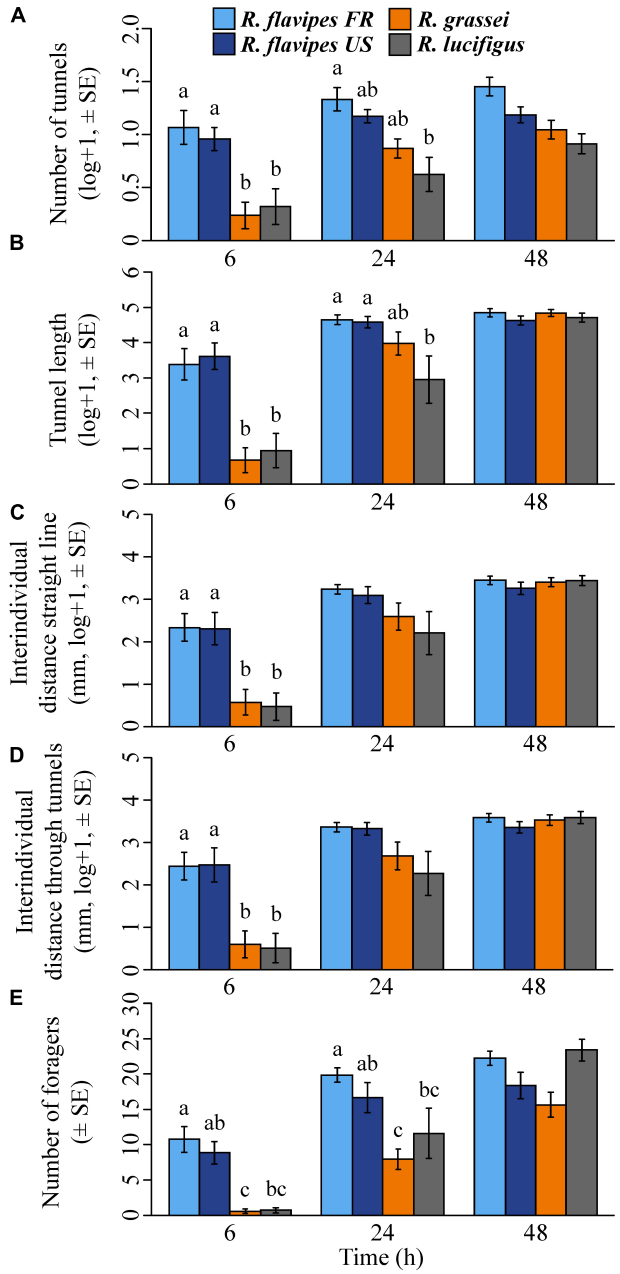

FIGURE 1 | The five variables measured at 6, 24, and 48 h. Number of tunnels **(A)**, total length of the tunnels **(B)**, distance between individuals in a straight line **(C)**, distance between individuals through tunnels **(D)**, and number of foragers (individuals dispersed away from the introduction area) **(E)**. Significant differences between species are indicated by different letters ($p < 0.05$).

test, $p < 0.04$). Similarly, PC1 was positively loaded by all five variables (**Table 1**). Interestingly, PC2 significantly segregated *R. grassei* and *R. lucifugus*. This difference primary stemmed from the number of tunnels and the number of foragers, as these variables were more loaded on PC2 (**Figure 3D**) than the three other variables, despite their loading values being lower than the threshold value of 0.8 (**Table 1**). At 24 h, *R. grassei* was characterized by a higher NT, while *R. lucifugus* was characterized by a higher NF.

At 48 h, the PCA was unable to discriminate the three species, as all colonies studied randomly segregated along each axis, regardless of their species or population of origin. Accordingly, there was no difference between species/populations at 48 h on PC1 (**Figure 3E**; ANOVA, $F_{3,53} = 5.59$, $p = 0.35$), which explained most of the variance observed (68.24%). At 48 h, only the tunnel length (TL) and the two interindividual distances (DL, DT) were associated with PC1 (**Figure 3F** and **Table 1**; all the loadings >0.917). Interestingly, PC2 at 48 h gave the same result as PC1 at 24 h, with it slightly segregating *R. flavipes* and the two non-invasive species, despite only the difference between *R. flavipes FR* and *R. lucifugus* being significant. Following Mardia's principle (Mardia et al., 1979), PC2 at 48 h was only marginally loaded by the number of tunnels (**Figure 3F**; loadings = 0.781).

DISCUSSION

The three species of *Reticulitermes* studied exhibited differences in their exploration behaviors for all five observed variables. Six hours after introduction, the two populations of *R. flavipes* (*US* and *FR*) showed a greater exploration ability compared to *R. grassei* and *R. lucifugus*. Foragers of *R. flavipes* were able to dig more tunnels with a longer total length, with individuals being more spatially dispersed and covering a larger exploration zone. Interestingly, the differences observed between species at 6 h almost disappear at 24 h, and are equal at 48 h, suggesting that *R. flavipes* more rapidly reaches adequate gallery size. These findings suggest that the enhanced exploration ability of *R. flavipes* may have played a role in its invasion success. The rapid construction of long tunnels may increase propagule pressure through a higher chance of human-mediated transport. Surprisingly, both the native and invasive population of *R. flavipes* have similar exploration capacities, suggesting that these exploration behaviors likely originated in its native range.

Both native and introduced populations of *R. flavipes* exhibit high exploration abilities for all five variables. Similar, yet different, tunneling patterns have been reported in invasive subterranean termites of the *Coptotermes* genus (Mizumoto et al., 2020). During experiments with food, *Coptotermes formosanus* constructs a low number of long tunnels, while *Coptotermes gestroi* constructs a high number of short tunnels (Grace et al., 2004; Lee et al., 2007). Longer tunnels are potentially associated with higher propagule pressure. This ability to colonize many pieces of wood also increases the chance of human-mediated transportation (Evans et al., 2011). Our data show that both populations of *R. flavipes* are characterized by a high number of long tunnels, 6 h after the introduction of foragers. These traits could represent advantages in terms of exploration capacities, food detection and foraging strategies, as described in termites and ants (Traniello, 1989; Hölldobler and Wilson, 1990; Traniello and Leuthold, 2000). Interestingly, the differences between species decrease over time and completely disappear at 48 h. The lack of differences between species at 48 h could be explained by the reduced size of the arena. Other experiments with similar two-dimension designs showed that the maximum tunneling distances are dependent on the size of the arena

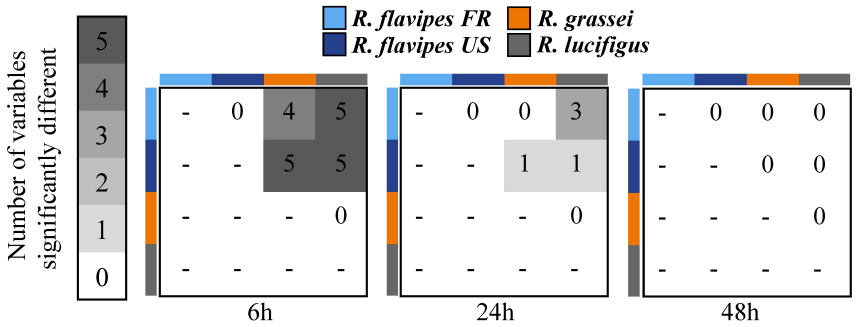

FIGURE 2 | Summary of the differences between the three species, reflecting the variations in the five measured variables at 6, 24, and 48 h.

(Nobre et al., 2007; Li et al., 2010; Chouvenc et al., 2011), as well as the group size (Su and Lee, 2009). However, our findings reveal that *R. flavipes* reaches the maximum/adequate gallery size faster than the two other *Reticulitermes* species, confirming the superior exploration ability of this species. Additionally, in our experimental design, colonies rarely built tunnels that met the edge of the arena, even if we cannot exclude that they detected the edges. Indeed, termites are able to use vibrational cues to estimate the size of a piece of wood (Evans et al., 2005). Nevertheless, removing the few colonies that hit the edge did not affect the results for any of the five variables. Another factor influencing foraging patterns is the caste ratio, since the presence of soldiers increases the survival of workers by reducing the stress caused by competitors (Tian et al., 2017). It could be interesting to test the group size/composition effect on the exploration behaviors in these different species, with a suitable design for a larger number of individuals.

This higher short-term exploration capacity for *R. flavipes FR* compared to *R. grassei* and *R. lucifugus* may also reflect differences in their life history traits. Indeed, *R. flavipes* and *R. grassei* are sympatric in the sampling area of the current study (Oléron) where they display differences in their life history traits. Both species exhibit extended families (i.e., the presence of multiple neotenic reproductives), but they differ in traits like parental care (Brossette et al., 2019), colony foundation (Brossette et al., 2017) and aggressiveness (Perdereau et al., 2011; Duarte et al., 2018). Feeding at multiple sites is a common trait for *R. flavipes*, while less than half of the colonies of *R. grassei* exhibit this trait in the studied population (Deheer et al., 2005). Exploiting multiple feeding sites leads to an increase in distance between individuals, as observed in *R. flavipes* in this study. Moreover, in accordance with our findings, introduced populations of *R. flavipes* have been previously found to inhabit larger foraging areas (up to 90,000 m^2) and to construct linear foraging tunnels (sometimes up to 320 m) compared to *R. grassei* (up to 70 m, respectively) (Deheer et al., 2005; Dronnet et al., 2005). Overall, *R. flavipes* exhibits a higher survival rate, a higher production of individuals, along with a higher total number of individuals during colony foundation compared to *R. grassei* (Brossette et al., 2017). Similar results have been observed in the eastern United States where *R. flavipes* and *Reticulitermes virginicus* are sympatric species. *R. flavipes* has higher foraging activities and is now considered as invasive in other United States locations, while *R. virginicus* remains endemic to eastern United States (Pitts-Singer and Forschler, 2000; Janowiecki and Vargo, 2021). As shown here, *R. flavipes* also possesses a higher short-term exploration rate compared to the two native *Reticulitermes* species. Altogether, these results may explain both the dominance of *R. flavipes* during interspecific competition and its invasive success. For *R. lucifugus*, no study has investigated its social structure or life history traits during colony foundation. Our study represents the first report on the exploration capacities of this understudied species despite its large geographical distribution in Europe (Kutnik et al., 2020). As shown in the results, differences between species are present at different time points after propagule introduction.

The native United States and the invasive French populations of *R. flavipes* exhibit similar exploration features, suggesting that the introduction of this species did not alter its foraging strategy. The establishment of an invasive species in a new environment is often facilitated by certain biological traits. Sometimes, such traits are already present in the native population and may represent a pre-disposition to invasion, as was described in plants for tetraploidy or biomass production (Henery et al., 2010; Van Kleunen et al., 2011). On the other hand, introduced populations sometimes exhibit post-introduction phenotypic changes in morphology, behavior, and/or life history traits. Finally, in some cases, successful invaders may simply occupy a vacant ecological niche within their novel environment(s), without exhibiting phenotypic changes when compared to native populations or greater competitive ability compared to native species. Specific traits favoring invasive success have also been described in social insects (Eyer and Vargo, 2021). For example, the perennial colony cycle and the low discrimination toward non-nestmates already present in the native ranges of *Vespula* wasps may have facilitated queen recruitment and the formation of highly polygyne nests in their invasive ranges (Kasper et al., 2008; Hanna et al., 2013). In the ant *Brachyponera chinensis*, the occurrence of highly inbred colonies in native populations may have acted as a pre-adaptive trait for invasiveness, mitigating the detrimental effect of inbreeding that introduced colonies commonly experience after a bottleneck event (Eyer et al., 2018). Here, the rapid and efficient exploration strategy pre-existing in the United States population of *R. flavipes* may have promoted its invasive success, by enabling

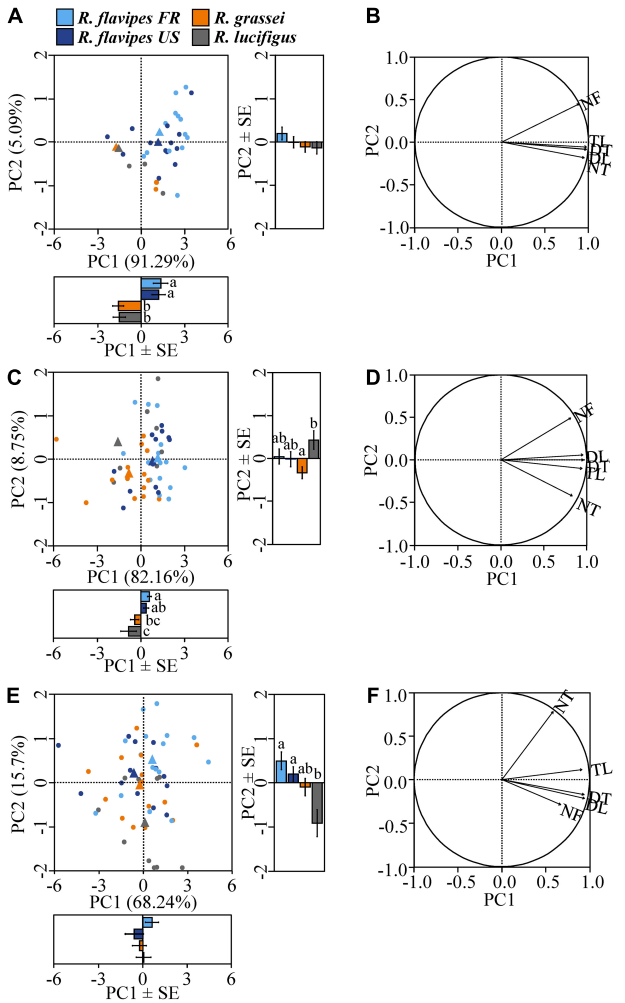

FIGURE 3 | Principal component analysis (PCA) of the colonies of the different species/populations at each observation time. PCA of the five variables and the effect of species/populations on PC1 and PC2 at 6 h **(A)**, 24 h **(C)**, and 48 h **(E)**. For each species/population, dots represent colonies and triangles represent centroids. Significant differences between species are indicated by different letters ($p < 0.05$). The two PC eigenvectors for the five variables at 6 h **(B)**, 24 h **(D)**, and 48 h **(F)**. The *x*-axis represents principal component 1 (PC1) while the *y*-axis represents principal component 2 (PC2) of the PCAs. NF, number of foragers; TL, tunnel length; DT, distance through tunnels; DL, distance in a straight line; NT, number of tunnels. Note that colonies which did not start digging, present identical PCA coordinates. It results in hidden points in **(A)** (11, 7, 3, and 1 colonies for *R. grassei*, *R. lucifugus*, and *R. flavipes FR* and *R. flavipes US*, respectively) and in **(C)** (1 colony for *R. grassei* and 3 for *R. lucifugus*).

the development of spatially expansive colonies spreading across multiple sites. In addition, it may favor invasiveness by increasing its ecological dominance and monopolization of resources. In its native range, *R. flavipes* exhibits a smaller foraging area (up to 800 m^2) and linear foraging tunnels (sometimes up to 76 m) compared to the introduced population (Vargo and Husseneder, 2009). Finally, this feature may increase its propagule pressure, by increasing the likelihood of human-mediated transport of distinct colony fragments. Moreover, in the *Reticulitermes* genus, workers are able to differentiate into neotenic reproductives (Myles, 1999), meaning that every piece of transported wood or soil with workers can become an independent functional colony (Evans et al., 2013). Interestingly, a recent molecular analysis suggests that extensive human-mediated jump dispersal is common in both the native and introduced ranges of *R. flavipes* (Eyer et al., 2021), which is consistent with the high exploration abilities observed in both of its ranges here. However, the widespread admixture within and across native and introduced populations through repeated introductions and potential re-invasion of the native range from the French population results in a lack of differentiation between native and introduced ranges (Eyer et al., 2021). It could therefore explain why no differences in exploration strategy were observed between the two tested populations of *R. flavipes*. Future experiments on different populations of *R. flavipes* in the United States will enable the investigation of possible adaptative traits favoring exploration to different environments.

Overall, this study provides new knowledge to better understand the establishment of *R. flavipes* in France, as well as the exploration characteristics that favor its invasive success. *R. flavipes* is prevalent in urban areas, while the two other species are mostly present in non-anthropized environments (Perdereau et al., 2019). Anthropized environments are subject to drastic changes caused by human activities. The rapid exploration of surrounding areas could represent a key factor in the establishment of *R. flavipes* colonies, and therefore a major advantage for its invasive success in urban areas. Our results therefore emphasize the need for early detection to prevent damages and to control expansion of *R. flavipes*. *R. flavipes* exhibits enhanced colony foundation characteristics compared to *R. grassei*, such as a significantly higher survival rate of alates (adultoid reproductive) and a higher production of eggs, larvae and workers (Brossette et al., 2017). Overall, our study highlights the greater short-term exploration capacity of the invasive species, *R. flavipes,* compared to the two other *Reticulitermes* species studied. Together with its capacity for developing numerous reproductives, reduced intraspecific aggression, rapid colony foundation, and elevated interspecific competitive ability (Perdereau et al., 2011; Brossette et al., 2017, 2019), this enhanced exploration activity may promote its invasiveness. Greater exploration activity not only increases species dominance and facilitates the monopolization of resources, but also increases propagule pressure, which is an essential component of dispersal.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: doi: 10.5281/zenodo.6023265.

AUTHOR CONTRIBUTIONS

CL, LP, AG, and FR designed the experiments. CL, LP, and FR coordinated the data collection. LP and AM collected the data. LP and CL performed the data analysis. LP, CL, and P-AE wrote

the manuscript with help of FR. All authors contributed to the article and approved the submitted version.

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Rescue Strategy in a Termite: Workers Exposed to a Fungal Pathogen Are Reintegrated Into the Colony

Megan N. Moran, Carlos M. Aguero, Pierre-André Eyer and Edward L. Vargo

Department of Entomology, Texas A&M University, College Station, TX, United States

Social insect colonies are characterized by an efficient division of labor, allowing high-value individuals (i.e., reproductives and brood) to be sheltered from tasks associated with increased risk of pathogen exposure, such as foraging or corpse disposal. This social organization helps limit the transmission of disease throughout the colony. Further, individuals can actively respond to imminent disease threats by altering their behaviors as a means of social immunity. In subterranean termites, although workers typically avoid detected pathogens, they can be attracted to pathogen cues when a nestmate is infected. Infected termites are usually groomed, but they may instead be cannibalized if the infection has already become lethal. The mechanisms governing these changes in behavior are unclear. We set out to examine *immediate* changes in individual behaviors, investigating the role that the infected individual plays in communicating its infection status to nestmates. We also assessed *gradual* changes in social organization after the re-introduction of an infected termite to the colony. Our results reveal that infected termites likely do not signal their infection status to nestmates through shaking behaviors and reduced movements, suggesting the occurrence of other mechanisms used in communicating infection. We also found that infected termites do not self-isolate and may travel to the densest part of the colony, where they can potentially benefit from grooming by large groups of nestmates. These results provide new insights into how individual changes in immune behaviors contribute to overall colony health, highlighting that, at early stages of infection, termites favor a rescuing strategy rather than isolation and/or cannibalization.

Keywords: ***Reticulitermes flavipes*, social immunity, social insect, Blattodea, rhinotermitidae, *Metarhizium***

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Brian R. Johnson, University of California, Davis, United States

***Correspondence:**
Megan N. Moran
megan.n.moran.14@gmail.com

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INTRODUCTION

The evolution of eusociality in animals has led to tremendous ecological success. This has reached a paradigm in social insects, which have rapidly diversified throughout different ecological niches (Wilson, 1990). However, living in large groups may entail several costs, especially in densely packed groups of closely related individuals. Disease can spread more easily as frequent interactions increase the chance of transmission (Hamilton, 1987; Schmid-Hempel, 1998; Godfrey et al., 2006; Brahma et al., 2021; Schmid-Hempel, 2021) and related individuals are more likely to suffer from the same disease agent

(Anderson and May, 1985; Shykoff and Schmid-Hempel, 1991; Nunn et al., 2006). Yet, social insect colonies are well protected against epidemic outbreaks, due to a variety of individual- and group-level defenses, such as allogrooming (Peng et al., 1987; Drees et al., 1992; Oi and Pereira, 1993; Rosengaus et al., 1998b; Hughes et al., 2002; Wilson-Rich et al., 2007; Yanagawa and Shimizu, 2007; Chouvenc et al., 2009b; Liu et al., 2019a; Cini et al., 2020), the transfer of antimicrobial substances through trophallaxis (Hamilton et al., 2011b), and corpse disposal (Sun and Zhou, 2013). These defenses, collectively referred to as social immunity, take advantage of an organized workforce in order to mitigate the costs of social living (Cremer et al., 2007; Cremer et al., 2018; Liu et al., 2019b).

Division of labor is the hallmark of social insect colonies, whereby individuals are allocated to different colony tasks. Within a colony, reproduction is the responsibility of one, or a few, individuals, while a larger workforce tends to all other needs. This social organization allows the reproductive caste to be protected from external threats, as some tasks, such as foraging, defense or corpse disposal, increase the risk of pathogen exposure (Durrer and Schmid-Hempel, 1994; Sun and Zhou, 2013; Stroeymeyt et al., 2014; Sah et al., 2018). Typically, colony members responsible for these risky tasks have reduced contact with high-value members of the colony (i.e., reproductives and brood), thus decreasing the chance that these valuable individuals will become infected (Wang and Mofller, 1970; Naug and Camazine, 2002; Naug and Smith, 2007; Stroeymeyt et al., 2018; Guo et al., 2020). Even within the worker caste, particularly dangerous tasks are handled by more expendable individuals. Many social insects exhibit age polyethism, such that younger individuals work inside the nest while older workers are responsible for more hazardous tasks (Seeley, 1982; Schmid-Hempel and Schmid-Hempel, 1984; Sun and Zhou, 2013; Natsopoulou et al., 2016). This serves to prolong the life of workers, and thus maximizes their contributions to the colony. Division of labor improves social immunity by constraining disease transmission throughout the colony.

While the organization of the colony workforce provides a measure of passive immune defense, individuals still actively respond to imminent disease threats. Selective pressures associated with disease are thought to have played a large role in the evolution of eusociality (Gadagkar, 1992). Consequently, many social insects are acutely sensitive to pathogen cues (Schmid-Hempel, 1998; Hussain et al., 2010; Tranter et al., 2014; Yanagawa et al., 2015; Cappa et al., 2019; Almeida et al., 2022) and, in some cases, can even discern the degree of pathogen virulence (Yanagawa et al., 2012). This strong detection ability allows social insects to adjust their behavior to reduce disease transmission risk. In several species of ants and bees, infected and contagious individuals self-isolate themselves, either by reducing their contact with nestmates or by leaving the nest entirely (Walker and Hughes, 2009; Chapuisat, 2010; Heinze and Walter, 2010; Bos et al., 2012; Stroeymeyt et al., 2018; Geffre et al., 2020; Alciatore et al., 2021).

In termites, the typical response to pathogen cues is avoidance or shaking alarm displays to warn nestmates (Rosengaus et al., 1999; Yanagawa et al., 2015; Bulmer et al., 2019). Shaking behaviors comprise an assortment of vibratory signals transmitted through the substrate and perceived by nestmates. They represent an effective form of communication in termites, especially in subterranean environments where visual and odorant signals can be less effective (Hill, 2009). Termites are therefore able to transmit different signals using shaking behavior, such as alarm signaling, caste identification and reproductive regulation (Eyer et al., 2021; Pailler et al., 2021). In addition, termites can exhibit different behaviors toward infected nestmates. Termites rely heavily on allogrooming to remove pathogenic spores from other workers (Rosengaus et al., 1998b; Chouvenc et al., 2009b; Davis et al., 2018; Liu et al., 2019a; Aguero et al., 2020). Although workers are typically repelled by olfactory pathogen cues, they may be attracted if those odors are presented alongside nestmate cues (Yanagawa et al., 2015). In some cases, workers will prevent the infected individuals from returning to the colony by sealing them inside a chamber (Epsky and Capinera, 1988). In the subterranean termite, *Reticulitermes flavipes*, when workers have incubated a lethal infection, the typical grooming response is replaced by cannibalization (Davis et al., 2018). It is currently unclear how termites determine when nestmates have developed lethal infections and can no longer be saved by grooming. If the infected individual is responsible for communicating its infection status, it may do so by either increased shaking alarms or reduced movement. A lack of movement, or moribundity, has frequently been observed in diseased termites, but it is unknown if this is a signal meant to communicate with nestmates or just a symptom of disease (Chouvenc et al., 2009b; Davis et al., 2018).

We set out to determine if pathogen-exposed workers of *R. flavipes* alter their behavior in the presence of nestmates. We examined immediate behavioral changes by measuring locomotion and shaking displays before and after reintroduction to a small group of nestmates. Then, we investigated gradual changes in behavior when infected workers return to a larger group within a nest. We used a fungal entomopathogen to infect termites and test if different incubation times affect these behavioral changes. Overall, our results assessed how individual immune behaviors relate to social immunity.

METHODOLOGY

Termite and Pathogen Preparation

In November 2019, groups of termites were collected from eight *R. flavipes* colonies in College Station, TX, United States. Collection points were located at least 15 m apart from each other to ensure that each group of termites came from a different colony (Vargo, 2003; DeHeer and Vargo, 2004; DeHeer et al., 2005). For each colony, some termites were dyed blue, so that they could be identified among undyed nestmates. Those termites were fed for 1 week on cellulose material containing Nile blue dye (Sigma, St. Louis, MO, United States), which is a fat-soluble stain commonly used to mark termites (Su, 1991; Davis et al., 2018; Aguero et al., 2020). Once the termites were dyed, pathogenic treatments were prepared from the conidia of the fungal pathogen, *Metarhizium*

robertsii, suspended in a 0.1% TWEEN® 80 solution (Sigma-Aldrich Chemie N.V, Netherlands) at a concentration of 1×10^7 conidia/mL (Aguero et al., 2021a). A similar preparation of this fungal strain at the same concentration resulted in successful infection and reduced survival compared to controls in *R. flavipes* (Aguero et al., 2021a). The spores of *Metarhizium* usually germinate and the hyphae that emerge penetrate the cuticle in the first 12–24 h. The fungus further develops inside the host body in the next couple of days, and usually kills the insect after 3–6 days (Schrank and Vainstein, 2010). The 0.1% TWEEN® 80 solution by itself was used as a control treatment. Individual termites were treated by 30 s of immersion in 0.5 mL in either a pathogen or a control solution. Treated individuals were individually moved to 60 mm diameter petri dishes lined with moistened filter paper and allowed to incubate for 15 min, 24 h, or 48 h. Experiments were started after the appropriate incubation time.

Immediate Changes in Behaviors

After incubation, a five-minute video of the Petri dish was recorded. Six total treatments were run (15-min incubation control, 24-h incubation control, 48-h incubation control, 15-min pathogen incubation, 24-h pathogen incubation, 48-h pathogen incubation). One worker was observed per treatment and two replicates were conducted for each of the four colonies (LB3, LC1, LC2, and LC5). Locomotion (i.e., time spent moving) and the number of shaking events of each individual termite were counted through blind observation by the same individual researcher. After 15 min, four undyed workers from the same colony were added to each Petri dish. In order to reduce the effects of this disturbance, an additional 15 min were allowed to pass before recording another five-minute video. Locomotion and number of shaking events of the focal termites were counted.

A single zero-altered negative binomial model (*ZANB*) (Zeileis et al., 2008) was used to determine the effects of both treatment and the presence of nestmate workers on immediate changes in locomotive behavior. The effect of the presence of nestmate workers on locomotion was also tested separately using a paired Wilcoxon test. A different zero-altered negative binomial model was used to test whether individual termites changed their shaking behavior once nestmate termites were added. This change in shaking behavior was also tested using a paired Wilcoxon test. However, the overall low number of shaking events was not sufficient to test for a potential effect of treatment.

Gradual Changes to Behaviors

Groups of 500 workers and five soldiers from each of four colonies (LB02, LB04, LB07, and LB08) were introduced into 25 cm × 25 cm planar arenas filled with moistened sand (**Figure 1**, similar to Chouvenc et al., 2011). The planar arenas contain a single entrance container connected to the arena through a small plastic tube. The side of the arena opposite to the entrance was lined with discs of filter paper, serving as food material on which the colony can establish its main chamber. Each plate was divided into four levels (A-D), so that observers could identify individuals in different areas of the arena (**Figure 1**). Termites were introduced through the entrance and allowed to tunnel through the plate for 1 month, which was enough time for the colony to settle and form stable tunnels and chambers. Unfortunately, the colonoids used in this study did not produce secondary reproductives after this one-month period, so there were no reproductives present in the planar arenas during the experiment. The entrance container was continuously supplied with filter paper (i.e., food source), so that foraging workers could be repeatedly collected and returned (which is impossible in the different levels of the plate arena).

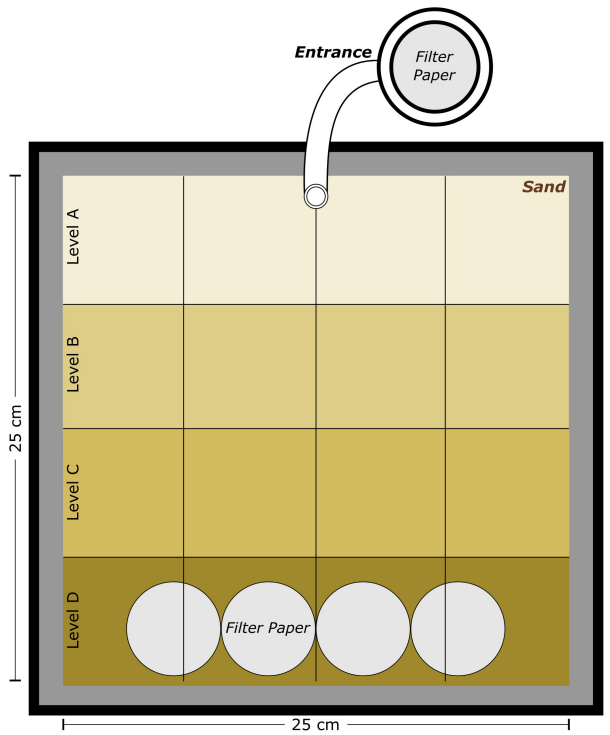

FIGURE 1 | Diagram of planar arenas. 500 workers and five soldiers from each colony were placed into the arena entrance, which leads into a 25 cm × 25 cm planar arena filled with moistened sand. The entrance was continuously supplied with filter paper, so that foraging workers could be repeatedly collected and returned. Each arena was divided into four levels (A-D). Level D contained filter paper discs that served as a food material on which the colony could establish its main chamber.

Four total treatments were run (24-h incubation control, 48-h incubation control, 24-h pathogen incubation, 48-h pathogen incubation). Each treatment was replicated by using 2–3 different colonies, resulting in a total of nine experimental replicates. All treatments were run simultaneously. After the colonies had settled for the one-month period, five workers were removed from the entrance of each plate. These workers were fed cellulose material containing Nile blue for 1 week then treated with either a pathogen or control solution. Treated workers were left to incubate for either 24 or 48 h before being reintroduced to the entrance container of the arenas. In order to assess changes in social organization, the number of nestmates in each level was counted through blind observation by the same individual researcher at 15 min before the reintroduction of treated individuals, as well as at 15 min, 1 day, and 7 days after

their reintroduction. In order to assess potential self-isolation, the number of dyed termites (i.e., infected or control treated) was also counted in each level at 15 min, 1 day, and 7 days after their reintroduction. To ease counting, each level was further split into 4 grid squares, which were later summed.

To analyze changes in social organization of the colonies, the distribution of nestmate workers among different levels was investigated using Fisher's exact tests for each treatment. We compared the distribution of nestmate workers found in different levels at each observation time to levels before the reintroduction of dyed termites (i.e., infected or control treated). To assess potential self-isolation, *U*-statistics permutation (*USP*) tests of independence (Berrett and Samworth, 2021) were used to determine if the number of dyed individuals differed between levels of the colonies. Different *USP* tests were performed separately for each treatment. Finally, two global Fisher tests (one for control data and one for pathogen data) were applied after combining data for the different observation times and incubation times. These tests were used to determine whether the dyed individuals (either control-treated or pathogen treated) were similarly distributed among levels compared to their nestmate workers. All analyses were performed in the statistical software R Studio Version 1.4.1717 (R Core Team, 2022).

RESULTS

When examining immediate changes to behavior, neither treatment (*ZANB*, $p = 1.0$ and $p = 0.529–0.936$ for zero-value and count data respectively) nor the presence of nestmate termites (*ZANB*, $p = 0.70$ and $p = 0.056$ for zero-value and count data respectively) were found to have a significant effect on immediate changes in locomotion when both of them were analyzed together (*ZANB*; **Figure 2A**). Consistently, a similar result was found when the presence of nestmate termites was investigated separately across all treatments, with no significant difference in locomotion before or after nestmate termites were added (*Wilcoxon test,* $p = 0.065$). However, there was a significant difference in locomotion before and after nestmate termites were added in the 15-min pathogen incubation treatment (*Wilcoxon test,* $p = 0.040$). Interestingly, a similar reduction in locomotion before and after nestmate termites were added was also observed in the 15-min incubation control treatment, despite not being significant (*Wilcoxon test,* $p = 0.092$). Additionally, the presence of nestmate termites did not significantly influence the number of shaking events observed using both the *ZANB* ($p = 0.591$ and $p = 0.411$ for both zero-value and count data respectively) and *Wilcoxon* test ($p = 0.670$; **Figure 2B**).

First, gradual changes within colonies after reintroduction of dyed termites were investigated through changes in social organization (i.e., abundance and distribution of nestmates among the different levels). Regardless of treatment, colony density concentrated away from the entrance of the plates, as workers were significantly more abundant in level D (60–80% of nestmate workers), suggesting this level represented the center of the colony (**Figure 3**). Similar patterns of worker abundance among levels were observed before and after reintroduction of control-treated or pathogen-treated individuals for the different observation times. No significant change in the distribution of nestmate termites was observed at any time after reintroduction of dyed termites compared to nestmate distribution before reintroduction (All Fisher's exact tests, $p = 1$; **Figure 4**). These results were observed for both incubation times of control treatment and both incubation times of pathogen treatment.

Second, gradual changes within colonies were investigated through potential self-isolation of infected termites. These results should be taken with caution due to the small sample size and associated observational difficulty. The count of dyed termites did not always match the number of reintroduced individuals. These discrepancies may have resulted from the death of infected workers or some being cannibalized or buried by nestmates. However, these discrepancies also occurred midway through the experiment and even for the control treatment. This suggests that missing termites may also be the result of the dye fading over time or termites hiding within tunnels or underneath the filter paper. After their reintroduction, the number of dyed individuals did not significantly differ between levels when examining each treatment separately (All *USP* tests, $p > 0.05$). This result was observed for both incubation times of control treatment ($p = 0.508$ and $p = 0.158$ for 24 h-treated and 48 h-treated individuals, respectively) and both incubation times of pathogen treatment ($p = 0.326$ and $p = 0.177$ for 24 h-treated and 48 h-treated individuals, respectively) (**Figure 3**). When incubation times and observation times were combined, the overall distribution of dyed individuals closely mirrored the distribution of nestmate workers for both control ($p = 0.235$) and pathogen treatments ($p = 0.385$; **Figure 4**).

DISCUSSION

We found that pathogen-exposed workers generally did not alter their locomotion or shaking behaviors in the presence of nestmates, regardless of the incubation time up to 48 h. We found reduced locomotion of isolated termites (control-treated and pathogen-treated individuals) shortly after nestmate workers were added in the 15-min incubation treatments. This behavioral change is probably used as a preventive inspection and grooming of reintegrated foragers.

Throughout the experiment, we found that the majority of the nestmate workers congregated within level D, which therefore represented the center of the colony. Interestingly, after reintroduction, the distribution of both control-treated and pathogen-treated individuals closely mirrored the distribution of their nestmates, indicating that they do not self-isolate after isolation or infection. In addition, the distribution of nestmate workers did not differ before and after the reintegration of infected workers. Overall, these results suggest that termites readily accept and care for infected individuals, generally adopting a rescuing strategy toward infected nestmates at the risk of an increased chance of spreading disease.

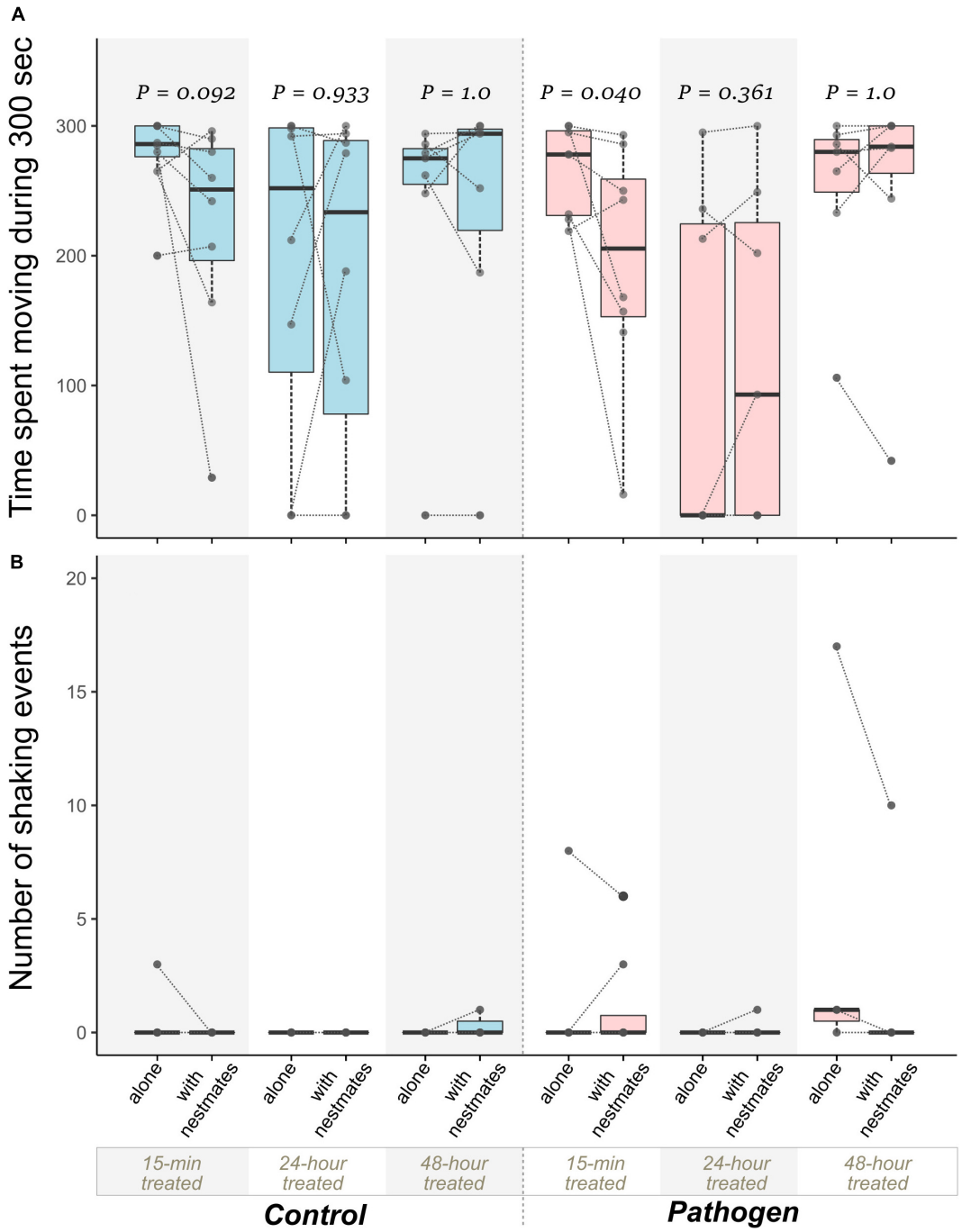

FIGURE 2 | Immediate changes in immune behaviors. Individual data points were connected with a line to illustrate the behavioral change of each individual. **(A)** Overall, treatment did not have a significant effect on immediate changes in locomotion (Wilcoxon test, $p = 0.065$). Reduced locomotion was observed shortly after nestmate workers were added in the 15-min incubation periods. For all other incubation periods, pathogen-exposed workers generally did not alter their locomotion in the presence of nestmate workers ($n = 4$ colonies). **(B)** The presence of nestmate workers did not significantly influence the number of shaking events ($n = 4$ colonies).

Although moving into dense areas of the colony may promote the spread of disease, it also increases the chance of being groomed by nestmates. Termites can self-groom, but it is much more efficient to be groomed by others (Rosengaus et al., 1998b; Yanagawa and Shimizu, 2007; Chouvenc et al., 2009b; Liu et al., 2019a). Thus, the cost for a colony to risk spreading disease by grooming, appears to be outweighed by the benefit of rescuing workers before they develop a lethal infection

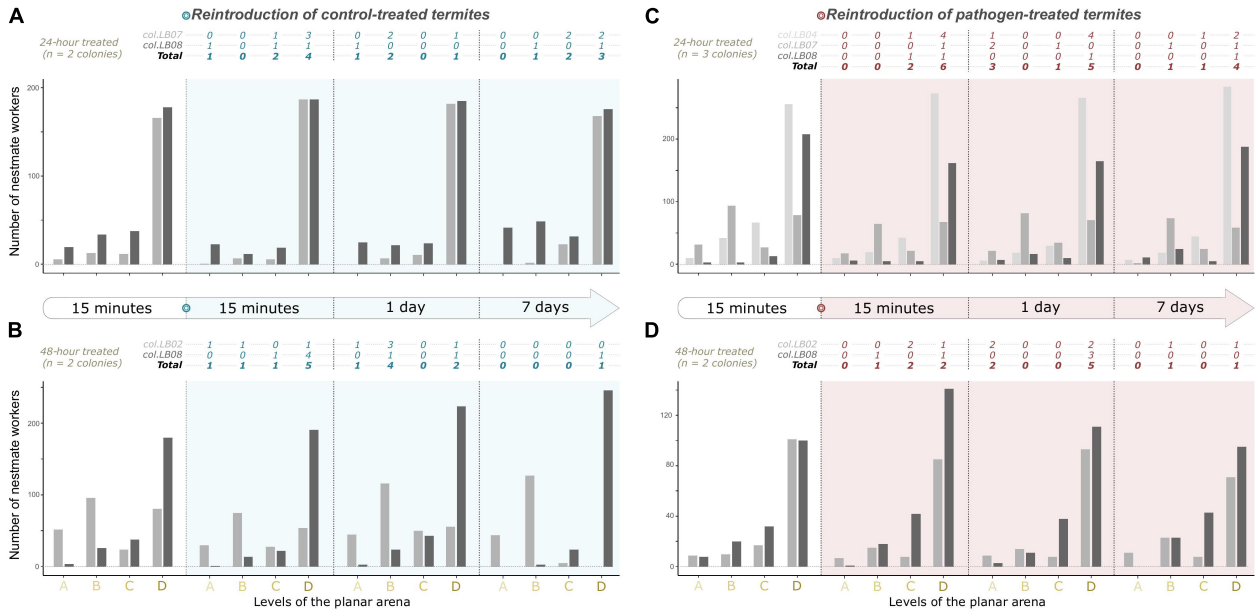

FIGURE 3 | Gradual changes in immune behaviors. Barplots depict the number of nestmate workers found in each level over time for the **(A)** 24-h incubation control, **(B)** 48-h incubation control, **(C)** 24-h pathogen incubation, and **(D)** 48-h pathogen incubation. N in each plot indicates the number of colonies tested for each treatment. The single dot on the observation time scale represents when the focal individuals were added (i.e., control-treated or pathogen treated). Regardless of treatment, colony density concentrated away from the entrance of the plates, as workers were significantly more abundant in level D (60–80% of nestmate workers). The rows of numbers on top of each barplot represent the number of dyed individuals (i.e., control-treated or pathogen treated) found in each level per colony. The bottom row is the summed total.

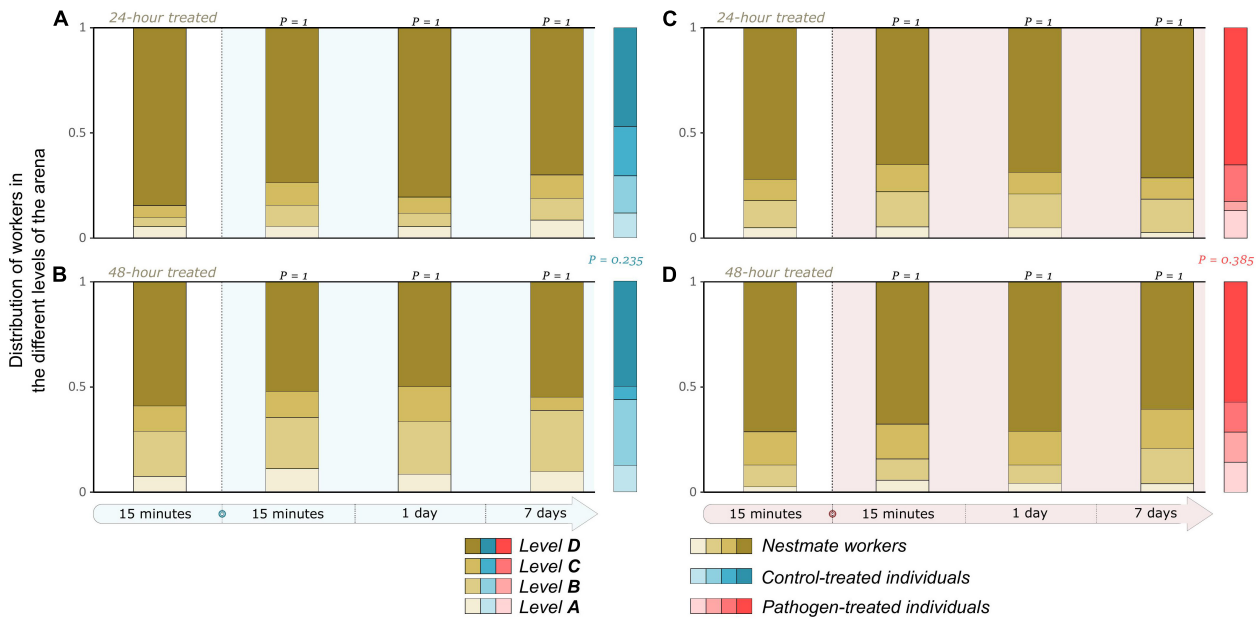

FIGURE 4 | Distribution of nestmate workers and focal individuals per level. Barplots represent the distribution of nestmate workers in different levels of the arena over time for the **(A)** 24-h incubation control, **(B)** 48-h incubation control, **(C)** 24-h pathogen incubation, and **(D)** 48-h pathogen incubation. The single dot on the observation time scale represents when the focal individuals were added (i.e., control-treated or pathogen treated). No significant difference was observed when the distribution of nestmate workers per observation time was compared separately to the distribution of nestmate works before focal individuals were added (*Fisher tests,* all $p = 1$). For each incubation time, colored barplots represent the overall distribution of focal individuals (i.e., combining observation times) among the different levels of the colonies from both the control and pathogen treatments. The overall distribution of dyed individuals closely mirrored the distribution of nestmate workers for both control ($p = 0.235$) and pathogen treatments ($p = 0.385$).

(Davis et al., 2018). Interestingly, similar results have been found in the clonal raider ant, where infected individuals are reintegrated within colonies and occupied a central place in the network of interactions among nestmates, leading to a general increase in physical contact toward infected individuals (Alciatore et al., 2021). Our findings also suggest that, similarly to this ant species, *R. flavipes* termites favor a general rescuing strategy toward infected nestmates, rather than avoidance. Additionally, termite nest material exhibits strong antimicrobial activity from feces (Rosengaus et al., 1998a), defensive salivary secretions (Bulmer et al., 2010; Hamilton et al., 2011a), and beneficial bacteria (Chouvenc et al., 2018; Aguero et al., 2021b). By traveling to more active parts of the nest, infected individuals may also be seeking areas with the strongest antimicrobial activity and may sanitize themselves in the process.

Even when a termite has developed a lethal infection and can no longer be saved, they do not become infective until the fungus has killed them and sporulates from their body. By seeking out nestmates, workers could be inviting cannibalistic responses for the safe disposal of their bodies before they can become infective. The termite gut serves an important role in termite immunity, as harmful spores are inhibited in the alimentary tract (Chouvenc et al., 2009b). Indeed, when an infected worker may no longer be saved from infection, the response of nestmates switches from grooming to cannibalization (Davis et al., 2018). Reduced movement has been suggested as the cue for nestmates to begin cannibalizing them (Chouvenc et al., 2009a; Davis et al., 2018). Additionally, shaking displays are used to signal pathogen presence and may also play a role in communicating infection status (Rosengaus et al., 1999; Yanagawa et al., 2015; Bulmer et al., 2019). We found no consistent changes in locomotion or shaking displays throughout observation times when infected termites were grouped with nestmates, which suggests that these behaviors are not used by workers to signal this infection to nestmates. These results were found under our experimental setup using early infected individuals; however, it is possible that changes in locomotion or shaking displays would be observed at later stages of the infection process, or that the dye used may influence shaking behavior. Nile blue was originally used to monitor termite colonies in the field and reportedly does not affect mortality, but it is unclear if it may affect behavior (Su, 1991). Additionally, further testing on a larger scale is necessary. However, reduced locomotion was observed after nestmate termites were added for the 15-min pathogen incubation treatment. Interestingly, this pattern was observed for both control and pathogen treated individuals held for a 15-min incubation period, although not significant for the control-treated group. The decrease in movement once nestmate workers were introduced may be attributed to nestmate worker inspection and grooming that naturally occurs once termites are faced with new individuals (Costa-Leonardo and Haifig, 2014). The occurrence of this pattern for both control-treated and pathogen-treated individuals suggests that isolation from the colony alone may already trigger this behavioral change, which may be used as a preventive inspection of reintegrated foragers. Potentially, if reduced locomotion does not rely on the presence of nestmates, then it may be a symptom of deteriorating health. This difference may not be possible to discern from behavioral studies and may require a more thorough analysis of physiological, chemical or transcriptional changes in infected individuals. For example, infected individuals of bees and ants exhibit change in cuticular hydrocarbons (CHCs) profiles suggesting their potential role in signaling immune status (Richard et al., 2008; Baracchi et al., 2012; Cappa et al., 2016; López et al., 2017; Pull et al., 2018; Cappa et al., 2019).

Behavioral changes after infection are not always the result of social immune defenses. Several pathogens can manipulate their host's behavior to benefit their own transmission. More complex examples of manipulation appear to require a degree of host specialization (Lafferty and Shaw, 2013). Carpenter ants infected with *Ophiocordyceps* fungi descend from arboreal nests to find optimal conditions for fungal growth (Hughes et al., 2011). Similarly, in honeybees, workers infected with Israeli acute paralysis virus show decreased aggression toward other colonies, increasing the chance of transmission to new hosts (Geffre et al., 2020). A reduction in movement is one of the most common examples of host manipulation, as it increases the chance of the current host being predated and further transmitting the disease (Lafferty and Shaw, 2013). If this is the case, the inhibitory strength of the termite gut may have accordingly evolved to prevent pathogen transmission during cannibalization (Chouvenc et al., 2009b).

We show that *R. flavipes* workers that are infected with a fungal pathogen do not self-isolate from the colony in the planar arenas. However, subterranean termite colonies and their spatial organization can be much more complex in nature. Foraging ranges can extend hundreds of square meters and mature colonies typically contain orders of magnitude more than the 500 individuals we used in our arenas (Vargo and Husseneder, 2009; Shults et al., 2021). While infected termites are uninhibited from dense pockets of workers, it is unknown if the same is true for reproductive chambers. Additionally, the concentration of pathogenic spores used in this study is much higher than what termites are expected to encounter naturally (Chouvenc et al., 2011; Loreto and Hughes, 2016). Lower pathogen titers are typically not used in studies of social immunity, as differences in colony survival are less likely to be seen. Potentially, termite workers may behave differently when exposed to less lethal pathogen concentrations. Expanding on these results by altering pathogen titers and tracking colony interactions on a smaller scale will increase our understanding of how individual behaviors translate to overall colony organization. Similarly, testing the reintegration of infected individuals at later stages of infection will allow us to tease apart whether infected and infectious individuals exhibit or elicit different behavioral responses. Finally, studying the connectivity network between every individual in the colony would permit testing whether the unrestrained contact of infected individuals also apply to highly valuable reproductive individuals and more susceptible juvenile individuals (Cole et al., 2020).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is available on Open Science Framework (DOI 10.17605/OSF.IO/6NDC9).

AUTHOR CONTRIBUTIONS

CA and EV: conceptualization. CA: data curation and methodology. MM and CA: formal analysis, investigation, and validation. EV: funding acquisition, project administration, and supervision. MM, CA, and P-AE: visualization. MM, CA, P-AE, and EV: writing—original draft. All authors have read and agreed to the published version of the manuscript.

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Transcriptomics on Social Interactions in Termites: Effects of Soldier Presence

*Masatoshi Matsunami[1,2†], Dai Watanabe[1,3†], Kokuto Fujiwara[3], Yoshinobu Hayashi[1,4], Shuji Shigenobu[5], Toru Miura[1,6] and Kiyoto Maekawa[3,7]**

[1] Graduate School of Environmental Science, Hokkaido University, Sapporo, Japan, [2] Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan, [3] Graduate School of Science and Engineering, University of Toyama, Toyama, Japan, [4] Department of Biology, Keio University, Yokohama, Japan, [5] National Institute for Basic Biology Core Research Facilities, Okazaki, Japan, [6] Misaki Marine Biological Station, University of Tokyo, Miura, Japan, [7] Faculty of Science, Academic Assembly, University of Toyama, Toyama, Japan

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***Correspondence:**
Kiyoto Maekawa
kmaekawa@sci.u-toyama.ac.jp

[†] These authors have contributed equally to this work and share first authorship

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The organization of social insect colonies requires sophisticated mechanisms to regulate caste composition according to colony demands. In termites, the soldier caste is responsible for the inhibition of soldier differentiation, but the mechanism underlying the regulation of soldier differentiation is still unclear. In this study, we performed transcriptome analyses to identify genes expressed in workers that fluctuated in the presence of soldiers in the subterranean termite *Reticulitermes speratus*. First, soldier differentiation was artificially induced *via* juvenile hormone (JH) application, and the inhibitory effects of soldier differentiation on soldier presence were evaluated. Second, transcriptomes were prepared from workers with or without soldiers under JH treatment, and expression analyses were performed to identify differentially expressed genes (DEGs) for each treatment. The expression levels of several DEGs were verified by quantitative real-time PCR. The results indicated that only a small number of DEGs were upregulated by the presence of soldiers. A homology search of DEGs and gene ontology (GO) analysis of the DEGs showed that some genes were responsible for the regulation of hormone levels, social interaction, and response to xenobiotic substances, suggesting that they could be involved in developmental arrest and pheromonal regulation in workers. Moreover, GO analysis indicated that the expression of many genes, including those involved in hormone metabolic processes, fluctuated with JH application. Suppression of soldier differentiation in the presence of soldiers could be accomplished by the expression of a large number of genes required for soldier differentiation.

Keywords: caste differentiation, soldiers, workers, transcriptome, juvenile hormone, *Reticulitermes*

Abbreviations: JH, juvenile hormone; SIFs, soldier inhibiting factors; DEGs, differentially expressed genes; GO, gene ontology; FDR, false discovery rate; CHC, cuticular hydrocarbon; GLM, generalized linear model.

INTRODUCTION

Social insects have multiple phenotypes (castes) in the same colony. The optimal composition of each caste and cooperation among individuals are needed for regular colony growth and maintenance (Wilson, 1971). Termites are major social insect groups, and their castes are normally discriminated as workers, soldiers, and reproductives (Roisin, 2000; Korb and Thorne, 2017). Termite caste differentiation is thought to be regulated by caste-specific gene expression through environmental stimuli that may trigger various hormonal conditions (Noirot, 1991; Watanabe et al., 2014). Because a distinctive sterile soldier caste is crucial for termite sociality and evolution, soldier differentiation has been extensively studied during the last two decades (Miura and Maekawa, 2020). Genes and cascades involved in specific weapon formations have been analyzed, and several important factors, including hormone-related and toolkit genes, have been clarified (e.g., Zhou et al., 2006; Toga et al., 2012; Masuoka et al., 2018; Sugime et al., 2019). The expression patterns of these factors can be affected by environmental stimuli *via* inter-individual (i.e., social) interactions (Watanabe et al., 2014). However, the effects of social interactions on gene expression and endocrine status have not yet been elucidated (Miura and Maekawa, 2020).

Termite soldiers are differentiated from workers *via* an intermediate stage called a presoldier. Presoldier and soldier formation is known to be inhibited by soldiers themselves and promoted by the reproductive caste in the same colony (Watanabe et al., 2014). Indeed, for the inhibitory mechanism by soldier existence, candidate primer pheromones [soldier inhibiting factors, SIFs (Park and Raina, 2005)], transferred from soldiers to workers, were identified in some species of *Reticulitermes* [γ-cadinenal and (-)-β-elemene; Tarver et al., 2009; Tarver et al., 2011; Mitaka et al., 2017]. These chemicals are terpenoids and are probably defense substances released from the frontal gland, which is developed in soldiers of phylogenetically apical termite lineages (Miura and Maekawa, 2020). Previous studies have also shown that live soldiers or whole extracts of soldiers had strong inhibitory effects on soldier differentiation (Tarver et al., 2011; Mitaka et al., 2017). Moreover, similar inhibitory effects were observed in the extracts of soldiers from more basal termite species (without frontal glands), which possess physical weapons such as enlarged mandibles and head capsule (Korb et al., 2003). Consequently, SIFs may consist of complex and lineage-specific compounds. However, because information about the internal changes of workers are lacking, it is unclear how the degree of inhibitory effects of SIFs is determined.

The percentage of soldiers is usually very small in natural colonies (Howard and Haverty, 1981), and thus soldier differentiation is not frequently observed compared to those of workers. However, soldier differentiation can be artificially induced by juvenile hormone (JH) or JH analog treatments with workers (Watanabe et al., 2014; Miura and Maekawa, 2020). In *R. speratus*, artificial presoldier induction rates were significantly decreased by the presence of soldiers compared to those without soldiers (Watanabe et al., 2011). Importantly, JH titers of workers with soldiers were significantly lower than those of workers without soldiers just 5 days after JH treatment. If workers are reared without soldiers, only small numbers of workers differentiate to presoldiers and soldiers (e.g., about 7% for 16 days in *Coptotermes formosanus*; Park and Raina, 2005). However, we never identify the soldier-destined workers before the presoldier molt normally in the mature colony. Consequently, to determine effectively whether gene expression changes are affected by the existence of soldiers (probably using SIFs), an artificial presoldier induction method is considered useful, and *R. speratus* workers should be investigated within 5 days after JH treatment.

Here, internal transcriptomic changes in JH-treated workers caused by coexisting soldiers were analyzed in *R. speratus*. The genome sequence and transcriptome of each caste/caste differentiation have been clarified for this species (Shigenobu et al., 2022; Saiki et al., submitted manuscript). Transcriptomes were prepared from worker individuals with or without soldiers within 5 days after JH treatment. Based on the differentially expressed genes (DEGs) observed and specific gene ontology (GO) terms detected, the molecular basis of physiological changes in workers with coexisting soldiers is discussed.

MATERIALS AND METHODS

Termites

Mature termite colonies (total: four) were collected in Furudo, Toyama Prefecture, Japan, in 2014. The nest logs were transported to the laboratory and kept in a plastic case under constant darkness. Sixth and seventh instar workers were selected for the following experiments based on the number of antennal segments and body size (Tsunoda et al., 1986; Takematsu, 1992). All experimental insects were maintained in an incubator at 25°C under constant darkness for at least 3 days before use.

Dish Assays for Induction of Presoldier Differentiation

Dish assays were performed in accordance with the procedure described previously (Tsuchiya et al., 2008; Watanabe et al., 2011; Masuoka et al., 2013). Briefly, filter papers (55 mm diameter; Advantec, Japan) were treated with 20 and 40 μg JH III (Santa Cruz Biotechnology, Dallas, TX, United States) dissolved in 200 μL acetone. Filter papers treated with acetone alone were used as controls. After the acetone evaporated, each filter paper was moistened with approximately 450 μL of distilled water and placed in a 65 mm Petri dish. Then, 20 workers were exposed to each filter paper along with 0 or 10 soldiers. Each category was replicated four times using individuals sampled from four different colonies. All dishes were kept in an incubator at 25°C in constant darkness. The number of dead individuals and differentiated presoldiers was checked every 24 h. If a dead worker was detected, it was immediately removed from the dish. If a soldier died, a live soldier was added from a separate dish kept in the incubator. On day 16, the induction rates of newly molted presoldiers were compared between dishes with 0 or 10 soldiers. Presoldier differentiation rates (mean ± S.D. values) were calculated from dishes replicated four times (20 workers per dish) and evaluated by the generalized Wilcoxon test (80

individuals in each JH III concentration) using the statistical software Mac Statistical Analysis, version 1.5 (Esumi, Japan). Statistical significance was set at $P < 0.05$.

Dish Assays for RNA Extraction

Dish assays for RNA extraction were performed using the method described in Section "Dish Assays for Induction of Presoldier Differentiation." Three days after treatment with 40 μg JH III (or acetone treatment as control), all workers in each dish were fixed with liquid nitrogen and stored at −80°C until RNA extraction.

RNA Extraction, Library Preparation, and Sequencing

Total RNA was extracted from four categories [(1) workers with 0 soldiers 3 days after acetone treatment, (2) workers with 10 soldiers 3 days after acetone treatment, (3) workers with 0 soldiers 3 days after JH treatment, (4) workers with 10 soldiers 3 days after JH treatment] using an SV Total RNA extraction kit (Promega, Madison, WI, United States). RNA extracted from 20 individuals without guts was used for each library. Four replicates derived from four different colonies (biological quadruplicates) were prepared (four libraries × four categories = 16 libraries). The amounts of total extracted RNA and DNA were quantified using a Qubit fluorometer (Life Technology, Eugene, OR, United States) and the quality was confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, United States). Total RNA (500 ng) was used for cDNA synthesis and purification using a low-throughput protocol with a TruSeq Stranded RNA LT Kit (Illumina, San Diego, CA, United States). A half-scale reaction of the standard protocol was used for library preparation. The quality and quantity of cDNA were validated using an Agilent 2100 bioanalyzer and a KAPA qPCR SYBR Green PCR Kit (GeneWorks, Thebarton, SA, Australia). Sixteen libraries were sequenced by single-end sequencing (101 bp) using Hiseq1500 (Illumina, San Diego, CA, United States). All reads were deposited in the DDBJ Sequence Read Archive (DRA) database under accession number DRA013774.

Identification of Differentially Expressed Genes

For each read, nucleotides with a low-quality score at the sequence ends and adapter sequences were removed using SolexaQA ver. 2.5 (Cox et al., 2010), and the cutadapt program version 1.2.1 (Martin, 2011), respectively. These reads were mapped against the *R. speratus* genome (Rspe OGS1.0; Shigenobu et al., 2022) using the TopHat program version 2.0.13 (Kim et al., 2013) with default settings.

The counting of reads and detection of DEGs were performed using Cufflinks pipeline version 2.2.1 (Trapnell et al., 2010). In this program, the count data were normalized and analyzed using the same algorithm implemented in DESeq (Anders and Huber, 2010). These counts were scaled using the median of the geometric means of fragment counts across all libraries. After normalization, pairwise comparisons were performed using cuffdiff command with "–frag-bias-correct" and "–multi-read-correct" options (Roberts et al., 2011; Trapnell et al., 2013). We used the following four schemes for comparison: (i) without JH and 0 soldiers vs. without JH and 10 soldiers, (ii) without JH and 0 soldiers vs. 40 μg JH and 10 soldiers, (iii) without JH and 10 soldiers vs. 40 μg JH and 10 soldiers, and (iv) 40 μg JH and 0 soldiers vs. 40 μg JH and 10 soldiers. Homology searches of DEGs obtained in (i) and (iv) (the same JH concentration and relatively small numbers of DEGs; see results) were performed by BLASTX using the NCBI non-redundant protein database (nr) (run on March 2022), and the top hit sequence was obtained for each DEG.

We also conducted a generalized linear model (GLM) analysis. Because Cufflinks pilelined did not generate raw count data, we re-mapped and counted our RNA-seq data. Briefly, we used the Bowtie program (Langmead et al., 2009) for mapping and RSEM v1.3.3 software (Li and Dewey, 2011) to estimate the relative abundance and expected read counts of all genes. The count data were normalized by the TMM (Trimmed Mean of *M* values) method in the edgeR software package (Robinson and Oshlack, 2010; Robinson et al., 2010; McCarthy et al., 2012). The adjusted count values were then used for the DEG analysis. Two model designs were used for DEG detection. In the first design, we considered three explanatory factors: soldier presence, JH treatment, and these interactions (Soldier presence × JH treatment). In the second design, in addition to these three factors, we used colony information as another explanatory factor. The threshold of statistical analysis is false discovery rate (FDR) cutoff of 0.05.

Annotation and Gene Ontology Enrichment Analysis

Since GO terms were only assigned to model organisms, we identified the fruit fly ortholog of each termite gene. All predicted termite amino acid sequences were searched against fruit fly (*Drosophila melanogaster*) amino acid sequences using BLASTP. The complete amino acid sequence datasets for the fruit flies were downloaded from Flybase version FB2012_04.[1] BLASTP searches were performed using termite genes as queries, and a 10^{-5} e-value cutoff was used against the fruit fly dataset. The top hit proteins were defined as orthologs of the focal termite gene.

Gene ontology enrichment analysis was performed using clusterProfiler software package (version 2.4.3, R version 3.3.3; Yu et al., 2012). We performed an enrichment test for GO terms by assuming a hypergeometric distribution. To prevent high FDR due to multiple tests, we also estimated the *q*-values for FDR control (Storey, 2002). For these analyses, we used a list of DEGs identified by pairwise comparisons. To identify enriched GO biological processes, we conducted an enrichment analysis of these DEGs, in which $p < 0.1$ and $q < 0.05$ were used as strict cutoff values.

Quantitative Real-Time PCR

To validate the transcriptome analysis, qPCR was performed for the identified DEGs (a total of three genes, see results). Individuals were sampled from five different colonies collected in Toyama Prefecture, Japan from 2019 to 2020. The dish assay

[1] https://flybase.org/

was performed following the method described above, and the presoldier induction rates were confirmed 16 days after the treatment. Total RNA was extracted from workers with 0 or 10 soldiers 3 days after treatment with 40 μg JH III using ISOGEN II (Nippon Gene, Tokyo, Japan). A total of 17–20 live workers (whole bodies) were used in each sample and homogenized using a Bead Mill 4 (Thermo Fisher, Waltham, MA, United States). Five replicates derived from five different colonies (biological quintuplicates) were prepared for each category. The amounts of RNA and DNA were quantified using a Qubit fluorometer, and the purity and quantity of the extracted RNA were determined by spectroscopic measurements at 230, 260, and 280 nm using a NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Tokyo, Japan). DNase-treated RNA (2 μg per sample) was transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative PCR (200 nM of each primer) was performed in biological quintuplicates using PowerUP SYBR Green Master Mix (Thermo Fisher) and QuantStudio 3 Real-Time PCR System (Thermo Fisher). According to the previous study (Miyazaki et al., 2021), to determine an internal control gene, the suitability of six reference genes, *EF1-alfa* (accession no. AB602838), *NADH-dh* (AB602837), *beta-actin* (AB520714), *GstD1* (gene ID: RS001168), *EIF-1* (RS005199), and *RPS18* (RS015150), were evaluated using GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). Specific primers were designed against each gene sequence using Primer3Plus (Untergasser et al., 2007; **Supplementary Table 1**). Statistical analysis, Welch's *t*-test, was performed using the statistical software Mac Statistical Analysis version 3.0 (Esumi, Tokyo, Japan).

RESULTS AND DISCUSSION

Presoldier Induction Rates

As shown in a previous study (Watanabe et al., 2011), presoldier induction rates after both 20 and 40 μg JH III treatments were significantly reduced by soldier presence (**Figure 1**). The JH titer (endogenous + applied JH III) levels in workers with 0 soldiers were shown to be higher than those with 10 soldiers 5 days after JH III treatment (Watanabe et al., 2011, 2014). Because we tried to effectively detect gene expression changes in workers before the reduction of JH III titer levels, total RNA was extracted from workers 3 days after 40 μg or without JH III treatments.

Numbers of Differentially Expressed Genes

We obtained different numbers of DEGs for the four pairwise comparisons (**Figure 2**). The number of DEGs between 40 μg and without JH III treatments in workers with 0 soldiers was 3,089. Of these genes, 1,516 and 1,573 were upregulated in 40 μg and without JH III-treated workers, respectively (**Figure 2**; **Supplementary Table 2**). Similarly, the number of DEGs between 40 μg and without JH III treatments in workers with 10 soldiers was 3,014. Of these genes, 1,567 and 1,447 were upregulated in 40 μg and without JH III-treated workers, respectively (**Figure 2**; **Supplementary Table 3**). In contrast, there were only 44 DEGs between soldier absence (26 upregulated genes) and soldier presence (18 upregulated genes) in acetone-treated workers (**Figure 2**; **Supplementary Table 4**). The expression levels of 171 genes were significantly different between soldier absence (142 upregulated genes) and soldier presence (29 upregulated genes) in JH III-treated workers (**Figure 2**; **Supplementary Table 5**). In particular, the expression of a large number of genes (approximately 3,000) in workers was fluctuated by JH/acetone treatments. These numbers of DEGs were similar to, but slightly larger than, those between JH analog (methoprene) and acetone-treated workers in *Coptotermes formosanus* (2,547 unigenes) (Du et al., 2020). *Reticulitermes* and *Coptotermes* are phylogenetically closely related to each other (Bucek et al., 2019), and inferred genome sizes (800–900 Mb) and total gene numbers (approximately 13,000–15,000) of both species are very similar (Maekawa et al., 2022). Consequently, these discrepancies may be due to the differences in treated chemicals [JH homolog (this study), synthetic juvenoid (Du et al., 2020)] and/or RNA-sequencing (RNA-seq) methods [genome-based assay (this study), *de novo* transcriptome assembly-based assay (Du et al., 2020)]. Most of these DEGs were also identified by GLM analysis (**Supplementary Tables 6**, **7**), suggesting that our analysis effectively listed genes which are related to soldier differentiation.

Quantitative Real-Time PCR

We selected three genes [cytochrome P450 (*Cyp4c3*; *RS013835*), alpha-amylase (*Amy-p*; *RS006137*), and multidrug resistance protein (*Mdr49*; *RS002816*)], which were listed as DEGs in JH III-treated workers with 0 and 10 soldiers (**Supplementary Table 5**). We focused on this category because it was expected that there would be large variations in gene expression levels among samples. RNA-seq analysis indicated that all these three genes were upregulated in workers with 10 soldiers. A suitable reference gene (*NADH-dh*) was selected for real-time qPCR analysis using GeNorm and NormFinder (**Supplementary Table 8**). Real-time qPCR analysis showed that high expression levels of the three genes examined were observed in JH III-treated workers with 10 soldiers (soldier presence), compared to those with 0 soldiers (soldier absence) (**Figure 3**). Although the statistical support of *RS002816* expression levels between soldier presence and absence was weak ($p = 0.106$; Welch's *t*-test), it should be noted that the *q* value of *RS002816* (0.012) was higher than that of the remaining two genes (0.007; **Supplementary Table 5**). Overall, the qPCR analysis supported the reliability of the RNA-seq results.

Differentially Expressed Genes Between Soldier Presence and Absence

We focused on DEGs specifically observed in workers with 0 and 10 soldiers under the same JH concentrations. Note that all DEGs discussed below were detected by both designes of GLM analysis (**Supplementary Tables 6**, **7**), except that *Cyp4c3* (*RS013835*) was not observed only in the first design. In the acetone treatment, three takeout protein genes (*RS013762*, *RS014812*, and *RS010477*) were highly expressed in workers with 0 soldiers, whereas a JH-inducible protein gene (*RS007835*) was

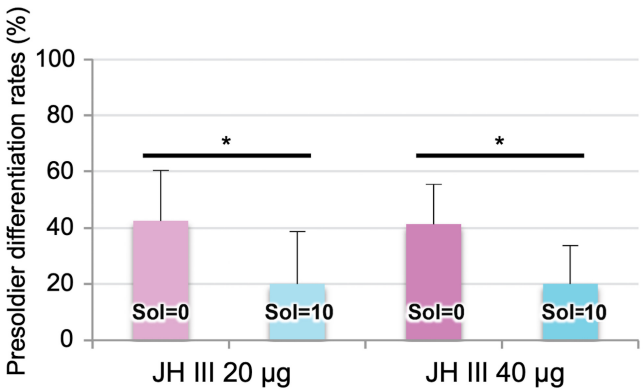

FIGURE 1 | Presoldier induction rates (mean ± S.D., biological quadruplicates) 16 days after the 20 or 40 µg juvenile hormone (JH) III application in *Reticulitermes speratus*. In both applications, presoldiers were more frequently differentiated from workers with 0 soldiers (soldier absence, "Sol = 0") than those with 10 soldiers (soldier presence, "Sol = 10") (generalized Wilcoxon test, $^*P < 0.05$).

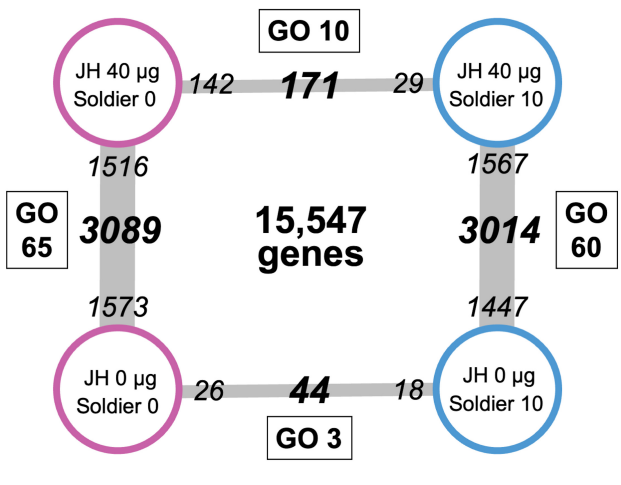

FIGURE 2 | Numbers of differentially expressed genes (DEGs, bold italic) between each category. Small italic numerals indicate the numbers of upregulated genes in each category. Gene numbers between juvenile hormone (JH) III and acetone treatments (3,014 in soldier presence, 3,089 in soldier absence) were much larger than those between soldier presence and absence (171 in JH treatments, 44 in acetone treatments). Numbers of significant gene ontology (GO) terms of the DEGs are indicated in squares.

highly expressed in workers with 10 soldiers (**Supplementary Table 4**). Takeout proteins are normally able to bind JH because they possess the JH binding protein domain conserved in insects (Noriega et al., 2006; Chamseddin et al., 2012). Although the functions of JH-inducible and takeout proteins are unanalyzed, there is a possibility that these work on the role of JH sequestration from the hemolymph of workers, such as JH binding proteins (e.g., hexamerin, Zhou et al., 2006). Alternatively, these proteins may be crusial for the change of the JH sensitivity in workers, which is similar to the case in *Pheidole* ants (Wheeler and Nijhout, 1984; Nijhout, 2003). In any case, these results support that JH levels in workers are affected by the three-day interaction with soldiers. Note that other JH binding proteins (including hexamerin) and JH biosynthetic/degradation genes, all of which were annotated in previous literature (Shigenobu et al., 2022), were not detected in our transcriptome analysis.

Two chemosensory protein genes (*RS000584* and *RS010442*) were highly expressed in workers with 0 soldiers (**Supplementary Table 4**) and were recognized as *RspeCSP1* and *RspeCSP7*, respectively (Shigenobu et al., 2022). Previous RNA-seq analysis in *R. speratus* indicated that both genes were highly expressed in the head compared to the remaining part of the body of workers (*RspeCSP7*) or workers and soldiers (*RspeCSP1*) (Shigenobu et al., 2022). Although their precise expression sites (antennae or other head parts) should be clarified, they may be involved in SIF-related social communication between soldiers and workers.

In the JH III treatment, the functions of some DEGs (total: 52) could not be identified based on the BLASTX search (hypothetical or uncharacterized proteins, no hit; **Supplementary Table 5**). Most DEGs shown in the acetone treatment described above (**Supplementary Table 4**) were not detected, but many genes involved in antimicrobial and xenobiotic responses and digestive enzymes were observed in workers with 10 soldiers [e.g., *Mdr49* (*RS002816*), prolixicin antimicrobial protein (*AttD*, *RS000201*), *laccase2* (*RS004166*), and *Amy-p* (*RS006137*)] and those with 0 soldiers [e.g., C-type lysozyme-3 (*RS003406*), toll-like receptor 6 (*RS012895*), and venom protease-like (*RS012253*)]. Interestingly, fatty acyl-CoA reductase (*RS002448*), a well-known soldier-specific gene (Maekawa et al., 2022), was highly expressed in workers with 10 soldiers. As this gene may be involved in the production of soldier-specific cuticular hydrocarbon (CHC) profiles (Wu et al., 2018; Maekawa et al., 2022), the presence of soldiers can induce changes in worker CHC profiles. *Cyp4c3* (*RS013835*) was also highly expressed in workers with 10 soldiers. Because *CYP4* is involved in the last step of CHC biosynthesis in *D. melanogaster* (Qiu et al., 2012), it may also support the above hypothesis. The soldier ratio applied in this study was quite high, and further analysis should be performed in colonies with an appropriate soldier ratio under natural conditions (about 2% in *R. flavipes*;

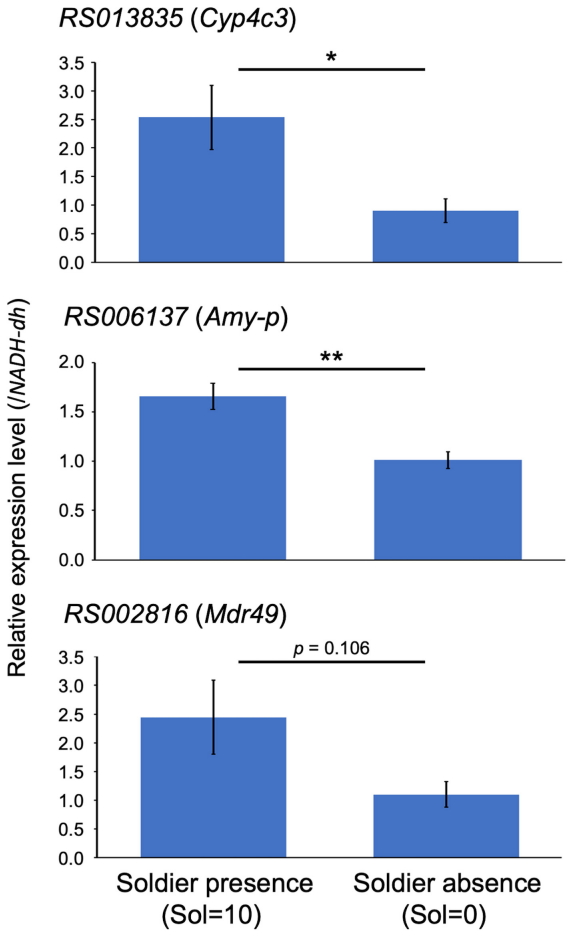

FIGURE 3 | Quantitative real-time PCR expression levels of the three genes (mean ± S.D., biological quintuplicates) in the 40 μg juvenile hormone (JH) III-treated workers with 10 soldiers (Soldier presence, "Sol = 10") and 0 soldiers (Soldier absence, "Sol = 0"). Each value was normalized to the expression levels of *NADH-dh* (**Supplementary Table 8**). Asterisks above the bars indicate significant differences (*$P < 0.05$, **$P < 0.01$; Welch's *t*-test).

Howard and Haverty, 1981) to clarify the general tendency of this hypothesis.

Finally, in JH III treatment, high expression levels of some members of the multigene family [beta-glucosidase (*RS004143*) and lipocalin (RS013912); Shigenobu et al., 2022] were observed in workers with 10 soldiers (**Supplementary Table 5**). Many paralogs of both genes have been identified in the *R. speratus* genome, and these paralogs showed different expression patterns among castes (Shigenobu et al., 2022). Beta-glucosidase is essential for cellulose digestion in termite workers (Watanabe and Tokuda, 2010), but *RS004143* was highly expressed in the thorax and abdomen of soldiers and reproductives (Shigenobu et al., 2022). Thus, *RS004143* may have a different role other than wood digestion in *R. speratus*. It is interesting to note that beta-glucosidase (called *Neofem2*) is involved in queen-recognition pheromones that probably function in the suppression of reproductive emergence in *Cryptotermes secundus* (Korb et al., 2009; Korb, 2016). Similarly, lipocalin is a member of the protein transporter family, but molecular phylogeny showed that *RS013912* was closely related to soldier-specific protein 1 (*SOL1*) identified in *Hodotermopsis sjostedti* (Shigenobu et al., 2022). SOL1 may function as a signaling molecule for defensive social interactions among colony members (Miura et al., 1999; Miura, 2005). We suggest that both *RS004143* and *RS013912* are involved in chemical communication, and their expression changes in workers are affected by different social circumstances, with or without soldiers.

Gene Ontology Enrichment Analysis of Differentially Expressed Genes

We performed GO enrichment analysis of the DEGs observed in each category (**Figure 2**). The number of GO terms detected between 40 μg and without JH III treatments in workers with 0 soldiers was 65 (**Supplementary Table 9**). Similarly, the number between 40 μg and without JH III treatments in workers with 10 soldiers was 60 (**Supplementary Table 10**). More than half of these terms (total: 38) were common (bold italic terms in **Supplementary Tables 9**, **10**), and four out of 38 terms (GO: 0016053, 0046394, 0072330, and 0032787; metabolic and biosynthetic processes of some molecules) were specifically observed during the worker-presoldier molt (Saiki et al., submitted manuscript). However, specific GO terms involved in hormone levels (e.g., GO: 0010817, 0042446, and 0045455) were detected only in workers with 10 soldiers (**Supplementary Table 10**). Moreover, the number of GO terms significantly detected between soldier presence and absence in the acetone-treated workers was only three (**Supplementary Table 11**); all of which were related to the regulation of hormone levels. These results clearly indicate that the JH levels in workers are affected by the presence of soldiers.

Finally, a total of 10 significant GO terms were observed between the presence and absence of soldiers in JH-treated workers (**Supplementary Table 12**). These terms included metabolic processes of some molecules (GO: 0006022, 1901071, and 0006040), chitin and cuticle development (GO: 0006030 and 0040003), and response to xenobiotic substances (GO: 0009617, 0042742, and 0050830). These results may be explained by the effect of the presence of soldiers on developmental arrest during JH-induced presoldier differentiation, which is generally accompanied by specific cuticle development *via* the tyrosine metabolic pathway (Masuoka et al., 2013; Masuoka and Maekawa, 2016).

CONCLUSION

Most workers treated with commercial JH III are differentiated into presoldiers, and living soldiers strongly inhibit the presoldier differentiation by rapid JH decrease soon after interaction with workers (Watanabe et al., 2011, 2014). This study aimed to understand the gene expression profiles of workers affected by coexisting soldiers. RNA-seq analysis supported that worker JH levels are affected by the presence of soldiers, probably by the

functions of JH binding and inducible proteins, regulatory factors of social interaction, and response to xenobiotic substances. Further gene function analyses of candidate targets are needed to clarify this possibility.

DATA AVAILABILITY STATEMENT

All sequence reads are available in the DDBJ Sequence Read Archive, accession numbers: DRA013774, DRX342915-DRX342930, and DRR357004-DRR357019 and in the BioProject Archive, accession number: PRJDB13353.

AUTHOR CONTRIBUTIONS

MM, DW, TM, and KM designed the study. DW, KF, and KM collected the samples. MM, DW, KF, YH, and SS performed the experiments. MM, KF, SS, and KM analyzed the data. MM, YH, TM, and KM drafted the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.924151/full#supplementary-material

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Body Size and Symmetry Properties of Termite Soldiers Under Two Intraspecific Competition Scenarios

Daniel Aguilera-Olivares[1†], Bárbara Torres-Guzmán[2], Alberto Arab[3†], Luis Flores-Prado[4†] and Hermann M. Niemeyer[5*†]*

[1] Departamento de Silvicultura, Universidad de Concepción, Concepción, Chile, [2] Departamento de Ciencias Ecológicas, Universidad de Chile, Santiago, Chile, [3] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC (UFABC), Santo André, Brazil, [4] Instituto de Entomología, Universidad Metropolitana de Ciencias de la Educación, Santiago, Chile, [5] Departamento de Química, Universidad de Chile, Santiago, Chile

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***Correspondence:**
Daniel Aguilera-Olivares
daguilera@udec.cl
Hermann M. Niemeyer
niemeyer@abulafia.ciencias.uchile.cl

†ORCID:
Daniel Aguilera-Olivares
orcid.org/0000-0003-0776-0275
Alberto Arab
orcid.org/0000-0003-0009-6658
Luis Flores-Prado
orcid.org/0000-0002-3373-8154
Hermann M. Niemeyer
orcid.org/0000-0003-2273-1608

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Single-piece nesting termites live and forage in the same piece of wood throughout their life, which limit their colony size. In certain species, more than one colony thrive in a given piece of wood (multicolonial substrate) and intraspecific competition become important in this limited resource, as has been reported in *Zootermopsis nevadensis* (Hagen, 1858) and *Neotermes chilensis* (Blattodea: Kalotermitidae) (Blanchard, 1851). The effects of such competition have been described mainly at population and colony levels rather than at the individual level. In eusocial insects such as termites, intraspecific competition constitutes a stress factor imposed to a colony as a whole and should also cause developmental instability in soldiers produced under such conditions. Investment in the production of soldiers involves a trade-off between colony maintenance costs and defense benefits. Hence, we hypothesize that body size and fluctuating asymmetry, two indicators of developmental instability, will increase when two or more colonies of *N. chilensis* share a piece of wood (high intraspecific competition scenario). Our results showed that soldiers developing in multicolonial substrates were indeed larger and more asymmetric than soldiers developing in unicolonial substrates. The large body size in a soldier could improve its chance to win a physical contest with a non-nestmate opponent; thus, despite the high cost to produce large soldiers in small colonies, larger soldier production could be an adaptative strategy to avoid being outcompeted. However, the effects of deviations from perfect symmetry on soldier performance are not clear.

Keywords: developmental instability, drywood termite, fluctuating asymmetry, Kalotermitidae, *Neotermes chilensis*, stress, Isoptera, Blattodea

INTRODUCTION

Intraspecific competition studies in social insects have focused mainly at the colony level (Passera et al., 1996; Thomas et al., 2005; Sanada-Morimura et al., 2006; Cronin et al., 2012; Lloyd and Poulin, 2014; Blight et al., 2016) and at the population level (Holway et al., 1998; Adams and Tschinkel, 2001; Korb and Linsenmair, 2001; Boulay et al., 2007; Grohmann et al., 2010;

Bourguignon et al., 2011; Perdereau et al., 2011; Tsuji, 2013; Pringle and Tarnita, 2017); however, the effects of intraspecific competition at the individual level are poorly studied.

Termites are eusocial species that live in colonies organized into three castes (Thorne, 1996; Korb and Hartfelder, 2008; Eggleton, 2011): (i) primary reproductives involved in reproduction, (ii) soldiers involved in defense against intruders, and (iii) true workers (or pseudergates) involved in nest keeping and taking care of juveniles and other castes and also, together with soldiers, in defense. Mandibles of termite soldiers are morphologically adapted to defense (Scholtz et al., 2008); hence, soldiers are unable to feed themselves and they depend on pseudergates or workers for survival (Haverty, 1977; Henderson, 1998). Consequently, investment in the production of soldiers involves a trade-off between colony maintenance costs and defense benefits (Noirot, 1989; Chouvenc et al., 2015). Pseudergates are undifferentiated and totipotent individuals who may develop into other castes such as soldiers or primary reproductives, or they may spend their whole life as pseudergates, depending on environmental conditions (Ogino et al., 1993; Roisin and Korb, 2011). Thus, environmental factors such as colony conditions (colony size, reproductive status, caste ratio, resource availability, etc.), temperature and seasonality significantly affect hormone and gene expressions (Scharf et al., 2007; Miura and Scharf, 2011) which trigger the differentiation from pseudergate to presoldier instar. This process may also be enhanced by ecological factors such as inter and/or intraspecific competition as a form of induced defense at the colony level (Passera et al., 1996; Aguilera-Olivares et al., 2017).

Termite species may be classified according to their nesting behavior in two main categories: separate-piece nesting termites, where foraging substrates and nesting substrate are different; and single-piece nesting termites, who spent their whole life in the same piece of wood (Abe, 1991; Shellman-Reeve, 1997). The colonies of separate-piece nesting termites are composed commonly by true workers and their size ranges from thousands to millions of individuals who can forage and move for thousands of square meters. On the other hand, colonies of single-piece nesting termites are characterized by the presence of pseudergates and colony size (usually no larger than a few thousand individuals) is limited by the volume of the piece of wood where they thrive (Abe, 1991; Shellman-Reeve, 1997; Korb and Hartfelder, 2008; Mizumoto and Bourguignon, 2021). If several colonies of single-piece nesting termites share the same substrate, inter and/or intra-specific competition is expected to occur, particularly when nesting resources become limited (Thorne et al., 2003; Ripa and Luppichini, 2004; Amarillo-Suárez et al., 2011).

Neotermes chilensis (Blattodea: Kalortermitidae) (Blanchard, 1851) is a single-piece nesting termite (i.e., it completes its life cycle within a finite resource) which uses dry scapes (stems of inflorescences) of the bromeliads *Puya alpestris* ssp. *zoellneri* [ex *P. berteroniana*—Zizka et al. (2013)] and *P. chilensis* (Molina, 1782) among its hosts (Aguilera-Olivares et al., 2015). In previous work at the same study site (Aguilera-Olivares et al., 2017), we reported that about half of the scapes were occupied by a single colony (unicolonial substrates) and the other half contained between 2 and 9 colonies (multicolonial substrates). Additionally, we observed galleries interconnecting two colonies within a scape which had been blocked with sawdust, probably following an agonistic interaction. Under this intraspecific competition scenario, the soldiers/non-soldiers ratio significantly increased. This implies that colonies exposed to competition have an inherently higher cost due to the increased production and maintenance of soldiers, and since resources are finite in this termite, an increased developmental stress is imposed on these soldiers. *N. chilensis* stands as an interesting species to study the effect of intraspecific competition in an eusocial insect because: 1) it lives on a limited resource; 2) it is not able to move to another resource, so it must compete when the nesting substrate is shared with other colonies; 3) due to low colony sizes (no more than 500 individuals) it is easier to study than other termite species with typical colony sizes in the thousands to millions of individuals; and 4) about 90% of individuals inside a colony are pseudergates, i.e. totipotential individuals which could develop as soldiers, alates or remain as pseudergates their whole life according environmental signals. Moreover, the soldier caste of *N. chilensis* appears as particularly relevant in terms of intraspecific competition at the individual level, especially the consequences of stress during development.

Developmental stability is the ability of an organism to produce its developmentally programed phenotype despite epigenetic perturbations; contrastingly, developmental instability occurs when an organism is unable to buffer those perturbations (Markow, 1995). In species with symmetric morphological traits, the developmentally programed phenotype is one with perfect symmetry of such traits; and any random deviations from this symmetry on individuals inside a population leads to fluctuating asymmetry (Palmer, 1994; Graham et al., 2010). These deviations may be associated with genetic stress such as hybridization (Graham and Felley, 1985; Handy et al., 2004), and with environmental stress such as food limitation (Swaddle and Witter, 1994), heat shock (Hosken et al., 2000), and intraspecific competition (Witter and Swaddle, 1994). In insects, there is evidence that high levels of larval density constitute an environmental stressor inducing fluctuating asymmetry (Clarke and McKenzie, 1992; Gibbs and Breuker, 2006; Beasley et al., 2013), which may be caused by the effects of intraspecific competition due to a reduction of food availability (Hunt and Allen, 2000). Nevertheless, the effect on fluctuating asymmetry of another scenario of intraspecific competition caused by a restriction in the use of a nesting substrate, as occurs when two or more insect colonies share the same nest, has not been assessed.

On the other hand, body size is a life-history trait which has been used in insects as an indicator of developmental stress (Warren et al., 2006; Couret and Benedict, 2014). Thus, the effect of factors such as altitude (Cushman et al., 1993; Smith et al., 2007; Hoiss et al., 2012), temperature (Bochdanovits and De Jong, 2003), and competition (Heinrich, 1993; Warren et al., 2006; Amarillo-Suárez et al., 2011; Wills et al., 2014; Korallo-Vinarskaya et al., 2015) during insect development are important predictors of body size outcome.

When interference competition occurs, body size generally determines who will dominate the resources; thus, larger individuals are frequently more successful than smaller ones (Heinrich and Bartholomew, 1979; Otronen, 1988; Heinrich, 1993; Zobel and Paxton, 2007; Bespalova and Helms, 2014). In termites, significantly higher fluctuating asymmetry has been detected in soldiers of *Coptotermes formosanus* Shiraki, which develop within incipient colonies (instable and stressful scenario) in comparison with soldiers which develop within mature colonies (more stable environment) (Chouvenc et al., 2014a). Furthermore, studies in three *Reticulitermes* (*R. speratus*, *R. virginicus,* and *R. flavipes*) and two *Cryptotermes* (*C. secundus* and *C. domesticus*) termite species have shown increased female-to-male ratio because female soldiers are larger than male soldiers and thus have a better chance to win in a physical encounter (Matsuura, 2006; Muller and Korb, 2008). In summary, intraspecific competition is a stressful scenario for termite colonies which could affect body size and symmetry properties of developing individuals and hence their performance.

The aim of this work was to study the effects of intraspecific competition at the individual level in an eusocial insect. If competition in the context of finite resources induces colonies to invest in larger and/or more numerous soldiers, it would directly impose a developmental stress on these individuals. We hypothesized that soldiers of *N. chilensis* developing in colonies that share a nesting resource with other colonies (high intraspecific competition scenario) would show significantly higher values of fluctuating asymmetry and they would be larger than soldiers whose colonies occur singly within a nesting resource (low intraspecific competition scenario).

MATERIALS AND METHODS

Species and Study Area

N. chilensis is an endemic termite from Chile distributed between 26 and 33.5°S (Ripa and Luppichini, 2004). The study area (Las Chilcas: 32°52′S; 70°52′W) is located within the sclerophyllous shrub community of central Chile (Gajardo, 1994) whose predominant species are *Adesmia arborea* Bert. ex Savi (Fabaceae), *Colliguaya odorifera* Mol. (Euphorbiaceae), *Echinopsis chiloensis* (Colla) H. Friedrich and G.D. Rowley (Cactaceae), *Puya chilensis* and *P. alpestris* ssp. *zoellneri* (Bromeliaceae). In the study area, *N. chilensis* builds its nests inside the dry scapes of *P. alpestris* ssp. *zoellneri*, with colonies containing up to 500 individuals and it is the only termite species present. Scapes were severed from the rest of the plant, brought to the laboratory in Santiago after being enclosed within a mesh to avoid loss of individuals, and maintained in a breeding room at 16±1°C under darkness for a maximum of 2 weeks until they were dissected.

Collection of Samples

A total of sixteen scapes of *P. alpestris* ssp. *zoellneri* were dissected and the number of colonies and of soldiers and non-soldiers were recorded. Eighteen colonies and fifty-three individuals in total were used in this study. Individuals collected were stored in 70% v/v alcohol. *Multicolonial soldiers*: five scapes had 2 or 3 colonies (mean ± sem: 2.75 ± 0.25 colonies per scape) some of which had soldiers (6.14 ± 1.62 soldiers per colony). A total of seven colonies from multicolonial substrates with 29–178 individuals (104.71 ± 23.3 individuals per colony) were used in the study. The analysis included 22 multicolonial soldiers (2–5 soldiers were extracted from each colony, 3.14 ± 0.46). *Unicolonial soldiers*: 11 scapes had a unique colony. In these scapes, colony size ranged from 48 to 492 individuals (136.1 ± 38.4 individuals per colony) and all had soldiers (7.91 ± 2.07 soldiers per colony). All the 11 colonies in unicolonial substrates were used in the study. The analysis included 31 unicolonial soldiers (2–4 soldiers were extracted from each colony, 2.82 ± 0.18).

TABLE 1 | Morphological traits used to evaluate body size and fluctuating asymmetry in soldiers of *N. chilensis* from unicolonial and multicolonial substrates.

		Morphological traits	Abbreviation	Zoom
Body size analyses	Mandibles	Right mandibular area	RMA	30x
		Left mandibular area	LMA	30x
		Right mandibular perimeter	RMP	30x
		Left mandibular perimeter	LMP	30x
		Interdental distance 1–2 (right)	IDR	30x
		Interdental distance 1–2 (left)	IDL	30x
	Head	Head area	HA	15x
		Head perimeter	HP	15x
		Head length	HL	10.5x
		Head maximum width	HMxW	15x
		Head minimum width	HMnW	15x
		Postmentum length	PmL	22.5x
		Postmentum width	PmW	22.5x
	Thorax	Pronotum length	PnL	18x
		Pronotum width	PnW	18x
		Mesonotum length	MsL	18x
		Metanotum length	MtL	18x
Fluctuating asymmetry analyses	Thorax	Prothoracic femur length	PFL	30x
		Prothoracic femur width	PFW	30x
		Prothoracic tibiae length	PTL	30x
		Prothoracic femur area	PFA	30x

Zoom used for taking photographs to measure traits are informed.

Measurement of Traits

Since termites have a non-quitinized abdomen, body size changes dramatically with hydration level. Hence, only quitinized traits were used (17 in total) to estimate body size (Muller and Korb, 2008; Johnson et al., 2011): six mandibular, seven head and four thoracic traits (described in **Table 1** and **Figures 1A–E**). On the other hand, the left and the right side of four traits were used to assess developmental instability through fluctuating asymmetry (**Table 1** and **Figure 1C**). One photograph was taken of each trait for body size estimation and one photograph was taken of each side of traits selected for the fluctuating asymmetry study. An Olympus® Trinocular Stereo Zoom Microscope Model SZ61 was used with an integrated MSHOT 30 camera; the zoom used for each trait is given in **Table 1**. Using the software M-shot Digital Imaging System (Micro-shot Technology Co, 2010), the traits

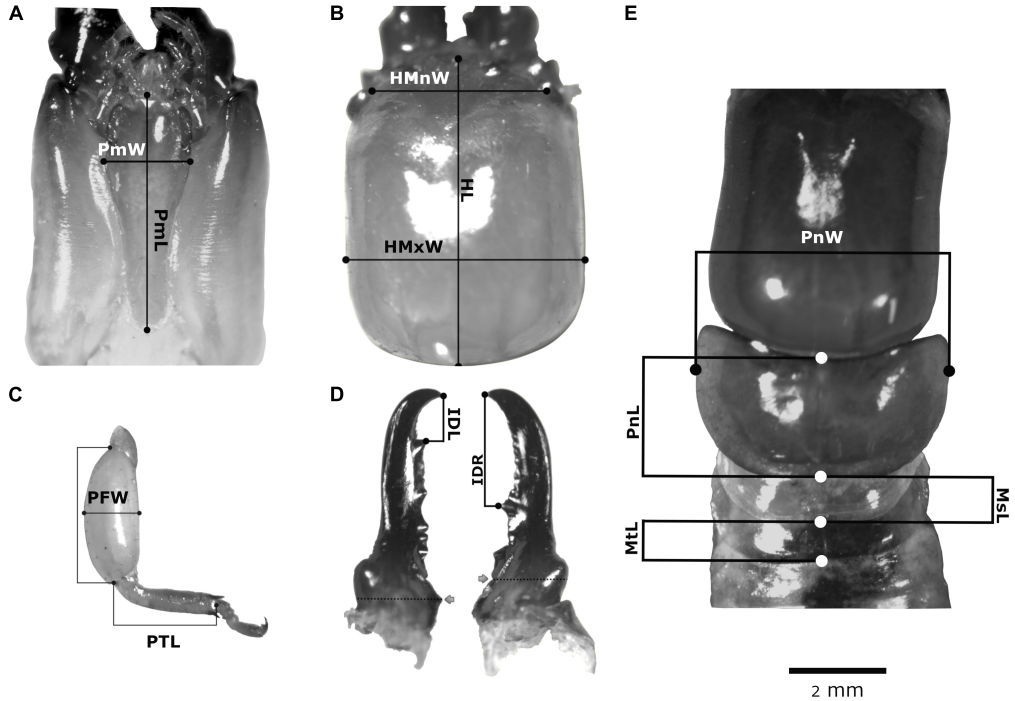

FIGURE 1 | Morphological traits used to evaluate body size and fluctuating asymmetry in soldiers of *N. chilensis* from unicolonial and multicolonial substrates. **(A)** Ventral view of the head; **(B)** dorsal view of the head; **(C)** prothoracic legs; **(D)** mandibles and **(E)** dorsal view of the thorax. Abbreviations used are described in **Table 1**.

were measured in triplicate in three different days to avoid bias and reduce errors.

Statistical Analyses

All the analyses and graphics were performed and generated using R version 3.4.4 (R Core Team, 2019).

Body Size

A Principal Component Analyses (PCA) was performed with the 17 traits measured using the FactoMineR package (Le et al., 2008) to retrieve a set of principal component variables that explained major differences in soldier morphology. The total contribution of each trait was calculated using the function *fviz.contrib*, the most contributed traits were selected as explained in **Supplementary Figure 1**. The principal components that most explained variation in soldier morphology were then used as predictor variables in Generalized Mixed Models (GLMM), in order to test significant differences in body size between soldiers from unicolonial and multicolonial substrates using the glmmTMB package (Magnusson et al., 2017). Coloniality (unicolonial and multicolonial substrates) was the fixed effect and colony size, i.e., the total number of individuals per colony, was the covariate. In order to control the indirect effect of the colony of origin, this parameter was used as the random factor in the models. The significance of the random effect was tested in reduced models. For each model, we assessed residual distribution using the DHARMa package in R (Hartig, 2021).

Fluctuating Asymmetry

The difference between the mean right side and the mean left side of each trait (R-L) was calculated. Following Palmer (1994), the following analyses were performed: (i) normality of the (R-L) distribution was assessed using the Kolmogorov-Smirnov test with Lilliefors correction to discard significant antisymmetry; (ii) significant deviation from zero was tested using a one-sample *t*-test to discard significant directional asymmetry; (iii) a regression was performed between R-L and trait size to discard size dependence; and (iv) a two-way ANOVA was performed with soldiers and side (triplicate data for each side) as factors to discard measurement error.

In order to assess deviations from perfect symmetry, two fluctuating asymmetry indexes were chosen (Palmer, 1994):

1) **FA1**= mean | R-L |, calculated for each four of traits in **Table 1**. We performed a PCA with the four FA1 index values obtained. We performed a GLMM to test significant differences in fluctuating asymmetry between soldiers from unicolonial and multicolonial substrates with PC1 and PC2 as predictor variables, similar to body size analyses described above.
2) **FA10** = σ^2, which gives an estimate of the variance between right and left sides after removing the effects of measurement errors and possible directional asymmetry. Thus, from the result of the two-way ANOVA mentioned above, the mean square of the interaction (MS_{int}), mean square of the error (MS_{error}), number of replicates (N_r),

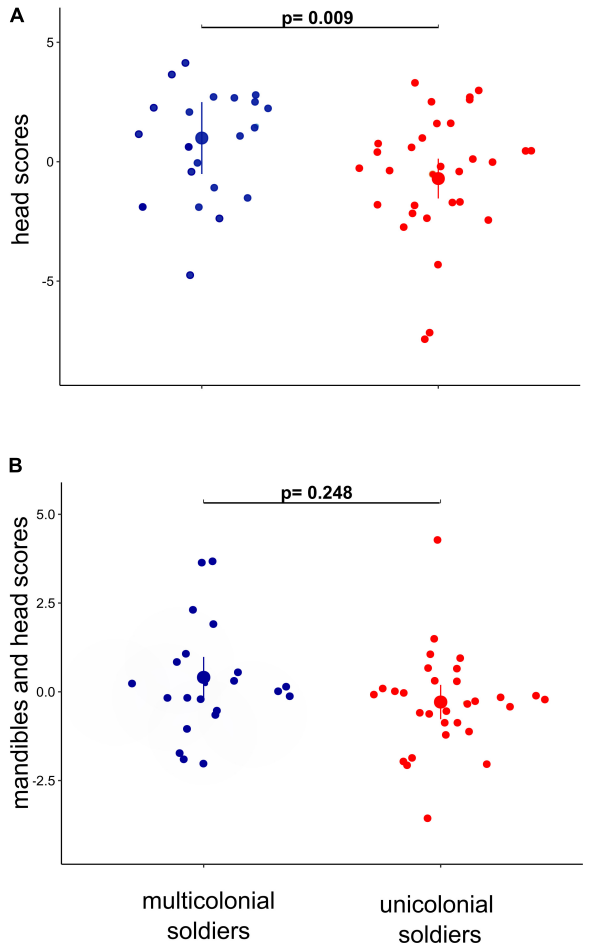

FIGURE 2 | Soldiers' head **(A)** and mandible/head **(B)** components among soldiers from multicolonial and unicolonial substrates. In the plot, the modeled means are represented by large circles, bars are the modeled 95% confidence intervals, and the model-adjusted individual response values are represented by the small dots. Metric scores were obtained from PCA of body size metrics. *P*-values indicate significance at $p < 0.05$.

number of soldiers (N_i), and number of sides (N_s) were used to calculate the variance (σ_i^2) for each trait (Equation 1) and their approximate degrees of freedom (Equation 2):

$$\sigma_i^2 = (MS_{int} - MS_{error})/N_r \quad (1)$$

$$\mathrm{df} = \frac{(MS_{int} - MS_{error})^2}{\left(\frac{(MS_{int})^2}{(N_s-1)(N_i-1)} + \frac{(MS_{error})^2}{N_s N_i (N_r-1)}\right)} \quad (2)$$

RESULTS

Body Size Analysis

Our preliminary PCA showed that the variation in morphology between soldiers from unicolonial and multicolonial substrates was mainly explained by 10 traits (RMA, RMP, IDR, PnW, PnL, LMA, HP, HA, HMxW, and HL) (**Supplementary Figure 1A** and **Supplementary Table 1**). These traits were selected and a second PCA was run, in which the first two principal components explained 77% of total variance of soldier morphology (**Supplementary Figure 1B** and **Supplementary Table 2**). The first principal component (PC1) was named "head scores" because head variables (head area, head perimeter, head length, and head maximum width) were the most contributory variables (51.6%) to this component (**Supplementary Figure 1B** and **Supplementary Table 2**). These variables showed strong positive correlations with this component (correlation coefficients from 0.81 to 0.90—**Supplementary Table 2**). High values of PC1 represent soldiers with larger heads. The second axis (PC2) was the "mandible and head scores" because mandible traits (right mandible perimeter, right mandible area, interdental distance 1–2 right) and a head trait (head length) were the most contributory variables (67.11%) to this component (**Supplementary Figure 1B** and **Supplementary Table 2**). These variables showed positive correlation with mandibular traits (correlation coefficients from 0.45 to 0.70 for RMP—**Supplementary Table 2**) and a negative correlation with the head trait (correlation coefficient −0.44—**Supplementary Table 2**). The GLMM on PC1 showed that soldiers from multicolonial substrates had larger heads than soldiers from unicolonial substrates ($z = -2.59$; $p = 0.009$) (**Figure 2** and **Table 2**). It was also found that colony size had a significant positive effect on head traits ($z = 3.49$; $p < 0.002$) but did not have a significant effect on the second principal component (**Table 2**). Similar results were obtained when the random effect was excluded from the models (**Table 2**). No significant deviations from model assumptions were detected (**Supplementary Figure 3A**).

Fluctuating Asymmetry Analysis

Preliminary tests showed non-significant antisymmetry (**Supplementary Table 3**), non-significant directional asymmetry (**Supplementary Table 4**), non-significant dependence of the trait on size (**Supplementary Table 4**); and non-significant measurement errors (**Supplementary Table 5**) in the four traits analyzed.

The first principal component (PC1) explained the 36.2% of variation among soldiers. Since prothoracic femur area, prothoracic femur length and prothoracic femur width FA1 index values had the highest factor scores for this principal component (**Supplementary Table 6**), we named it "prothoracic femur" component (**Supplementary Figure 2**). The second principal component (PC2) explained the 27.7% of variation among soldiers and prothoracic tibia length FA1 index values had the highest factor score (**Supplementary Table 6**); hence, this principal component was named "prothoracic tibia" (**Supplementary Figure 2**). GLMM showed that soldiers from multicolonial substrates had significantly higher fluctuating asymmetry indexes than soldiers from unicolonial substrates in prothoracic femur component ($z = -2.22$; $p = 0.027$); however, non-significant differences ($z = 1.57$; $p = 0.117$) were found in the prothoracic tibia component (**Table 2** and **Figure 3**). Colony size had non-significant effect on both components (**Table 2**). Similar results were obtained when no random factors were included in the model (**Table 2**). No significant deviations from model assumptions were detected (**Supplementary Figure 3B**).

TABLE 2 | Fixed effects of the GLMM analyses for body size metrics (green shadow) and fluctuating asymmetric indexes 1 (FA1) (purple shadow) of soldiers of *N. chilensis* from multicolonial and unicolonial substrates.

	Analyses	Estimate	SE	*z*-value	Pr (>| z|)
Without Random Factor	***PC1 scores of body size metrics***				
	Coloniality (fixed effect)	−1.70	0.62	−2.72	**0.007**
	Colony size (covariate)	0.01	0.003	3.29	**0.001**
	PC2 scores of body size metrics				
	Coloniality (fixed effect)	−0.36	0.40	−0.90	0.366
	Colony size (covariate)	−0.002	0.002	−0.94	0.349
	PC1 scores of FA1				
	Coloniality (fixed effect)	−0.92	0.32	−2.94	**0.003**
	Colony size (covariate)	−0.0001	0.001	3.49	0.794
	PC2 scores of FA1				
	Coloniality (fixed effect)	0.45	0.29	1.57	0.117
	Colony size (covariate)	0.001	0.001	0.79	0.428
Random factor: Soldier nested in Colony	***PC1 scores of body size metrics***				
	Coloniality (fixed effect)	−1.70	0.65	−2.59	**0.009**
	Colony size (covariate)	0.01	0.003	3.49	**0.002**
	PC2 scores of body size metrics				
	Coloniality (fixed effect)	−0.86	0.39	1.16	0.248
	Colony size (covariate)	−0.38	0.42	−0.89	0.374
	PC1 scores of FA1				
	Coloniality (fixed effect)	−0.86	0.39	−2.22	**0.027**
	Colony size (covariate)	−0.0001	0.001	−0.22	0.830
	PC2 scores of FA1				
	Coloniality (fixed effect)	0.45	0.29	1.57	0.117
	Colony size (covariate)	0.001	0.001	0.79	0.428

Colony size was used as covariate. All analyses were run without any random effect or with soldier nested in colony origin as random effect. Probabilities under 0.05 are highlighted in bold.

Finally, the FA10 index showed a significantly more fluctuating asymmetry in soldiers from multicolonial substrates than in soldiers from unicolonial substrates in all femur traits (**Table 3**).

DISCUSSION

Many abiotic and biotic factors may affect the normal ontogenetic development of insects (Palmer, 1994; Markow, 1995; Graham et al., 2010; Couret and Benedict, 2014). In this work, termite colonies were collected from a common site; hence, those present in unicolonial and multicolonial substrates were exposed to the same abiotic factors. Additionally, *N. chilensis* was the only termite species present in the study site and *N. chilensis* termites were the only insects found inside the scapes. Hence, intraspecific competition operating on a termite colony as a whole is expected to be the most important stress factor that may differentially affect unicolonial and multicolonial soldier individual development. All the indexes and analyses used in this work showed significantly higher levels of fluctuating asymmetry in soldiers developed in multicolonial substrates than soldiers developed in unicolonial substrates. Thus, our results showed that the presence of neighboring colonies generated developmental instability in soldier differentiation from pseudergates.

When two or more colonies share a nesting substrate, several scenarios could follow, e.g., their galleries could meet and colonies could fuse, they could fight against each other with the possible consequence of the elimination of one of them, or they could avoid each other. In exotic invasive species, nestmate recognition mechanisms generally fail because genetic diversity in the population is low and relatedness among nestmates is high. This may lead to colony fusion, as has been shown in termites (Perdereau et al., 2010, 2011; Lee et al., 2019) and ants (Holway et al., 1998; Eyer et al., 2018). On the other hand, in native species, where genetic diversity in the population is high and relatedness between colonies is low, colony fusion is rare (Shelton and Grace, 1996; Deheer and Vargo, 2004; Smith et al., 2012). In the population of the native species *N. chilensis* under study, the mean relatedness of nestmate pairs was shown to be 0.465 ± 0.0085 (mean ± sem), corresponding closely to that expected for full siblings (r = 0.50) and the relatedness between non-nestmate pairs was close to zero (Aguilera-Olivares et al., 2015). This suggests that if two or more colonies meet within the nesting substrate, colony fusion would be rare or inexistent. In fact, nestmate recognition was reported in *N. chilensis*, soldiers being more aggressive toward non-nestmates than toward nestmates from different castes (Aguilera-Olivares et al., 2016a,b). These observations suggest that individuals from two colonies of *N. chilensis* within a scape either fight and eliminate opponent

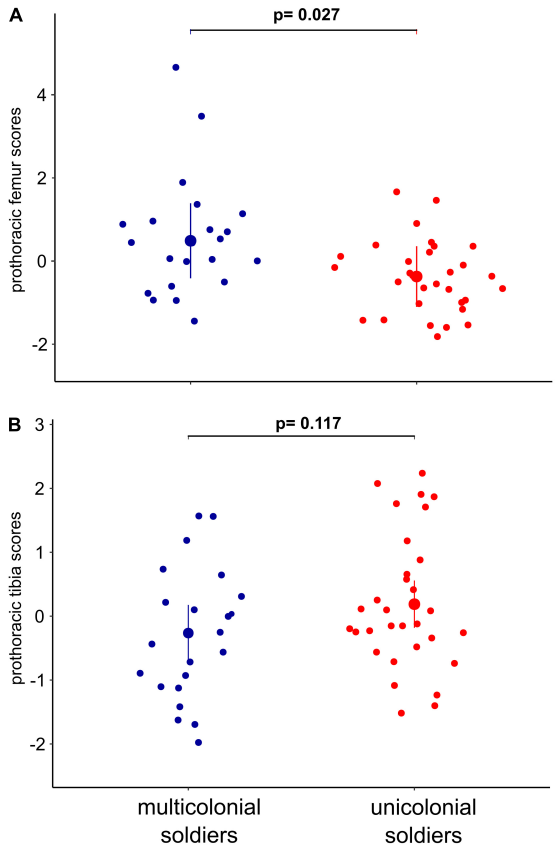

FIGURE 3 | Soldiers' prothoracic femur **(A)** and prothoracic tibia **(B)** principal components from FA1 indexes among soldiers from multicolonial and unicolonial substrates. In the plot, the modeled means are represented by large circles, bars are the modeled 95% confidence intervals of each mean, and the model-adjusted individual response values are represented by the small dots. Scores were obtained from PCA of fluctuating asymmetry indexes (FA1). *P*-values indicate significance at $p < 0.05$.

TABLE 3 | Fluctuating asymmetry index 10 (FA10) of four morphological traits of soldiers of *N. chilensis* from multicolonial and unicolonial substrates.

Morphological trait	Soldier	FA10 (σ^2)	Statistics
Prothoracic femur length	Multicolonial	0.00231	$F_{(20, 27)} = 2.20$
	Unicolonial	0.00105	**$P = 0.029$**
Prothoracic femur width	Multicolonial	0.00291	$F_{(20, 19)} = 5.65$
	Unicolonial	0.00052	**$P < 0.001$**
Prothoracic tibia length	Multicolonial	0.00487	$F_{(30, 20)} = 1.50$
	Unicolonial	0.00731	$P = 0.172$
Prothoracic femur area	Multicolonial	0.01635	$F_{(21, 19)} = 2.12$
	Unicolonial	0.00771	**$P = 0.049$**

P-values under 0.05 are highlighted in bold.

colonies or avoid them. Additionally, the colony of origin had a very slight if any effect when its inclusion or exclusion as random factor was considered in the models; thus, despite the fact that individuals inside a colony of *N. chilensis* are closely related, there is enough variation among soldiers to be considered independent samples, suggesting that the response to intraspecific competition in our analyses is independent of their degree of relatedness.

Body size is a life-history trait that has been used in insects as an indicator of developmental stress (Warren et al., 2006; Couret and Benedict, 2014). Intraspecific competition as a stress factor has been described in literature (Heinrich, 1993; Warren et al., 2006; Amarillo-Suárez et al., 2011; Wills et al., 2014; Korallo-Vinarskaya et al., 2015). In territorial species where interference competition occurs with physical encounters displayed, larger individuals have better chances to win a fight and monopolize resources than smaller individuals (Price et al., 2011; Bespalova and Helms, 2014; Holland et al., 2021). In our study, we demonstrated that soldiers from multicolonial substrates had significantly larger heads than those from unicolonial substrates, strongly suggesting that in a competitive scenario, colonies produce larger soldiers which increase the chance to win fights and monopolize resources. Furthermore, Muller and Korb (2008) showed that females of *Zootermopsis nevadensis* (Hagen, 1858), a single-piece nesting termite, develop more often into soldiers than males because they are significantly larger, which is an advantage when blocking holes/galleries or fighting with other individuals (Zobel and Paxton, 2007). In our work, quitinized traits of *N. chilensis* that showed differences in size between uni- and multicolonial substrates are closely related to defensive tasks. Thus, head traits (head area, head perimeter, head length, and head maximum width) could be related to the blocking of galleries with the head and to more developed muscles to produce stronger bites (Bespalova and Helms, 2014). When the scape used by two or more colonies is depleted and the tunnels dug by members of different colonies finally meet, all of these traits could improve the chance to win a fight in a hypothetical scenario of interference intraspecific competition (Heinrich and Bartholomew, 1979; Otronen, 1988; Heinrich, 1993; Zobel and Paxton, 2007; Bespalova and Helms, 2014).

Soldiers totally depend on workers or pseudergates for survival (Haverty, 1977; Henderson, 1998); thus, the soldier production represent a trade-off at colony level between benefits (colony defense) and costs (investment in soldier nurturing) (Noirot, 1989; Chouvenc et al., 2015). Additionally, in incipient colonies some larvae must develops into soldiers instead of workers in order to maintain a relatively stable soldier proportion, which could have consequences in colony growth as a result of a reduction of worker number (Chouvenc et al., 2015). In *Coptotermes* species, a subterranean and separate-piece nesting termite, incipient colonies with low numbers of workers and hence a reduced nurturing capacity produce smaller and more asymmetric soldiers (i.e., low quality soldiers produced at low cost) as an strategy to keep a stable caste ratio which produces a delay on the growth of colonies (Chouvenc and Su, 2014b; Chouvenc et al., 2015, 2017); while soldiers from mature colonies which contain high number of workers and hence exhibit high nurturing capacity, are larger and symmetric (high quality soldiers produced at high cost) (Chouvenc and Su, 2014b; Chouvenc et al., 2017). In single-piece nesting termites, such as *N. chilensis*, colony survival is directly related with its capacity to get and defend resources; thus, despite the high cost to produce large soldiers in small colonies, larger soldier production could be an adaptative strategy to avoid being outcompeted.

CONCLUSION

This study shows for first time the effect of intraspecific competition at the individual level in an eusocial insect. Soldiers of *N. chilensis* which developed from pseudergates in a high intraspecific competition scenario (multicolonial substrates) were larger and exhibited more asymmetric traits than soldiers which developed in a low intraspecific competition scenario (unicolonial substrates). While the large body size in a soldier could improve its chance to win a physical contest with a non-nestmate opponent, the effects of deviations from perfect symmetry on soldier performance are not clear. In single-piece nesting termites, such as *N. chilensis*, colony survival is directly related with its capacity to get and defend resources; thus, despite the high cost to produce large soldiers in small colonies, larger soldier production could be an adaptative strategy to avoid being outcompeted. Having shown the effects of intraspecific competition on body size and asymmetry of soldiers, it would be interesting to test the fitness consequences of larger body size and fluctuating asymmetry in soldiers of *N. chilensis* and its consequences for colony life. Additionally, the effects could be tested in other single-piece nesting termites such as the worldwide distributed (except in Asia) *Cryptotermes brevis* (Walker, 1853) and in *Z. nevadensis*, where multicolonial substrates have been reported.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DA-O, LF-P, BT-G, and HN: study conception and design. BT-G and DA-O: material preparation and data collection. AA, BT-G, and DA-O: statistical analyses. DA-O: writing first draft of the manuscript. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.882357/full#supplementary-material

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Life and Death of Termite Colonies, a Decades-Long Age Demography Perspective

Thomas Chouvenc, Paul M. Ban† and Nan-Yao Su*

Department of Entomology and Nematology, Fort Lauderdale Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Fort Lauderdale, FL, United States

A eusocial insect colony represents a complex biological entity that must ensure degrees of perennity once it reaches maturity (production of dispersing imagoes over many successive years) to optimize its reproductive success. It is known that a subterranean termite colony invests differentially in different castes over time and adjusts colony functions depending on colony internal and external conditions over many years of activity. However, the current study demonstrates that *Coptotermes formosanus* Shiraki field mature colonies go through dramatic demographic changes and breeding structure shifts, even many years after they have reached reproductive success. By analyzing the changes in age demography of *C. formosanus* colonies from four field sites, we here provide a new perspective on how a colony may function over decades, which reveals that each colony demographic trajectory is unique. In a way, throughout its life, a termite colony displays its own "demographic individuality" that drives its growth, its foraging ability, its competitiveness, its age demography, its senescence and ultimately its death. This study is therefore a narrated story of the life -and death- of different *C. formosanus* field colonies over decades of observation.

Keywords: *Coptotermes formosanus*, colony perennity, reproduction, colony senescence, succession

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****Correspondence:***
Thomas Chouvenc
tomchouv@ufl.edu

†Deceased

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INTRODUCTION

Animal societies have the ability to form complex groups of cooperating individuals, where a distinct degree of organization was reached in eusocial taxa (often referred to as "superorganisms") (Wheeler, 1911; Emerson, 1939; Seeley, 1989; Hölldobler and Wilson, 2009; Boomsma and Gawne, 2018). Eusociality involves a reproductive division of labor within the social context of overlapping generations and cooperative brood care (Wilson, 1971). This major transition has evolved multiple times with remarkable degrees of evolutionary convergence among independent taxa (Mueller et al., 2005; Howard and Thorne, 2010; Harrison et al., 2018). Eusocial insects are important ecological drivers, with ants and termites combined representing more than half of the terrestrial arthropod biomass (Eggleton, 2020), while displaying remarkable degrees of biodiversity and phenotypic innovations (Hölldobler and Wilson, 2009; Chouvenc et al., 2021).

In the life cycle of superorganisms, eusocial insects such as ants and termite colonies have decoupled the default linear reproductive process of organisms (birth → juvenile development → adult maturation → reproduction), where the production of sterile individual helpers during colony growth is analogous to the development of the soma line, while the

emergence of reproductively competent imagoes is limited to matured colonies as an analogy to the germ line (Oster and Wilson, 1978; Boomsma and Gawne, 2018; Ramsay et al., 2021). The establishment of the reproductive division of labor is therefore tied to two distinct types of reproductive investments playing at two different degrees of organization and at different times within the colony life cycle, especially for taxa relying on independent colony foundation strategies (Cronin et al., 2013). First, is the internal reproductive cycle of individuals in the colony aiming at producing sterile helper castes that contribute to the growth and defense of the colony. Second, is the external reproductive cycle of the colony aiming at producing reproductively competent alates that disperse and spread their genetic material to the next generation outside of the colony (Emerson, 1939; Moritz and Southwick, 2012; Shik et al., 2012; Boomsma and Gawne, 2018). Thus, independently from the life cycle of each individual within a colony, eusocial insect colonies must go through their own life cycle from birth (=colony foundation) → juvenile development (=colony ergonomic growth) → adult maturation (=production of dispersing reproductive caste) → to sexual reproduction (=dispersal flight events) (Nutting, 1969; Oster and Wilson, 1978; Porter and Tschinkel, 1985; Chouvenc and Su, 2014; Nalepa, 2015).

The theoretical framework of the ergonomic optimization of eusocial insect colony growth and their reproductive perennity provided a comprehensive background to explain the differential resource investment into castes throughout the life cycle of a social insect colony (Oster and Wilson, 1978; Houston et al., 1988). The demography of a social insect colony may change upon its variable intrinsic metabolic needs and extrinsic environmental fluctuations (Porter and Tschinkel, 1986; Kaspari and Vargo, 1995; Hou et al., 2010; Cook et al., 2011; Chouvenc and Su, 2014; Fewell and Harrison, 2016). In addition, many eusocial taxa can display diverse and plastic breeding strategies (Bourke and Ratnieks, 1999; Heinze and Keller, 2000; Vargo et al., 2003; Vargo, 2019; Eyer and Vargo, 2021). Such characteristics explain why Oster and Wilson (1978) considered eusocial insect colonies as "highly coordinated growth machines" that are optimized through selective pressures to maximize colony fitness outcomes.

Notably, the study of social insect colony demography has primarily been driven by the determination of colony size (i.e., the number of individuals in the colony), as it often defines the colony ability to acquire resources and thrive in a competitive environment (Michener, 1964; Su and Scheffrahn, 1988a; Haagsma and Rust, 1995; Tschinkel et al., 1995; Naug, 2009). Reciprocally, the optimization of resource acquisition and utilization through colony ergonomic growth and colony reproductive output influences colony size in a feedback loop (Oster and Wilson, 1978; Dornhaus et al., 2012). Therefore, acquiring demographic data about social insect colonies can provide a unique insight about its reproductive status, its breeding system, its ecological impact, its competitive ability, and other aspects that would inform how a eusocial insect colony functions as a superorganism (Oster and Wilson, 1978; Tschinkel, 1991; Schmid-Hempel, 1992; Vargo and Husseneder, 2009). However, the determination of colony size and other demographic variables from field populations is inherently challenging often because of its destructive nature and the resulting inability to follow demographic changes over time (Darlington, 1984; Tschinkel, 1998), which often results in the reliance on theoretical models to explain long term ecological patterns (Oster and Wilson, 1978; Shik et al., 2012; Su, 2013).

In subterranean termites (Rhinotermitidae), colony access is limited owing to their cryptic lifestyle and their widely spread underground foraging territory (King and Spink, 1969), which have presented additional challenges for identifying general patterns about colony demography and ecology. Such limitation has resulted in a reliance on mark-recapture protocols, with limited predictive power from the inference process (Su and Scheffrahn, 1988a; Grace, 1990; Thorne et al., 1996; Evans et al., 1998; Su and Scherer, 2003; Arab et al., 2005; Su, 2013). The quest for subterranean termite colony size estimate initially aimed at establishing termite control protocols (Su et al., 1991; Su and Scheffrahn, 1998; Su, 2001, 2019), which left aside a rather important aspect of termite colony functionality: their inherent age cohorts, their caste demographic composition, and their reproductive status over time. In theory, such colony demography must be highly variable throughout the life of the colony (Oster and Wilson, 1978). Unfortunately, to our knowledge, no study was able to gather data with comprehensive information about termite colony age demographic changes over time, except for a limited observation from Grace et al. (1995).

Termite colony age demography is a direct result of two opposite factors: (1) colony natality rate, which relies on queen(s) oviposition and quality of brood care, and (2) colony mortality rate, which depends on environmental threats on foraging individuals and the inevitable individual senescence (Nakajima et al., 1963; Šobotník et al., 2012; Chouvenc and Su, 2014; Du et al., 2017). Similar to human demographic studies of countries that use age pyramids to determine how natality and mortality shape the age structure of a population, which impact economic trends (Goldstein, 2009), an analogous investigation of individual age composition in termite colonies can directly reflect on the statuses of their general demography, health, reproductivity and resource management efficiency. To our knowledge, such study has not been done because of the need to follow the population age structure over the lifetime of a colony in the field. Therefore, making any interpretation about the demography of large perennial eusocial insect colonies remains primarily speculative or theoretical (Oster and Wilson, 1978; Su, 2013).

Arguably, the seminal work of Oster and Wilson (1978) established the foundation for understanding the fundamental shifts required in colony demography throughout the life cycle of the colony (foundation, ergonomic growth, reproduction) for fitness maximization, but they omitted to discuss the phase that eventually all social insect colonies have to go through: colony senescence that involves the loss of critical colony functions, which ultimately leads to colony death. Paradoxically, individual senescence (or lack thereof) in social insects has been extensively investigated in the context of the superorganism (Rueppell et al., 2007; Kramer and Schaible, 2013; Giraldo and Traniello, 2014; de Verge and Nehring, 2016; Kramer et al., 2021), where sterile individuals are disposable and often not

as long-lived as reproductive castes (Keller and Genoud, 1997; Lemanski and Fefferman, 2018; Giraldo et al., 2021). However, the longevity of individuals directly impacts their ability to maintain colony functions (resource acquisition, colony maintenance, brood care) as a high return on investment for the colony (Moret and Schmid-Hempel, 2009; Dornhaus et al., 2012; Chouvenc et al., 2015a; Lemanski and Fefferman, 2018; Bernadou et al., 2021). Conversely, beyond honey bee colony collapse research (Ellis et al., 2010; Dainat et al., 2012; Evans and Chen, 2021), colony senescence is a poorly studied aspect of the life of eusocial insects, especially in termites (Lepage and Darlington, 2000; Chouvenc et al., 2013a).

Often, observations of "natural" termite colony death in the field may arise from accidental events such as flooding, droughts, predation, or inherent colony senescence (Collins, 1981; Lepage and Darlington, 2000). It has been suggested that colony senescence could be initiated by a reduction or loss of reproduction, which leads to an absence of brood ultimately resulting in the accumulation of old individuals within the colony (Bodot, 1969; Darlington and Dransfield, 1987; Darlington, 1991; Grace et al., 1995; Chouvenc and Su, 2014). Studies on the use of chitin synthesis inhibitor (CSI) baits against subterranean termite colonies implied that the rapid death of the brood and the accumulation of old workers was analogous to an accelerated senescence of the colony, combined with a progressive starvation of all dependent castes (Chouvenc, 2018, 2020; Kakkar et al., 2018; Chouvenc and Lee, 2021). This recent research has provided clues on what the end of colony life may look like from a demographic perspective. However, beyond such limited observations, information on colony demographic changes over time and colony senescence in subterranean termites in the field remains fragmentary.

Finally, the colony life cycle and the inherent molting processes of subterranean termites add another layer of complexity when it comes to how to interpret demographic changes over time (Grace et al., 1995; Chouvenc and Su, 2014). Subterranean termites molt periodically until they die from a series of factors (age, injury, predation, disease, competition, resource limitation). Each time a worker molts, it grows slightly bigger which means that when comparing two groups of termites, the relative weight of individuals can be used as a proxy for the determination of the relative age of the two groups (Nakajima et al., 1963; Chouvenc and Su, 2014; Su et al., 2017). Similarly, high reproduction would lead to an influx of young individuals, while low reproduction would lead to an accumulation of old individuals over time. Therefore, if one could monitor the changes in the average weight of individuals throughout the life of a subterranean termite colony, it can be used as proxy to estimate the relative age of a termite group, which in turn could inform the natality and mortality rate for the entire colony over time.

In 1985, the University of Florida Ft Lauderdale Research and Education Center subterranean termite laboratory was established by NYS to address the rising problems resulting from the recent establishment of *Coptotermes formosanus* Shiraki (Blattodea:Rhinotermitidae) in Florida (Koehler, 1980), an invasive termite species in the United States, with a major structural pest status (Lax and Osbrink, 2003; Rust and Su, 2012; Chouvenc et al., 2016; Evans, 2021). To study the foraging behavior of this invasive species, field ground traps were established on properties with active termite infestation and were monitored by PMB. In addition, such traps were used to collect large numbers of termites to provide biological material for laboratory experiments. As a result, more than 250 studies were published using such source material between 1986 and 2009 from this laboratory. However, as termites were collected, a series of measurements were recorded, resulting in the acquisition of a vast database from more than 15,000 field traps over the span of 24 years from more than 120 different sites. This database was digitized between 2009 and 2014, and it was recently partially curated by TC. Therefore, as a result of the existence of this dataset, it is now possible to look into the fundamental demographic changes throughout the life of subterranean termite colonies and reveal aspects of their ecology that were previously unsuspected. The current study is the first of an upcoming series of demographic studies that will use this subterranean termite dataset to answer a set of questions that emerged during its initial analysis. In this first study, we focused on age demography of colonies over time (using worker, soldier and nymph average weights as proxies), and highlighted the senescence processes of large mature colonies, with a multidecade monitoring approach of four distinct field sites with *C. formosanus* activity.

MATERIALS AND METHODS

Termite Collection and Processing Protocols

Termite ground traps (often referred in the termite literature as "bucket trap") were installed at locations with known *C. formosanus* activity, following the protocol established by Su and Scheffrahn (1986). However, this protocol was modified over the years to minimize potential disturbances and increase the number of termites collected, while streamlining the termite processing method when brought back to the laboratory. Paradoxically, while this modified termite trapping method was used for more than 20 years of termite collection, it was never fully redescribed properly. We here provide an updated overview of this procedure.

Wooden stakes (*Picea* sp.) were installed in the ground of properties with known *C. formosanus* activity and monitored periodically (1–3 months). When foraging termites were found feeding on a wooden stake, a hole around the stake was dug in the ground, a bottomless bucket (18 cm diam × 20 cm height) was installed, a bundle of wood was placed at the bottom of the hole and a lid was placed on top of the plastic container. A bundle of wood consists of five 15 × 10 × 0.3 cm pieces of wood (*Picea* sp.), each separated by two 8 cm plastic straws, and bundled all together by two plastic-coated metal wires tied around it. By twist-tying the wires, the straws compressed to the point of creating a ∼2 mm gap between each wood layer. The wood for each bundle was oven-dried at 73°C for 3 days and weighted prior to assembly. A serial number was attributed to each bundle using a 2 × 4 × 0.2 cm plastic label stappled on one of the sides of the wood bundle. Periodically, the bundles

were collected from the field traps, and replaced with new bundles for ongoing monitoring. The retrieved bundles were then individually processed in the laboratory using the Tamashiro et al. (1973) method, which uses moist wooden tong-depressors as ramps for termites to sort themselves out from debris, toward a clean wooden slab. It allows for a rapid processing of a large numbers of termites while limiting injury levels. The remaining wood material from the bundle was cleaned up, oven-dried for 3 days and weighted again to determine net wood consumption. The rationale behind this overall standardized protocol using wood bundles was to create a space (provided by the compressed straws) that favors termite aggregation and access to feeding surfaces when in the bucket (= "termite space," Chouvenc et al., 2011), to maximize the number of termites collected, but also to expedite termite processing and minimize damage done to termites in the laboratory by simply cutting the two wires and sorting wood debris and straws from termites.

Between 1986 and 2009, a total of 15,016 wood bundle samples from ground monitoring traps located at more than 120 independent sites were collected and processed. In the current study, data from a selection of four distinct sites located in Hallandale, FL were used, here referred as sites *Cf1*, *Cf2, Cf3,* and *Cf4*, for a total of 3,491 cumulated traps. Here, "site" refers to a delineated monitored area, but depending on the context, may reflect the demographic changes of a single colony, or in some cases, a succession of multiple colonies, as explained in the results. These four sites were selected from the rest of the dataset because of their relatively long sampling timeline compared to all other sites (10, 11, 22, and 24 years respectively). The rest of the unused data will be used in follow-up studies that will focus on aspects of termite demographics not discussed here-in. Over the years of monitoring, new wooden stakes were regularly placed on surveyed sites in order to maintain ongoing observation of termite colonies from multiple trap locations, in case of temporary or definitive loss of foraging termite activity from one or more traps. Wood bundles were collected and replaced from field traps periodically (frequency varied greatly, see below), resulting in a large collection of groups of termites from the field. With few exceptions of triple mark-recapture procedures for colony size estimations as the ones performed in Su and Scheffrahn (1988a) and to confirm that different traps at a site were accessed by a single colony, termites were never returned to their colony of origin, but were instead used for laboratory experiments for over two decades. Such protocol implies that from the 10–24 years of monitoring, millions of termites were removed from established colonies. While this protocol inherently altered colony demographics each time termites were collected, we here argue that its impact may have been negligeable, as it was estimated that less than 0.5% of the overall termite colony population were sampled on a monthly basis (Su and Scheffrahn, 1988a; Chouvenc and Su, 2014).

Data Acquisition

The termite population obtained from a single wood bundle sample represents a snapshot of its foraging demographics at a given foraging location. By accumulating several snapshots over many years, it provided a trend in foraging population dynamic of a given colony at any given time. Each time a wood bundle was brought back from a field trap, it was processed in the laboratory, all castes were separated to individual containers, and a series of variables were recorded: Site of origin, trap #ID at the site, date placed in the ground, date collected from the ground, initial wood weight, final wood weight, and the number and the total mass of workers, soldiers, nymph, alates and secondary reproductives, if any. Data about the presence of a brood (eggs/larvae) was not found in the trap records, but it is unknown if the presence of a brood was overlooked or it was never found at foraging sites [although, the brood in *Coptotermes* is expected to be found at the central part of the nest, not at foraging sites, according to Kakkar et al. (2017), who used laboratory colonies with primary reproductives only].

Workers represented the largest proportion of the individuals collected, so in order to determine the number of workers without having to count them individually, a subsampling protocol was established to estimate the total number of workers from the processed wood bundle. Five groups of 10 workers were randomly subsampled from the pooled workers and the total weight of each group of 10 workers was noted to calculate the average worker weight from each subsample. Then, the average weight of an individual worker from a given wood bundle was determined by averaging the five worker weight subsample averages, indirectly providing information (standard deviation = weight heterogeneity within a given trap) on the potential variability of worker weights within a given foraging trap. Once the average worker weight was determined, the total worker mass was then used to estimate the total number of workers from a wood bundle. For all other castes, all individuals were counted, and the average individual weight was inferred from the total mass of each caste.

Data Curation

Data acquisition quality was variable over the 24 years of trap collection. While the protocol was standardized in the early days of termite trap collection, the manual labor to process the traps and record the variables were partially performed by many different temporary technicians over the years. As a result, some variables were acquired inconsistently, weight calculations were sometimes inaccurate, and notes sometimes lack context to make sense of it. In addition, as the initial records were manually collected in notebooks, the subsequent digitalization process possessed inherent typos. Therefore, by cross-referencing all resources available, the dataset was carefully curated to ensure the reliability of the data obtained and outliers were all checked for validity. A majority of suspicious outliers resulted from a simple misplaced decimal or from typing an adjacent number on the keypad. By checking back on the original written records, each of them was corrected accordingly. However, despite a thorough curation process, many trap records were unusable owing to their lack of reliability (undecipherable handwriting, orders of magnitude outliers with no clue on how wrong the calculations were

made, entry too incomplete to make sense out of it, etc.). Thus, out of the 3,491 wood bundles collected from traps at the four sites of interest, 3,067 trap records could be used with confidence in this study, while 424 unreliable trap records were removed from the analysis. Finally, the wood consumption data only was partially recorded over the years, explaining some of the discrepancies in the number of traps used across variables in the results.

It is here important to emphasize that the data was acquired with the initial purpose of informing on the quality of the termites collected from the field prior to using them for experimentation in the laboratory (1986–2009). There was no initial tangible intent to compile all entries from all these years and to perform the analysis presented in the current study. Therefore, while data collection could have been more rigorous in hindsight, additional variables could also have been acquired (worker instar composition, dry weight, molting frequency, sex determination of individuals, distances between traps, etc.), but because of limited time and resources, the need to focus on the experiments at that time, and no plan for a multidecade analysis, we are limited to what was collected.

Variables of Interest

The average weight of individual castes was used as a proxy to determine the relative developmental stage of the foraging population at the time of the sampling, which directly reflects on which instars were present, and by extension, reflected on the general demography of the colony (Grace et al., 1995; Chouvenc and Su, 2014; Su et al., 2017, see "development pathway context" overview below). Therefore, the variability of the age composition of individuals over time was the primary focus of the study.

While colony size could have been an important variable to obtain, the number of termites collected at each sampling event cannot be used as a proxy to estimate the actual population size of colonies. *Coptotermes formosanus* colonies can reach populations of several millions, can fluctuate extensively over time, and colony size estimation protocols for a single point in time is resource and time consuming, which prohibits the scaling of the process to repeated measurement (King and Spink, 1969; Su and Scheffrahn, 1988a; Su, 2013). In addition, the overall data on caste ratios, total number of termites collected, feeding activity over time, and on other aspects this dataset can inform on termite colony functioning will be discussed in future papers. However, in special "demographic transitions" described in the current study, some of these variables were inspected to provide additional elements for interpretations.

Developmental Pathway Context to Interpret Colony Age Demographics

A background about developmental pathways in *Coptotermes* is first necessary to place age demography data in a relevant context (**Figure 1**). In *Coptotermes*, eggs (E) hatch into first instar larvae (L_1), which then molt into a second instar larva (L_2). Here, despite hemimetabolous development, the use of the "larva" term follows the analogous function of immature individuals as found in social Hymenoptera, while "nymph" refers to an individual on the imaginal line (wing buds present), as per Thorne (1996). Second instar larvae (L_2) can then molt into a first instar worker (W_1 = nutritionally competent individual that start performing various tasks in the nest), which then successively molt into older worker instars ($\rightarrow W_2 \rightarrow W_3 \rightarrow \ldots$) until they die of old age (W_{6+}), and workers progressively grow bigger as they molt into older instars (Shimizu, 1962; Higa, 1981; Du et al., 2016). Thus, a group of workers with a relatively small average weight indicates a group of relatively young workers, and vice versa. Also, weight fluctuation may have nutritional and moisture components, but we assume it to be negligeable in the context of this study and was not determined at the time of sampling. It was also shown that foraging termites display degrees of age polyethism, as older workers tend to forage further away from the central part of the nest than younger workers (Du et al., 2017; Su et al., 2017; Lee et al., 2021). It therefore explains why the demographic profile of a colony may vary among traps on any given sampling date, as the age cohort of foragers are not homogeneously distributed throughout the colony foraging territory (Lee et al., 2022). For a given colony with multiple monitored foraging sites, it can therefore be inferred that a trap with a majority of old workers is located further away from the central part of the nest than a trap with a majority of relatively young workers (Shimizu, 1962; Su et al., 2017; Lee et al., 2022). In mature colonies of *C. formosanus*, soldiers (S) are produced from various worker pathways through a presoldier stage (PS), which depends on the availability of workers at the time they initiate differentiation into soldiers: $W_1 \rightarrow PS_2 \rightarrow S_2$, $W_2 \rightarrow PS_3 \rightarrow S_3$, and $W_3 \rightarrow PS_4 \rightarrow S_4$. Contrary to workers, the weight of soldiers cannot be used as a proxy for their relative age, as the soldier caste is final and no longer molts. Instead, the later the soldier pathway, the bigger the soldier, so the average weight of soldiers indirectly informs on the relative worker demographics at the time the individuals engaged into soldier development (Park and Raina, 2003; Chouvenc and Su, 2014). Nymphs (N) are produced seasonally toward the production of imagoes (alates) for dispersal flight events (Chouvenc et al., 2017). In *Coptotermes*, nymphs are produced from second instar larva ($L_2 \rightarrow N_1 \rightarrow N_2 \rightarrow \ldots \rightarrow N_6 \rightarrow$ Alate) and can be found at foraging sites (Albino and Costa-Leonardo, 2011). Late instar nymphs may also be the source for the production of secondary reproductives (Chouvenc and Su, 2014). Relatively few alates were collected in this study, as it is possible that alates move above ground (trees) prior to their dispersal flights and may only be present in traps for a short period.

Statistical Analysis

For all monitored sites (*Cf1*, *Cf2*, *Cf3,* and *Cf4*), the worker average weight, the soldier average weight, and the nymph average weight were scatter-plotted over time, where one datapoint represents the average weight of one trap. For each caste, an average weight trend was generated using a rolling average function (period = 3, i.e., previous, current, next sampling event) to soften sharp fluctuations and outliers over time. The generated figures are purely descriptive of the demographic changes that occurred at these four foraging sites

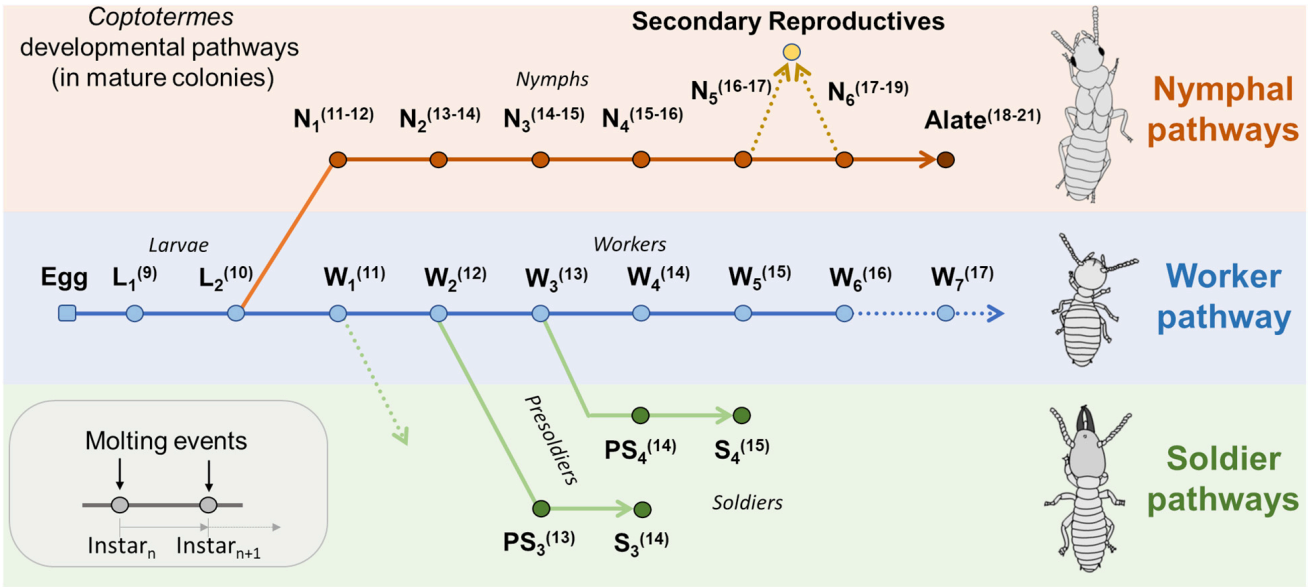

FIGURE 1 | Developmental pathways expected in mature colonies of *Coptotermes formosanus*, modified from Chouvenc and Su (2014). L_n, Larva instar; W_n, Worker instar; N_n, Nymph instar; PS_n, Presoldier type, and S_n, Soldier type. Values in parenthesis$^{(n)}$ indicate the expected number of antennomeres for each given instar.

over time. Next, for each site, the relationship between the average weight of workers and the weight of soldiers and nymphs, respectively, were all positively correlated (Pearson correlation, $p < 0.001$ for all), so a simple linear model (*lm*, *linear*) was used in R v 4.1.0 (R Core Team, 2021) using the default and *ggplot2* packages.

Then, the seasonal fluctuations of caste weights were investigated by pooling the data from several years with cyclical trends into a single combined annual dataset (1989–1993 for *Cf1*, 1993–1996 for *Cf2*, 1997–2008 for *Cf3*, and 1988–1997 for *Cf4*). From these combined annual datasets, for workers and soldiers, weights followed sinusoidal seasonal function (*lm*, *sin*). However, nymph weight followed a different seasonal pattern, progressively increasing from May to January, but then plateauing or decreasing from January to May as they eventually go through imaginal molt. A cubic polynomial function was therefore applied (*lm*, *poly3*) on the combined nymph datasets (best fit).

To investigate the demographic changes during critical transitions, additional parameters were produced. The standard deviation (=heterogeneity of age cohorts within a given trap) associated the average worker weight values (obtained from five subsamples of 10 workers) was scatter-plotted with the average worker weight, and 95% ellipses were generated with *ggplot2*, using pre-transition, transition, and post-transition values as factors of the ellipses for visual representation. This analysis allowed us to determine if there was a strong heterogeneity of worker weight among subsamples during a demographic transition, implying a potential colony fusion event, a territorial takeover from a neighboring colony, or a long hiatus in oviposition activity. Finally, when comparing two different time periods, data from each period were independently pooled and compared using *t*-tests or ANOVA, depending on the number of factors.

Notes About Data Interpretation

In this paper, our ability to interpret demographic events from the dataset fundamentally relies on the established literature about *Coptotermes* foraging ability (Shimizu, 1962; Nakajima et al., 1963; King and Spink, 1969; Su and Scheffrahn, 1988a; Grace et al., 1995), on the recent advancements in our understanding of *Coptotermes* biology at the colony level (Messenger et al., 2005; Chouvenc and Su, 2014; Du et al., 2016, 2017; Su et al., 2017; Kakkar et al., 2018; Chouvenc, 2019) and on our direct observation of large mature colonies reared in our laboratory for a decade (Chouvenc, 2022). However, to our knowledge, the dataset provided in this study is beyond the timescale and the population scale of any previous study on any social insects. The complexity of the demographic variation of colonies over time maintains degrees of challenges on potential interpretative scenarios. In addition, the data acquired through field traps are reflecting a demographic fraction of the foraging populations at the time of collection. These data represent a temporary window within the life of termite colonies and remains mostly fragmentary. Therefore, there is an inherent speculative approach to result interpretation in the current study. However, they are all rooted in our own experience of rearing subterranean laboratory colonies to maturity (Chouvenc, 2022), and in the extensive literature about *Coptotermes* foraging ability in the field and in the laboratory. Ironically, a voluminous section of such literature was produced over several decades by using the termites referred to in the current study (virtually everything published from Su and colleagues between 1986 and 2009).

RESULTS AND DISCUSSION

Sampling Metadata

Sampling frequency varied greatly among sites and across time. A summary of the overall data acquired for each of the four sites provides an overview of the sampling effort and the resulting termite activity profile of each surveyed site (**Table 1**).

Demographic Observations Over Time

Each site displayed a unique demographic profile in the population structure of foraging termites over time. Such observations provide a novel understanding about age demographic succession within single colonies, as the average age or developmental status directly depends on the mortality and natality rates of individuals. The following section provides comments on the observations made for each site, which reflects on how colonies display unique demographic profiles over time. However, the sites are described in order of interpretative complexity, where the information provided from the first site informs aspects of the second site, etc. Finally, the interpretation of demographic changes is provided linearly through the chronology of events for each colony, so it is highly recommended to refer to the figures when indicated throughout the following sections, as the visual representation of the data is indispensable to follow our interpretations.

Site *Cf1*, a Simple Perennial Trajectory With Final Senescence

This site apparently contained a single termite colony during the time of observation (1988–1997). The worker average weight (**Figure 2A**) was relatively stable between 1988 and 1993, with noticeable seasonal fluctuations that followed a sinusoidal function ($F_{2,307} = 90.73$, $p < 0.001$, $R^2 = 0.372$) (**Figure 2B**). Such seasonality was previously noticed in *Coptotermes* (Waller and La Fage, 1987a) where workers tend to be bigger in winter months than in spring-summer months. In 1993–1994, a drop in worker's weight reflected a relatively progressive change in the age structure of foragers, with the presence of many younger workers, potentially indicating an increase of either egg production from primary reproductives, an addition of secondary reproductives, and/or the progressive death of many old individuals, resulting in an overall rejuvenation of the population. However, starting in 1995, the colony progressively accumulated old workers until 1997 when the colony finally collapsed and died (as a result, monitoring was terminated), supporting the observations by Grace et al. (1995) and Chouvenc (2018) that senescent colonies primarily contain old individuals. Such observation reveals that reproduction stopped relatively suddenly, with the putative death of the queen(s) in late 1994, as it was previously observed that in absence of reproduction, *Coptotermes* workers and soldiers can survive for 3–4 years in laboratory and then die of old age (Chouvenc et al., 2013b). Alternatively, queens may stop laying eggs several months prior to their death (Bess 1970), implying that reproduction may have stopped while the presence of the live queen could have temporarily inhibited the production of secondary reproductives (Costa-Leonardo et al., 2004; Chouvenc and Su, 2014), although the socio-physiological processes involved in the emergence of replacement or supplementary reproduction in *Coptotermes* remain poorly understood.

The weight profiles of soldiers followed closely the ones from workers throughout the observation (**Figures 2A,C**), including some degree of seasonal fluctuations ($F_{2,307} = 21.41$, $p < 0.001$, $R^2 = 0.122$) (**Figure 2D**) but less pronounced than the one observed in workers. Notably, the magnitude of the final increase of weight in soldiers after 1995–1997 was not as marked as the one observed in workers [worker average weight/soldier average weight ratio before January 1995 $= 1.04 \pm 0.06$ ($n = 69$), after January 1995 $= 1.21 \pm 0.04$ ($n = 13$), $t = 8.82$, $df = 80$, $p < 0.001$]. This reflects that as the termites were aging in the absence of population renewal during colony senescence, W_5 and higher worker instar accumulated in the colony and as soldier replacement is presumably limited to pathways initiating from W_3 maximum (Chouvenc and Su, 2014), the production of "bigger" soldiers was not possible through W_4 and higher. Therefore, toward the end of colony senescence, the production of soldiers may be terminated by default (absence of $< W_5$), preventing the ∼1:1 average weight ratio between the two castes observed throughout the rest of the senescence of the termite colony, because of the progressive accumulation of old workers. Nymphs were produced by the colony at the *Cf1* site between 1988 and 1995 (**Figure 2E**), confirming that by the time the observation started, the colony already reached maturity and was at least 5 years-old (Chouvenc and Su, 2014). Nymph weight followed a seasonality, with relatively small nymphs being observed at foraging sites around May of each year. These nymphs progressively increased their weight as they went through successive molts to mature, until reaching a plateau between Jan and May of the following year pattern (**Figure 2F**, $F_{3,128} = 26.37$, $p < 0.001$, $R^2 = 0.382$) as they eventually turned into alates and exited the colony via dispersal flights (April-June). Our observation confirms that in *Coptotermes* mature colonies, nymphs can be found at foraging sites year-round (Albino and Costa-Leonardo, 2011). Remarkably, nymphs were absent from the colony from July 1995 until the death of the colony in 1997. This nymph absence highlights that with the lack of reproduction starting around late 1994, the brood that initiated nymphal development in 1994 was able to proceed to maturity into imago in 1995. However, the subsequent absence of eggs and larvae in 1995 shut down possibilities for the nymphal pathway to occur in the following years (Raina, 2006), ultimately resulting in a nymphless colony throughout senescence.

A strong positive relationship (*lm*, $F_{1,620} = 879.0$, $p < 0.001$, $R^2 = 0.586$) (**Figure 3A**) was found between the weight of worker and soldier throughout the period of observation. A weaker relationship was found between workers' weight and nymphs' weight (*lm*, $F_{1,258} = 151.1$, $p < 0.001$, $R^2 = 0.369$) (**Figure 3A**). This discrepancy can be explained by the fact that soldiers are produced through the pool of available workers and both their developmental status are inherently tied. In addition, the changes of their demographic representation at foraging site partially depends on a sinusoidal seasonal fluctuation, while being continuously produced throughout

TABLE 1 | Summary of the trap sampling metadata.

Site	*Cf1*	*Cf2*	*Cf3*	*Cf4*
Number of trap records used	622 trap records	525 trap records	854 trap records	1,066 trap records
Number of sampling events	82	104	203	191
Duration of survey	10 years	11 years	22 years	24 years
Time frame	1988–1997	1986–1996	1988–2009	1986–2009
Total number of Trap locations at site	17 traps	16 traps	15 traps	18 traps
Active traps at any time (min-max)	7.58 ± 3.25 traps (2–14)	5.05 ± 2.10 (1–10)	4.21 ± 2.46 (1–12)	5.58 ± 2.61 (1–13)
Time between each trap collection	35.44 ± 25.22 days	45.00 ± 37.08 days	46.00 ± 27.00 days	42.01 ± 24.41 days
Long pause in sampling	None	None	219 days in 1989, 335 days in 1998	308 days in 1986, 279 days in 1997, 441 days in 1999
Wood consumption (from x number of traps with data)	55.90 kg (from 380 traps)	72.06 kg (from 356 traps)	102.17 kg (from 658 traps)	156.99 kg (from 801 traps)
Consumption rate (g of wood/trap/day	3.90 ± 2.65 g	6.70 ± 3.79 g	3.73 ± 2.70 g	5.05 ± 3.09 g
Number of workers collected	1,203,945	1,768,573	2,346,084	2,785,858
Number of soldiers collected	103,626	86,824	164,748	203,303
Number of nymphs collected	9,283	10,354	70,275	69,236
Number of Supplementary Reproductives collected	8	11	1,284	505
Number of alates collected	1,070	8	2,940	14,208
Average number of individual collected per month	11,168	15,944	9,982	10,278
Notes	Simple perennial trajectory with final senescence	Perennial trajectory with a reproductive shift	Reproductive shifts and long-term inbreeding	Complex demography, rapid senescence, territorial replacement, perennity, reproductive shift to inbreeding

the year through overlapping generations. On the contrary, nymphs are initiated yearly during a limited period of time and then progressively matured throughout the following year. During this time, many larvae that would have contributed to the production of workers are instead initiating a nymphal developmental pathway, reducing the pool of individuals that would otherwise sustain the production of workers and soldiers. Therefore, nymph initial production ($L_2 \rightarrow N_1$) may directly contribute to a temporary increase in the average age of workers in the following months while nymphs continue to mature. It is therefore possible that the seasonality fluctuations in the workers age demographic are partially the result of temporary larval investment into nymphal production and not just from environmental fluctuations (Waller and La Fage, 1987b).

In addition, *Coptotermes* late instar nymphs were suggested to be a non-dependent caste because of their ability to directly feed on wood at foraging sites (Crossland and Su, 2006; Albino and Costa-Leonardo, 2011). We here also argue that their presence at foraging site is an efficient strategy for the colony to limit the cost of the inherent nutritional requirements for alate production, by bringing the developing nymphs directly to where the resources are located. Some of the relatively young nymphs show up in May at foraging sites at 6–9 mg, already as advanced nymphal instars. It implies that the initial development of nymphs most likely starts much earlier, safe around the central area of the nest, while later instar nymphs move to foraging sites. Therefore, it establishes that the developmental process from N_1 to alate takes much more than a year to complete, implying that the alate production cycles for two consecutive years largely overlap.

Finally, only 8 secondary reproductives were haphazardly collected as singletons at site *Cf1* between 1989 and 1994. Their presence was not visibly associated with any demographic events but their relative rarity cannot exclude that other secondary reproductives may have been present in the colony at the central part of the nest or other non-monitored foraging sites. Notably, no secondary reproductive was found in the colony after 1995 when the colony accumulated extremely large worker and soldier individuals (**Figures 2A,E**, **3A**), reinforcing the argument that in the absence of larvae, the nymphal pathway necessary to produce secondary reproductive is shut to the senescent colony, sealing its final demise (Nakajima et al., 1963; Lenz et al., 1988; Myles, 1999; Raina, 2006; Chouvenc and Su, 2014).

Site *Cf2*, a Perennial Trajectory With a Reproductive Shift

This site was monitored for 11 years, from 1986 to 1996 (**Figure 4A**), but sampling events between 1986 and 1996 were irregular, providing a relatively poor resolution in workers' demographic changes. However, in 1991–1992, old workers progressively accumulated in the colony, followed by a sudden rejuvenation of the worker pool in 1993 (here referred as "transition A"). Following this transition, a drastic drop in worker's weight was observed until 1996, with weak sinusoidal seasonal patterns (**Figure 4B**, $F_{2,201} = 4.89$, $p = 0.008$, $R^2 = 0.047$). As seen in *Cf1*, the soldier weight closely followed

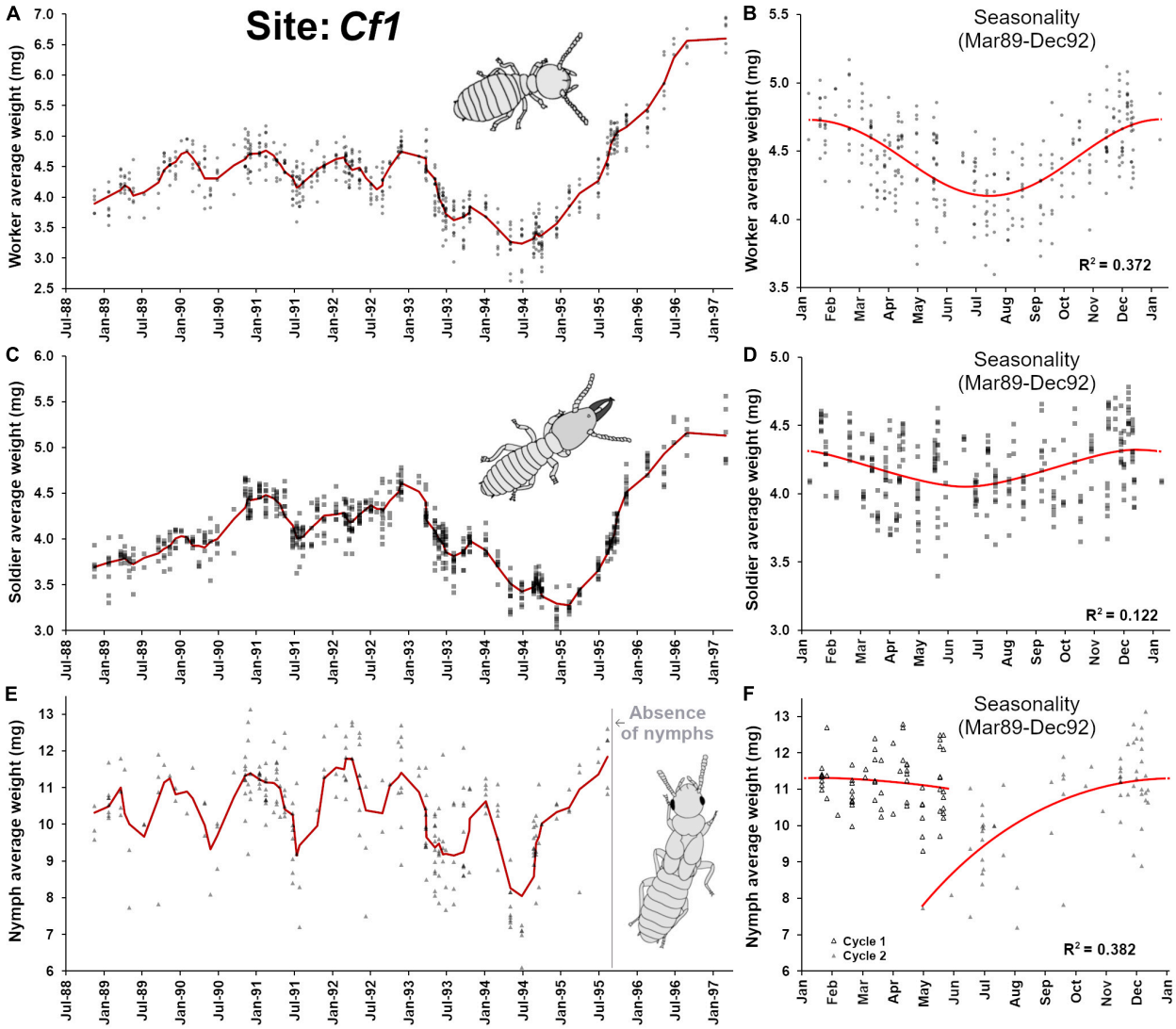

FIGURE 2 | Colony demographic changes for *Coptotermes formosanus* at site *Cf1*. Each data point represents an average caste weight from a field trap. For the 1988–1997 demographic trends of each caste, the red line represents the rolling average function (period = 3) **(A)** Workers' average weight between 1988 and 1997. **(B)** Workers' average weight seasonality, following an annual sinusoidal function ($F_{2,307} = 90.73$, $p < 0.001$, $R^2 = 0.372$). **(C)** Soldiers' average weight between 1988 and 1997. **(D)** Soldiers' average weight seasonality, following an annual sinusoidal function ($F_{2,307} = 21.41$, $p < 0.001$, $R^2 = 0.122$). **(E)** Nymphs' average weight between 1988 and 1997. **(F)** Nymphs' average weight seasonality, with an annual growth that follows a polynomial function (*poly3*, $F_{3,128} = 26.37$, $p < 0.001$, $R^2 = 0.382$). Note the absence of nymphs at foraging sites after the 1995 dispersal event and throughout the colony senescence.

the weight variation observed in workers (**Figure 4C**), although an apparent delay of ∼3 months can be seen in variation patterns. Again, this reflects that soldier production and replacement are driven by the worker pool available at the time of individual workers initiating soldier differentiation (Park and Raina, 2003; Liu et al., 2005; Chouvenc and Su, 2014), for which additional time is needed for the full presoldier-soldier differentiation. Contrary to workers, the seasonality of the change in soldier weight was strongly marked (**Figure 4D**, $F_{2,201} = 161.4$, $p < 0.001$, $R^2 = 0.616$). Nymphs were haphazardly collected from traps from 1987 to 1992, with a notable absence of nymphs throughout 1991, but their presence could then reliably be confirmed after transition A (**Figure 4E**), with a distinct seasonal production (**Figure 4F**, $F_{3,115} = 87.2$, $p < 0.001$, $R^2 = 0.695$), as observed in *Cf1*. Finally, similar as *Cf1*, the trends of relationships between weight of workers and the weight and soldiers ($R^2 = 0.654$) and of nymphs ($R^2 = 0.167$). It confirms that larvae are engaging in the nymphal developmental pathway within a narrow window of time and mature over the following year, while worker and soldier developments are tied and partially depend on the pool of larvae available at the time of their differentiation. Note that contrary to *Cf1*, no final senescence and colony death was observed in *Cf2*, and nymphs were associated even in traps with relatively old workers (**Figure 3B**).

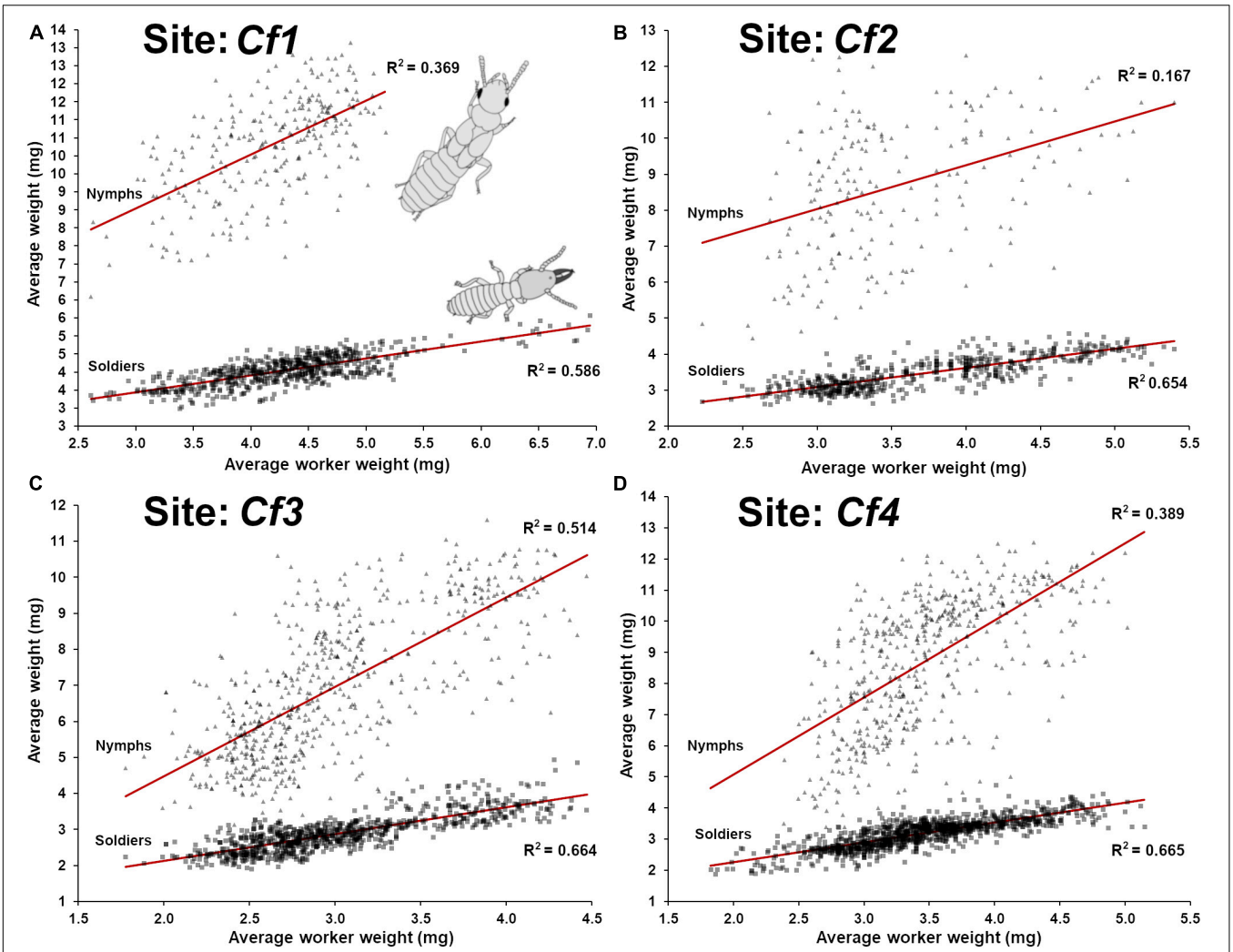

FIGURE 3 | Relationship between the average weight of workers and the average weight of soldiers and nymphs in field traps throughout the time of a site observation. **(A)** Site *Cf1* (1988–1997), worker-soldier (*lm*, $F_{1,620} = 879.0$, $p < 0.001$, $R^2 = 0.586$) and worker-nymph (*lm*, $F_{1,258} = 151.1$, $p < 0.001$, $R^2 = 0.369$) relationships. **(B)** Site *Cf2* (1986–1996), worker-soldier (*lm*, $F_{1,523} = 989.6$, $p < 0.001$, $R^2 = 0.654$) and worker-nymph (*lm*, $F_{1,197} = 38.6$, $p < 0.001$, $R^2 = 0.167$) relationships. **(C)** Site *Cf3* (1988–2009), worker-soldier (*lm*, $F_{1,852} = 1{,}687$, $p < 0.001$, $R^2 = 0.664$) and worker-nymph (*lm*, $F_{1,611} = 646.7$, $p < 0.001$, $R^2 = 0.514$) relationships. **(D)** Site *Cf4* (1986–2009), worker-soldier (*lm*, $F_{1,1064} = 2{,}116$, $p < 0.001$, $R^2 = 0.665$) and worker-nymph (*lm*, $F_{1,508} = 323.2$, $p < 0.001$, $R^2 = 0.389$) relationships.

Two additional notable observations were made: one during transition A and one during the final sampling event. First, the within-trap worker average weight heterogeneity during demographic transition A (January 1993 – September 1993) was higher (0.256 ± 0.131a, $n = 36$) than the heterogeneity prior (November 1988 – December 1992) to the transition (0.181 ± 0.100b, $n = 187$, $p < 0.01$) and the heterogeneity after (October 1993 – August 1996) the transition (0.145 ± 0.071b, $n = 203$, $p < 0.01$) (ANOVA, $F_{2,423} = 24.82$, $p < 0.0001$, Tukey HSD, different letters in text indicate $\alpha = 0.05$ significant difference). Transition A followed a period where older workers and larger soldiers accumulated, with a temporary absence of nymphs in 1991 but a resumption of the presence of nymphs at the foraging sites in 1992 was observed. Combined, these observations imply that the termite colony may have gone through a temporary senescence, with a putative absence or reduction of oviposition for almost a year (starting in late 1990). During transition A, groups of foragers composed primarily of both old and young individuals but with few middle-aged individuals could be observed (high weight heterogeneity). Such situation may be explained by a relatively sudden resumption of oviposition within the colony through the presence of secondary reproductive (three of them were found in 1993, while none of them were collected prior to transition A). An alternative scenario remains possible, where the aging colony at *Cf2* fused with a younger neighboring colony (Su and Scheffrahn, 1988b; Vargo and Husseneder, 2009; Husseneder et al., 2012; Lee et al., 2019; Vargo, 2019), resulting in a rapid drop in worker and soldier

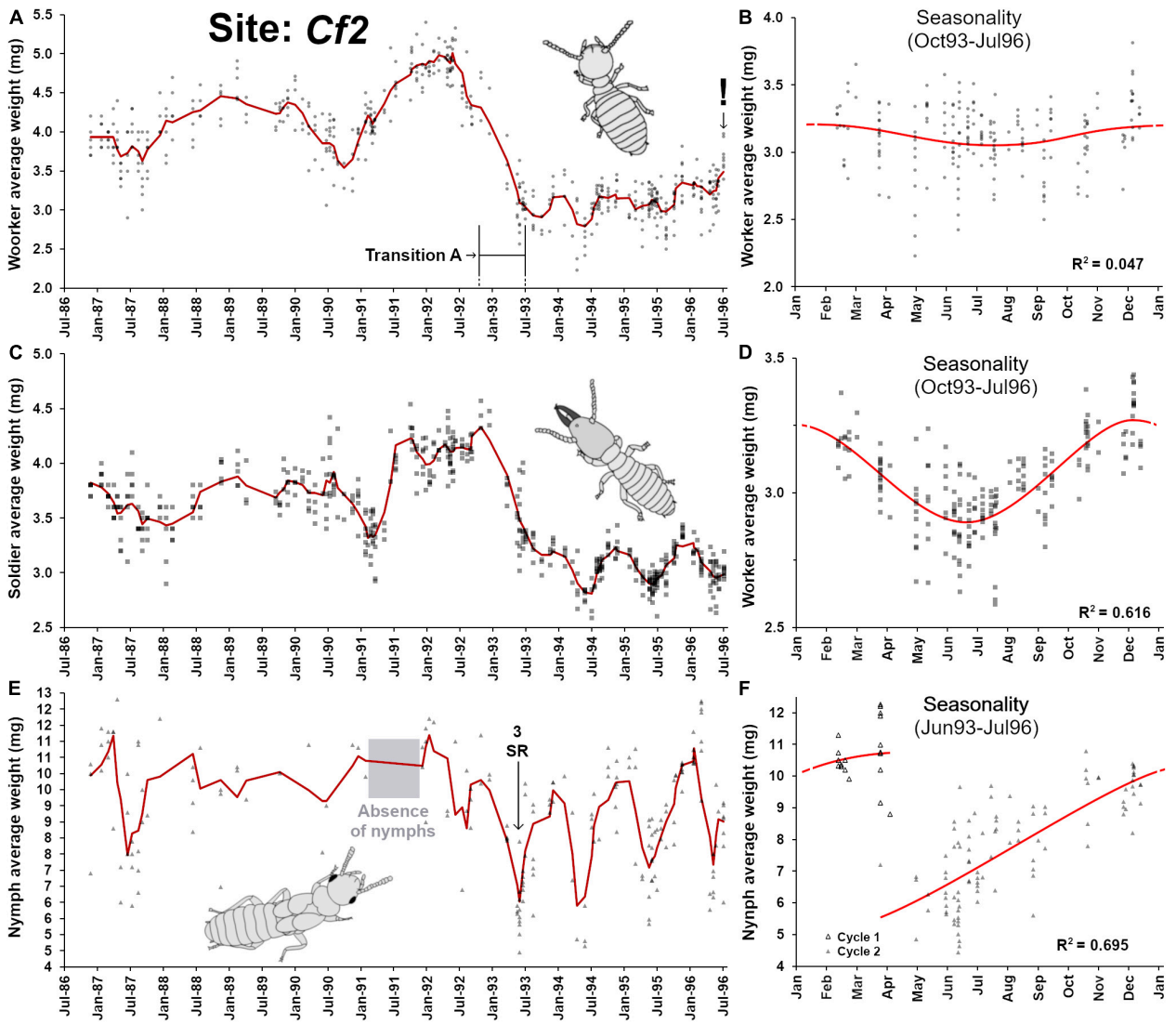

FIGURE 4 | Colony demographic changes for *Coptotermes formosanus* at site *Cf2*. Each data point represents an average caste weight from a field trap. For the 1986–1996 demographic trends of each caste, the red line represents the rolling average function (period = 3). **(A)** Workers' average weight between 1986 and 1996. **(B)** Workers' average weight seasonality, following a weak annual sinusoidal function ($F_{2,201} = 4.89$, $p = 0.008$, $R^2 = 0.047$). **(C)** Soldiers' average weight between 1986 and 1996. **(D)** Soldiers' average weight seasonality, following an annual sinusoidal function ($F_{2,201} = 161.4$, $p < 0.001$, $R^2 = 0.616$). **(E)** Nymphs' average weight between 1986 and 1996. **(F)** Nymphs' average weight seasonality, with an annual growth that follows a polynomial function (*poly3*, $F_{3,115} = 87.2$, $p < 0.001$, $R^2 = 0.695$). "Transition A" represents a major reproductive shift within the colony, with a hiatus in reproduction, followed by the re-establishment reproduction via secondary reproductives (=SR).

weights. However, the resumption of the presence of nymphs prior to transition A may make this alternative scenario unlikely, but not mutually exclusive.

The second notable observation relates to a written note found in the records by PMB on August 1st 1996 saying "Sentricon baits found on site, termite collection terminated." This note was important, as it indicated that the owner of the property had commercial subterranean termite hexaflumuron bait stations (Su, 1994) installed, and that the site could no longer serve as a source for termite experiments in the laboratory. Unfortunately, the opportunity to observe the accelerated senescence of a CSI-baited colonies in the field with the rapid accumulation of old workers (Chouvenc, 2018; Gordon et al., 2022) was missed. However, the last sampling event still revealed the initial colony demographic shift that would be expected with the loss of the brood and young workers (Chouvenc and Lee, 2021), with a sudden rise of the soldier ratios, from an average of 4.26 ± 3.6% ($n = 28$) in the three months before the implementation of the CSI baits, to 32.44 ± 13.47% ($n = 8$) on August 1st 1996 (t-test, $df = 34$, $t = 10.18$, $p < 0.001$), and with the sudden increase in workers' weight from an average of 3.25 ± 0.21 mg ($n = 28$) in the three months before the implementation of the CSI baits, to 3.68 ± 0.20 mg ($n = 8$) on August 1st 1996 (t-test, $df = 34$, $t = 5.29$, $p < 0.001$). Therefore, despite the lack

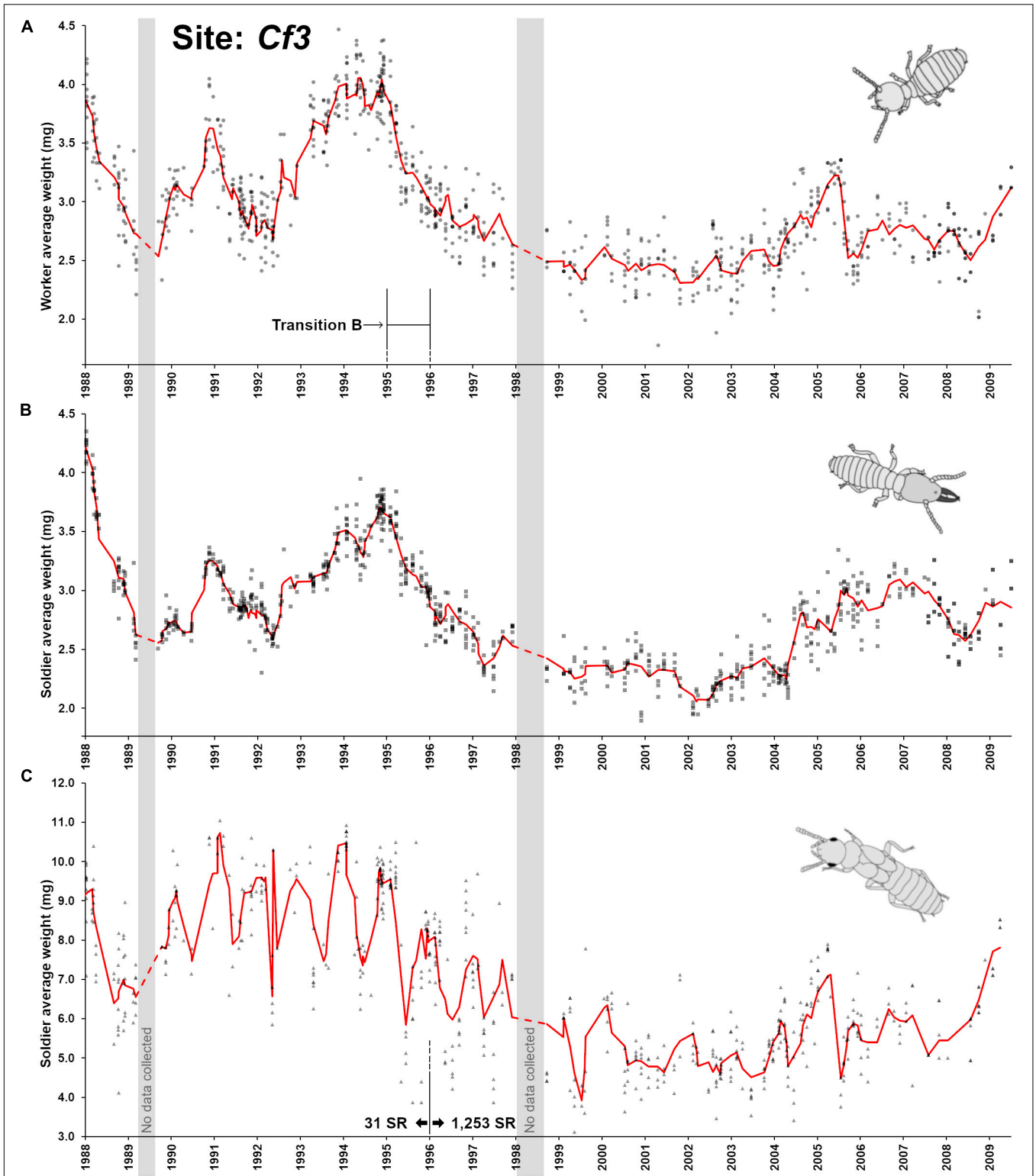

FIGURE 5 | Colony demographic changes for *Coptotermes formosanus* at site *Cf3*. Each data point represents an average caste weight from a field trap. For the 1988–2009 demographic trends of each caste, the red line represents the rolling average function (period = 3). **(A)** Workers' average weight between. **(B)** Soldiers' average weight. **(C)** Nymphs' average weight between. "Transition B" represents a major reproductive shift within the colony with the ongoing production of secondary reproductives (=SR, 1,253 produced after 1995) which may have resulted in cycles of inbreeding. No clear annual demographic cycles could be found in workers and soldiers.

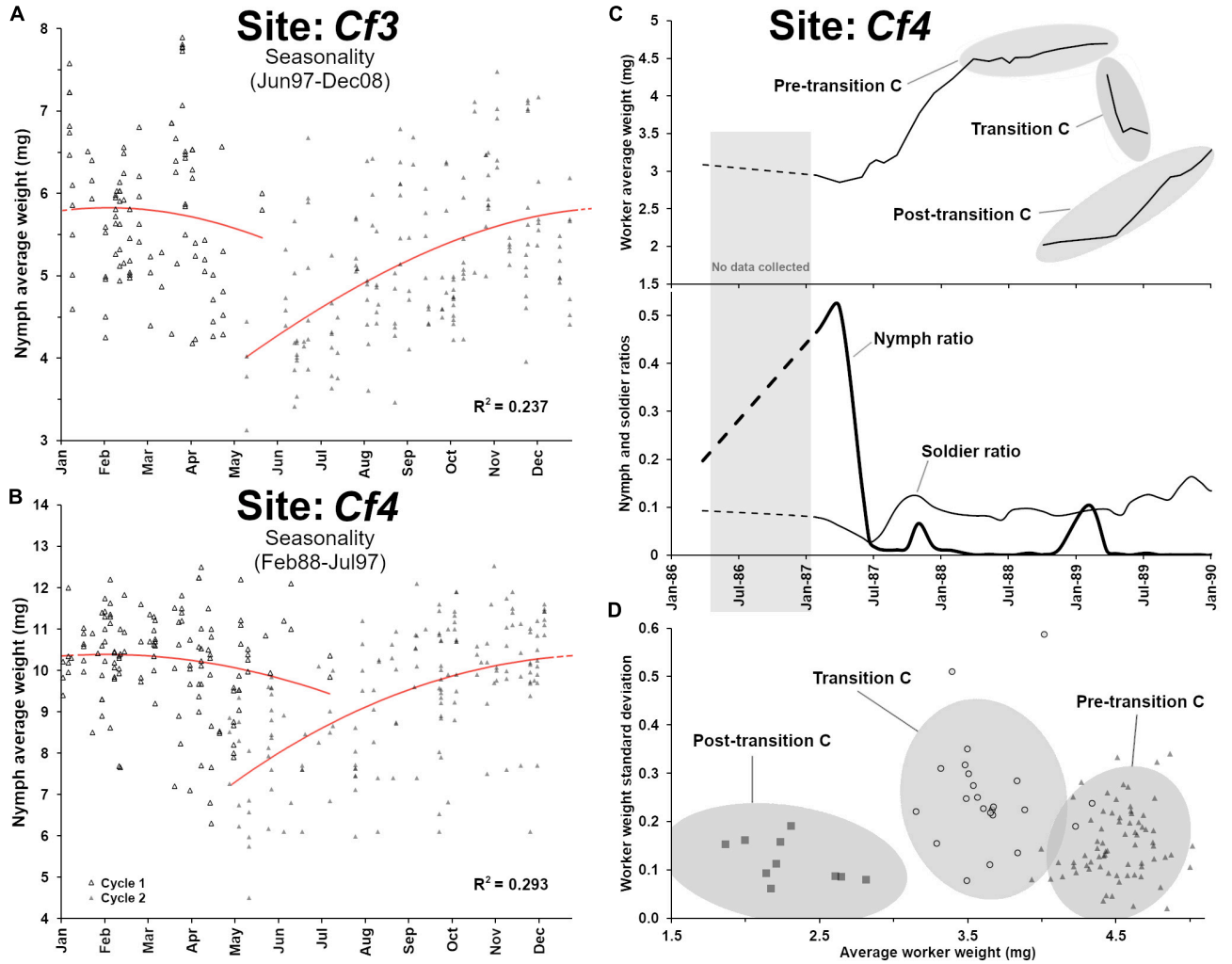

FIGURE 6 | Additional demographic analyses from site *Cf3* and site *Cf4*. **(A)** Nymphs' average weight seasonality from *Cf3*, with an annual growth that follows a polynomial function (*poly3*, $F_{3,263} = 27.3$, $p < 0.001$, $R^2 = 0.237$). **(B)** Nymphs' average weight seasonality *Cf4*, with an annual growth that follows a polynomial function (*poly3*, $F_{3,302} = 41.8$, $p < 0.001$, $R^2 = 0.293$). **(C)** Details of the demographic "transition C" from *Cf4*, showing a high nymph ratio event prior to the senescence of the colony (pre-transition C). **(D)** Weight heterogeneity among worker groups (with 95% ellipses) during transition C, (heterogeneity = 0.25 ± 0.11a) was higher than pre-transition C (0.15 ± 0.07b) and post-transition C (0.11 ± 0.05b) (ANOVA, Tukey *post hoc*, $\alpha = 0.05$), suggesting a temporary colony fusion process with a young neighboring colony, during the colony senescence of the initial colony at site *Cf4*.

of final observation of colony collapse when exposed to CSI baits (Su, 1994, 2019), all the early signs of accelerated colony senescence were confirmed.

Site *Cf3*, Successional Reproductive Shifts and Long-Term Inbreeding

Site *Cf3* differed from the two previous sites on many aspects. First, the time frame of observation was over 22 years (1988–2009), giving a long-term perspective of termite colony activity at this site. Second, the demographic of the colony at this site went through a major shift during a 1995–1996 transition (here referred as "transition B"). Finally, no seasonality was found in worker and soldier weight throughout the observation (**Figure 3C**). Despite the visual compression of demographic changes from the long observation period (**Figure 5A**), a remarkable series of aging/rejuvenating cycles in the worker pool was visible. As the colony observation was initiated in 1988–1989, it was already going through a rapid decline in workers' age demographic, as it was going through a phase of increased oviposition, phasing out an old cohort of workers. Such a pattern indicates that the colony had already reached maturity for several years, as confirmed by the already established presence of nymphs. Then, a slowdown of oviposition resulted in aging cohorts of workers in late 1990. Another rejuvenation period occurred in 1991, followed by another period of reduced oviposition with an accumulation of aging cohorts of workers through 1994. Then, this cycle came to an end with a fundamental

shift observed in 1995 ("transition B"), with a prolonged period of high oviposition that lasted for more than 15 years, resulting in a semi-permanent state of a young worker demographic at foraging sites throughout *Cf3*. The aging/rejuvenation cycle resumed briefly in 2005 and in 2009, with a temporary accumulation of relatively old workers. The fate of the colony is unknown, as collection was terminated in 2009. Soldier colony demographics followed the patterns observed in workers over the years (**Figure 5B**). Contrary to *Cf1* and *Cf2*, no seasonality fluctuations could be observed in *Cf3* workers and soldiers (sinusoidal functions, $p < 0.41$) throughout the 22 years monitoring period, suggesting that various other factors (environmental and internal) may have influenced population demographics of this colony beyond seasonality and nymph production cycles, as observed in *Cf1* and *Cf2*.

Nymphs were produced periodically each year (**Figure 5C**) with a marked seasonality in their maturation cycle (**Figure 6A**, $F_{3,263} = 27.3$, $p < 0.001$, $R^2 = 0.237$). However, nymph demographics displayed two distinct weight patterns (*t*-test, $t = 24.95$, $df = 611$, $p < 0.001$) from before transition B (8.69 ± 1.4 mg, $n = 231$) and after transition B (5.87 ± 1.3 mg, $n = 382$), as nymphs produced after the transition were much smaller (**Figure 5C**). Alates produced by *C. formosanus* can vary in size depending on the timing of dispersal within the season and the colony of origin (Chouvenc et al., 2017), but the current observation also indicates that it may depend on the colony breeding structure, as reflected in the small nymph weight after transition B. The change in breeding structure was marked by the number of secondary reproductives found in the colony over time: while 31 supplementary reproductives were haphazardly collected in field traps (12 out of 412 traps) at the *Cf3* site between 1988 and 1996, a total of 1,253 supplementary reproductives were collected between 1996 and 2009 (188 out of the 442 traps in this period). The large number of secondary reproductives was associated with relatively small workers, soldiers and nymphs. In addition, secondary reproductive were also much smaller after transition B, from averages of 5.43 ± 0.76 mg, $n = 12$ prior to the transition, to an average of 3.99 ± 0.44 mg, $n = 188$ after the transition, ($t = 10.38$, $df = 198$, $p < 0.001$). Reproductive functionality and sex of these secondary reproductives were not reported in the records, but *Coptotermes* mature colony may accumulate both functional and non-functional brachypterous neotenics (Myles, 1999; Costa-Leonardo et al., 2022). While the relatively small weight of workers and soldiers can partially be explained by the high reproductive rate of the colony from the large number of supplementary queens in the colony after 1996, the small weight of nymphs cannot. Instead, we suggest that transition B led to a long cycle of inbreeding within the colony (Vargo, 2019), resulting in developmental limitations for all castes, with smaller morphs produced as proposed by Husseneder et al. (2005). This observation in *Cf3* also highlights that over time, colony demographic traits and breeding structure can be altered to extremes, as a colony may resort to various reproductive strategies throughout its lifetime, to ultimately optimize its fitness while being constrained by its ongoing demographic status.

Site *Cf4*, a Succession of Complex Demographic Shifts

This last site was observed for the longest period of time (1986–2009) and displayed the most complex demographic changes when compared with the three previous sites, with a potential succession of established colonies over time. However, the information obtained from *Cf1*, *Cf2,* and *Cf3* sites provided us with some key elements that guided our interpretation of the demographic changes over time at the *Cf4* site (**Figure 3D**, **Figure 7**). The first obvious observation is that the *C. formosanus* population at this site went through two major demographic transitions, one around 1989 (referred as "transition C") and another one around 2002 (referred as "transition D"). As explained below, site *Cf4* may actually reflect the succession of two distinct colonies and one dramatic breeding structure shift over the decades. Worker age demography in 1986 appeared to be relatively young but a rapid accumulation of old workers occurred in 1988 (**Figure 7A**), with a similar trend for soldiers (**Figure 7B**). Remarkably, the colony produced a large number of small nymphs in 1987 (**Figures 6C**, **7C**) with an average of more than 50% of nymphs in traps. This event happened just prior the rapid accumulation of old workers. Out of the 15 foraging traps collected between January and April 1987, 12 of them had > 40% nymphs, and 4 among these traps had > 90% of nymphs. This aberrant nymph ratio was never observed again at any point in time across all four sites. The large accumulation of nymphs and the rapid aging of the worker cohort both suggests that colony suddenly lost its reproductive ability and that most individuals were getting ready to "jump the ship" with most (if not all) larvae differentiating into nymphs instead of workers and soldiers. Such 'last swarm' strategy was not previously reported in *Coptotermes* (Osbrink et al., 2016; Chouvenc, 2020) but was observed in other lower termite species when resources are exhausted, or when reproduction is terminated, as most competent individuals turned into alates in a last fitness boost attempt (Gulmahamad, 2002; Korb and Lenz, 2004). However, out of season dispersal flight events of *Coptotermes* have been reported for colonies in the process of being baited by a CSI formulation (Su, 1994), as a possible analogous last-ditch effort to disperse one last time. In the case of *Cf4*, as most larvae engaged in nymph differentiation in early 1987, it directly competed with the renewal of the worker and soldier populations, contributing to the rapid to aging cohorts of the sterile castes. No secondary reproductives were found between 1987 and 1989, supporting a complete absence of reproduction at this point.

While the 1987–1989 cascading demographic events should have led to the final senescence of the colony by 1990, as observed in *Cf1*, something else happened for *Cf4* in 1988 that prevented such observation. In late 1988, a single trap at site *Cf4* suddenly switched workers and soldiers demography, from $\sim$ 4.5 mg individuals to $\sim$ 2.0 mg individuals, between two consecutive sampling events (**Figure 6C**), confirming that a neighboring colony with a relatively young demography took over this foraging trap. Then, during the 1989 transition C event, the majority of foraging traps temporarily displayed highly heterogenous worker age groups (**Figure 6D**), implying that the

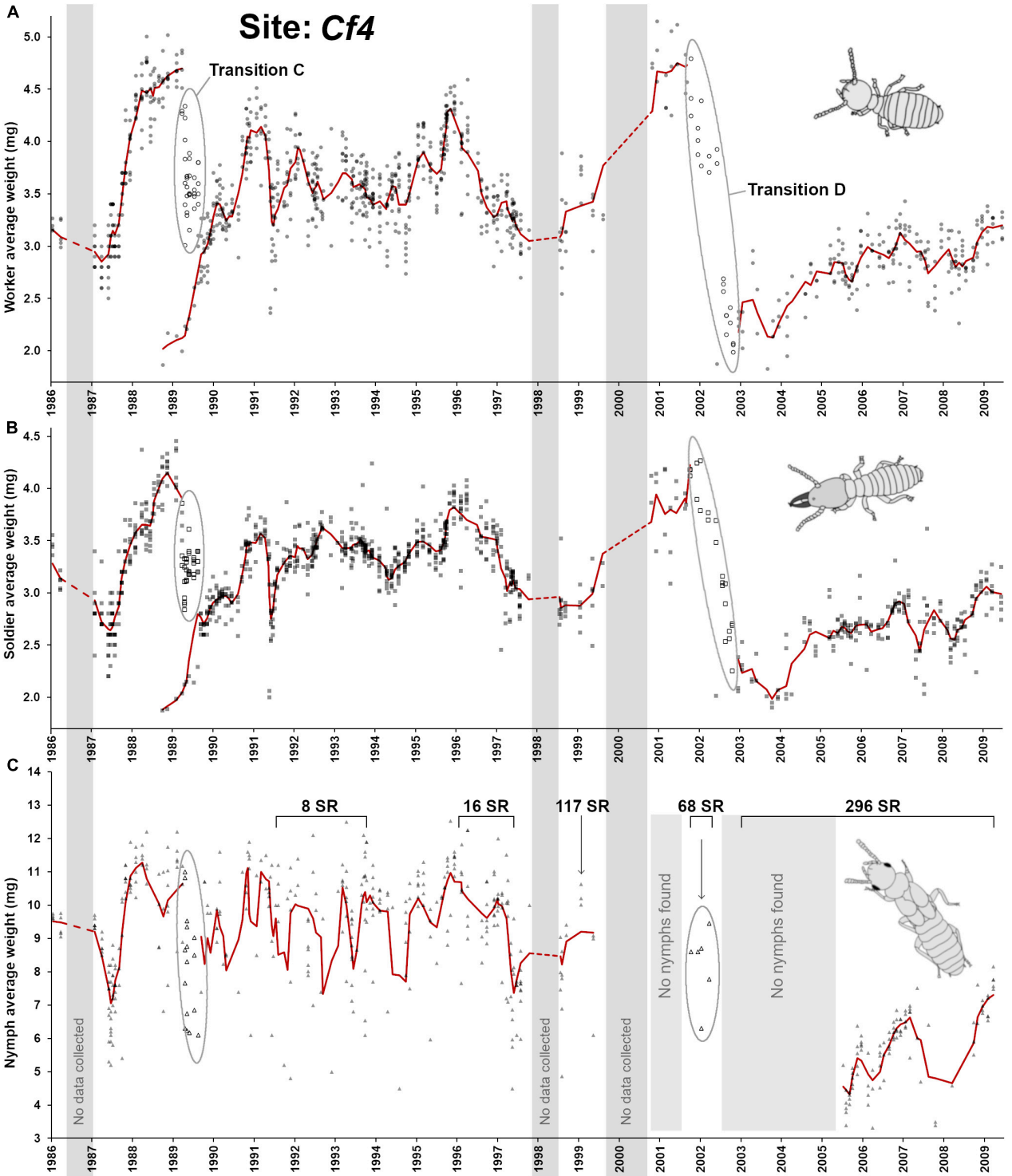

FIGURE 7 | Colony demographic changes for *Coptotermes formosanus* at site *Cf4*. Each data point represents an average caste weight from a field trap. For the 1986–2009 demographic trends of each caste, the red line represents the rolling average function (period = 3). **(A)** Workers' average weight. **(B)** Soldiers' average weight. **(C)** Nymphs' average weight. "Transition C" represents colony senescence and simultaneous colony fusion with a young neighboring colony. "Transition D" represents a generational demographic turnover followed by putative cycles of inbreeding associated with secondary reproductives = SR), and a temporary absence of nymph production prior to colony "re-maturation" (2003–2005).

two colonies were in the process of fusion (Su and Scheffrahn, 1988b), with a mix of old and young workers, leading to an intermediate average worker weight with a relatively high standard deviation within each trap. This mixed population was temporary, as all workers and soldiers from the initial colony eventually died of old age, leaving all traps with a uniformly "young" population during the post-transition C from the newly established colony (**Figure 6D**, ANOVA, *post hoc* $\alpha = 0.05$). Therefore, the transition C event provides a remarkable example of a weakening senescent colony that was simultaneously being taken over by a relatively young neighboring colony (Husseneder et al., 2005; Messenger and Su, 2005; Messenger et al., 2005; Lee and Su, 2011; Bernard et al., 2017), which involved a temporary colony take-over process (Lee et al., 2019).

From 1990 to 1996, the newly established colony went through various cycles of aging/rejuvenating cohorts of workers and soldiers (**Figures 7A,B**), similar to what was observed at previous sites, with a cyclical production of nymphs (**Figure 6B**, *poly3*, $F_{3,302} = 41.8$, $p < 0.001$, $R^2 = 0.293$). However, although some nymphs were found in 1989-1991 in the newly established colony, their numbers were very few when found (often < 10 nymphs per trap at best, with most traps displaying no nymphs). The number of nymphs found then increased substantially starting in 1992. The initial small weight of workers and the reduced number of nymphs therefore confirmed that the colony was relatively young and in the process of maturing (Chouvenc and Su, 2014) during and after transition C. Between 1990–1995 only 8 secondary reproductives were haphazardly collected, putatively as supplementary reproduction to the primary queen and king. However, a demographic shift occurred after a rise of the workers' weight in 1995 suggesting the potential loss of the primary reproductives, or a long pause in oviposition. Supporting such interpretation is the accumulation of secondary reproductives in 1996 (16 collected that year), which resulted in a drop of the weight of soldiers and workers for two consecutive years (until 1998), as oviposition resumed.

The succession of events during 1998–2000 is unclear because of fragmentary sampling. However, key elements allow us to propose a solution to this puzzle. First, the weight of soldiers and workers increased continuously between 1998 to 2001, indicating a potential colony senescence. Second, there was a complete absence of nymphs between 1999 and 2004 also supporting ongoing senescence of the colony. Finally, 117 secondary reproductives were collected in a single trap in late 1998, and 68 secondary reproductives in early 2002, potentially indicating an attempt to resume oviposition. Interestingly, a few nymphs (<200 total) were produced and found in traps in early 2002 (transition D), at the same time as the 68 secondary reproductives, confirming that at least a small egg production occurred in the previous year and the oviposition resumption attempt was not a complete failure. Although a de- novo nymph production was proposed (Raina et al., 2004), i.e., workers differentiating into tertiary reproductives (Thorne, 1996), this scenario remains unlikely in *Coptotermes* (Lenz et al., 1988; Myles, 1999; Chouvenc and Su, 2014). With transition D, oviposition restarted, which resulted in the rapid rejuvenation of the worker pool, as old workers also died of senescence. However, the small worker/soldier weight and the lack of nymphs from 2003 to 2005 could instead imply that transition D reflected the taking over by a new juvenile neighboring colony, as observed in transition C, which would then finally mature with the first production of small nymphs toward alates for the 2006 season. Several elements however indicate that this alternative interpretation is unlikely. First, during 2002–2009, 296 additional secondary reproductives were found throughout this period, including in 2003–2005 when no nymph could be found. The development of functional secondary reproductives is often not possible in young *Coptotermes* colonies (Lenz and Runko, 1993; Costa-Leonardo et al., 2004; Chouvenc et al., 2015b). Second, just like we found in *Cf3*, the weight of secondary reproductives dropped dramatically after the transition D, from an of average 5.45 ± 1.46 mg, $n = 24$, to an average of 3.38 ± 0.80 mg, $n = 77$ ($t = 8.91$, $df = 99$, $p < 0.001$). Third, contrary to the newly established colony after transition C, which rapidly accumulated workers and soldiers between 3.5 to 4.5 mg (in < 2 years), sterile castes after transition D remained relatively small (2.0–3.0 mg) for > 6 years of observation. Fourth, nymphs were particularly small after transition D, with a shift from 9.29 ± 1.86 mg, $n = 418$, to an average of $5.65 \pm 0.1.25$ mg, $n = 101$ ($t = 18.69$, $df = 517$, $p < 0.001$). Finally, while wood consumption and the number of termites collected were not analyzed in this study because of the high variability over time and because it will be the focus of a future study, wood consumption rate at *Cf4* changed notably after transition D, from 5.92 ± 3.12 g/d/trap, $n = 579$, to 2.78 ± 1.37 g/d/trap, $n = 222$ after the transition ($t = 14.49$, $df = 799$, $p < 0.001$), suggesting a potential reduced efficiency in wood foraging. All of these combined observations support that the colony was undergoing cycles of inbreeding (Husseneder et al., 2005) from secondary reproductives that accumulated since the early 2000s, as we previously concluded for site *Cf3* after transition B. For *Cf4*, transition D resulted in a colony that most likely lost a large portion of its population, with a 2002 temporary senescence. Then, a delayed resumption of reproduction occurred through cycles of inbreeding. Without a critical mass of individuals, we here suggest that it temporarily lost its ability to produce nymphs (2003–2005) as the colony lost its "mature" status by temporarily displaying the profile of a juvenile colony (Chouvenc and Su, 2014), which it then regained in 2006 as the colony grew back to a critical mass of individuals and started producing small nymphs and alates again (=colony "re-maturation"), until 2009 when the monitoring ended.

CONCLUSION

A subterranean termite colony is a complex biological entity that must thrive through various challenges once it has reached maturity (production of alates). Oster and Wilson (1978) provided a theoretical framework on how eusocial colonies may achieve degrees of perennity once they have completed their ergonomic growth and reached maturity, by investing differentially in different castes over time and adjusting colony functions depending on colony internal and external conditions over many years of activity. However, the current study indicates

that colonies may go through dramatic demographic changes and breeding structure shifts, even many years after they have reached maturity, to maintain perennity and maximize fitness output. In a way, throughout its life, a colony displays its own demographic individuality, as the life of a colony is marked by internal and external events that drives its growth, its foraging ability, its competitiveness, its age demography composition, its senescence and ultimately, its death.

In this study, the single colony at site *Cf1* reflected a simple perennial trajectory, as the colony sustained annual production of alates for at least a decade and went through cyclical seasonal variations of worker, soldier and nymph age structures. Eventually it lost reproductive abilities, and entered senescence with the accumulation of old individuals, lost colony essential functions, and ultimately died within 3 years. Site *Cf2* introduced the concept of a temporary senescence within the life of a termite colony, as oviposition may be temporarily halted or highly reduced, which could have led to colony death. However, secondary reproductives were successfully produced which resulted in a rejuvenation of the population, phasing out old cohorts of sterile helpers, while resuming nymph production. Site *Cf3* then showed that a subterranean colony can maintain its foraging activity and alate production for more than two decades. It also showed that the large accumulation of secondary reproductives, after the putative loss of primary reproduction and temporary senescence, can lead to rapid colony rejuvenation in conjunction with extensive cycles of colony inbreeding, resulting in the production of relatively small individuals across castes. Finally, site *Cf4* displayed the most complex and dramatic colony demographic shifts, which involved a massive nymph production prior to a rapid colony senescence, in conjunction with a young neighboring colony take-over. The new colony then went through a decade-long perennial productivity before losing reproduction, which led to a critical attempt to resume oviposition with many secondary reproductives, almost fail to do so, but eventually managed to survive through cycles of inbreeding after losing a critical mass of old individuals. It temporarily lost its maturity status for a few years by demographically behaving like a juvenile colony, then eventually resumed nymph production toward a return to a perennial mature colony functioning. This study also provides new insights on the colony senescence process in subterranean termite field colonies. Colony senescence is not always straightforward from a demographic perspective and this study provides diverse scenarios where final colony collapse and death may sometimes be avoided by establishing secondary reproduction, which depending on the internal context, can be a chaotic transition and may not always be successful. Alternatively, colony senescence and collapse can be accelerated when neighboring colonies take over the foraging territory of the dying colony.

Regardless of the demographic changes and reproductive outcomes over the many years of the life of a mature colony, this study highlights that each colony may attempt different strategy to ultimately optimize its fitness. *Cf1* went through a quiet death after many years of alate production. *Cf2* was able to establish a stable colony productivity after a temporary loss of reproduction. *Cf3* avoided colony senescence by massively producing secondary reproductives and imposing cycles of inbreeding, which still resulted in a large production of alates that would disperse and engage in sexual reproduction, potentially negating the temporary effects of inbreeding. Finally, on *Cf4*, the initial colony went through an alate production hail Mary, the second established colony took over and engaged in a perennial reproductivity, then narrowly avoided colony collapse by finally establishing secondary reproduction successfully.

To conclude, this study provides novel elements on how subterranean colonies function over time and revealed that each colony demographic trajectory is unique through their perennity. It also showed that a single sampling event of a colony only provides limited information, which lack the complex historical context of what was the demographic status before and after such single data snapshot. The dataset used in this study allowed us to reframe such demographic context over decades of observation. Yet, this analysis represents a fraction of the comprehensive dataset collected by PMB, which remains to be further curated and analyzed in future studies for a set of specific questions. Finally, while we acknowledge the inherent speculative nature of data interpretation in this study, owing from the uniqueness of the dataset, all interpretations were carefully considered and deemed as the most likely scenarios, when taking all of the available evidence. However, we encourage future researchers that may be skeptical of such interpretations, to subject them to further testing, and confirm or refute them. Regardless, we hope this study can be used, either way, as a building block toward a better understanding of social insect colony demographic complexity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

N-YS initiated the field plots and established the initial termite collection protocol. PMB coordinated the data collection. TC performed the data curation, data analysis, figure production, and first manuscript writing. All authors read and approved the final manuscript, except PMB, whose co-author status is a tribute to his essential contribution to the field of termite research.

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José F. Fontanari,
University of São Paulo, Brazil

REVIEWED BY
Ervin Humprey Duran-Bautista,
University of the Amazon, Colombia
Caroline Franco,
University of Oxford, United Kingdom

*CORRESPONDENCE
Alberto Arab
albertoarab@gmail.com

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Termites are the main dung removals in a degraded landscape in Brazil

Frederico R. Alves[1], Daniel Aguilera-Olivares [2], Mauricio Martins Rocha[3] and Alberto Arab [4]*

[1]Programa de Pós-Graduação em Ciências Ambientais, Universidade Federal de Alfenas, Alfenas, Brazil, [2]Departamento de Silvicultura, Universidad de Concepción, Concepción, Chile, [3]Independent Researcher, São Paulo, Brazil, [4]Centro de Ciências Naturais e Humanas, Universidade Federal do ABC (UFABC), Santo André, Brazil

Termites are one of the most relevant groups for recycling nutrients and keeping the flow of energy in ecosystems. Although their role as lignocellulose decomposers is the focus of studies, they also act as dung recyclers, but their importance in this process is poorly understood. Here we performed manipulation experiments to determine dung removal by termites in forest remnants and cattle pastures in a fragmented Atlantic Forest landscape. We used wire bags of different mesh sizes placed along transects in three forest fragments and pastures for 10 days to compare the contribution of termites and other coprophagous macrodetritivores to dung removal. Our results indicated that termites removed more dung in pastures than in the forest fragments. In addition, dung beetle exclusion significantly reduced the percentage of dung removal within forest fragments, but not on pastures, indicating termites are important dung recyclers in pastures.

KEYWORDS

Isoptera, forest fragmentation, cattle dung, Atlantic forest, beetles, ecological roles

Introduction

Most natural environments have been currently modified by human population growth causing different degrees of habitat loss (Fahrig, 2019). This loss in turn impacts biodiversity and causes the disruption of several ecological processes (Veldkamp et al., 2020). Due to a growing human population, cattle production has increased significantly over the years, which contributed about 12–18% of total greenhouse gas emissions, mainly through gut fermentation and excreta. In addition, livestock dung is also involved in eutrophication and acidification of natural ecosystems (Cai et al., 2021).

Termites (Blattodea) are ecosystem engineers, modulating the balance of carbon between the soil and the atmosphere through lignocellulose decomposition mediated by symbiotic microorganisms (Brune, 2014). The construction of tunnel networks and nests by these insects alters the physical and chemical properties of the soil through bioturbation and deposition of organic material, promoting growth and modifying the

community structure of plants in the surrounding soils (Jouquet et al., 2011; Ashton et al., 2019; Griffiths et al., 2021; Myer et al., 2021). Basal groups of termites (called *lower termites*) are primarily xylophagous and rely primarily on flagellate protozoans for lignocellulose digestion (Brune, 2014). However, the evolutionary success of the Termitidae (the most diverse and numerous family of termites, called *higher termites*) is attributed to the loss of protozoans and the acquisition of specialized lignocellulolytic bacterial lineages that allowed diet diversification, including wood, grass, soil, litter, lichen, and fungi (Bourguignon et al., 2011; Brune and Dietrich, 2015). In tropical environments, termites are the main macrodetritivores, decomposing half of deadwood in rainforests and more than 30% of the litter in savannas (Veldhuis et al., 2017; Griffiths et al., 2019; Sundsdal et al., 2020).

Dung removal is an important ecological function provided by some macrodetritivores that benefits to the environment through the mobilization of carbon and nitrogen into the soil, reduction of greenhouse gasses and ammonia (Cai et al., 2021), and fly pest suppression (Nichols et al., 2008; Sitters et al., 2014). Dung removal have been usually attributed to dung beetles (Scarabeinae) (Nichols et al., 2008); however, several termite species are also known to feed on a wide range of herbivore mammal dung; the majority of these termites belong to the grass and litter feeding guilds (Freymann et al., 2008) and are likely attracted to the fibrous plant material, nitrogen, and water content of feces (Anderson and Coe, 1974; Freymann et al., 2008).

Brazil is one of the largest producers of livestock in the world (OECD and FAO, 2021). Expanding cattle production requires additional land and water, in addition to increasing excrement output. The destruction of forests through the expansion of grazing land for livestock is one of the main drivers of decreased biodiversity (Sano et al., 2008; Freitas et al., 2010). The loss of coprophagous organisms, particularly termites and dung beetles (Braga et al., 2012; Cancello et al., 2014), may bring further issues to the environment due to their key role in removing dung and the other indirect ecosystem services they exert that were listed above (Nichols et al., 2008; Cai et al., 2021). However, there is no information on how habitat degradation simultaneously affects these organisms and their ecological role in dung removal. In this study, we established a manipulative experiment to quantify and compare the relative contribution of dung removal by termites and other coprophagous macrodetritivores to pastures and forest fragments in an Atlantic Forest landscape.

Materials and methods

Study site

We conducted this study in the rural areas of Alfenas (21°25′45″ S; 45°56′50″ W), a municipality in the southern part of the Brazilian state of Minas Gerais (**Supplementary Table 1**). This region was originally covered by Atlantic Forest but agricultural development resulted in a largely fragmented landscape (**Figure 1**). The Atlantic Forest is considered a biodiversity hotspot due to its high diversity and endemism (Myers et al., 2000). However, it is a domain severely fragmented and threatened with less than 28% of the original areas remaining (Rezende et al., 2018). Experiments were conducted during January–March 2014 in three forest fragments and three deforested areas converted in cattle pastures. These sites were selected using digital image processing of the satellite Sino-Brazilian CBERS-2B with a resolution of 20 m following two criteria: (i) Similarity of spectral attributes such as color and texture and (ii) the presence of natural cover forest in a circular buffer of 1 km radius from the center of selected sites. Forest fragments contains semi decidual vegetation (Carneiro et al., 2014) while the pastures, dominated by *Brachiaria* grasses were not exposed to cattle grazing to avoid interference with dung removal experiments. Sampling did not involve any endangered species and the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), a Brazilian Ministry of the Environment's enforcement agency, provided authorization for termite sampling (SISBIO n° 33269). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Termites associated with cattle dung

Fresh dairy (115 samples) and dry-cow dung pats (183 samples) were collected at cattle grazing pastures 1 km apart from the experimental areas. Termites were carefully removed using forceps and brought to the lab where they were identified to the lowest possible taxon.

Dung removal

We set up this experiment using circular arenas of 60 cm in diameter limited by a fence of nylon with 20 cm in height to avoid dung transportation outside the arenas. In the study site, we found that, apart from termites, the most abundant coprophagous macrodetritivores were dung beetles (Alves, 2015). To assess dung removal by termites, we used 3 mm wire mesh to produce "only termite accessible dung bags" because termite species found in cattle dung have a body width of 1.52 ± 0.39 mm (mean ± SD) (Araujo, 1970; Coles De Negret and Redford, 1982; Redford, 1984; Krishna et al., 2013). Because the smallest dung beetle species found in this study have body width of 3.47 ± 0.34 mm (N = 24) (**Supplementary Table 2**), 3 mm dung bags allow termites to feed on the dung, effectively excluding dung beetles (Howison et al., 2016). Each bag was filled with 200 g of a mixture of

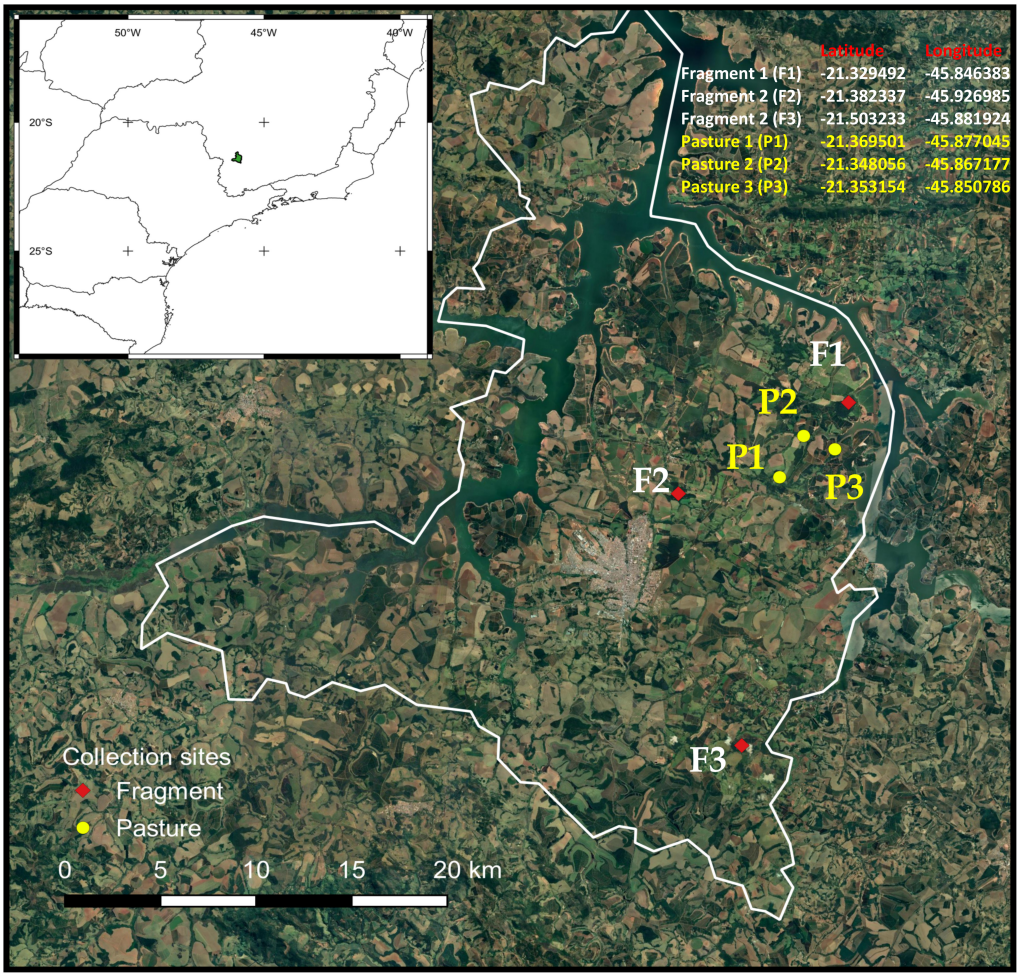

FIGURE 1
Map of study site and experimental design. Dung removal was evaluated in the Alfenas region of the southeastern Brazil, in the state of Minas Gerais.

herbivore (cow) and omnivore (pig) dung, obtained from local farmers and placed at the center of the arenas. Both cow and pig dung are effective attractants to a wide range of coprophagous organisms dependent on the dung of different mammal feeding guilds (Nichols et al., 2008). Two additional experiments with "dung bags" of 1 mm (macrodetritivores inaccessible) and 20 mm (macrodetritivores accessible) wire mesh were used in combination with "only termite accessible dung bags" to evaluate the contribution of all coprophagous macrodetritivores to dung removal (**Table 1**).

Each sample was protected from rain by covering it with a 30 cm diameter plastic plate placed 15 cm above it, as a roof. The arenas were placed along three 120 m transects at each habitat type. Transects were placed at the center of the site and perpendicular to one of the borders. In each transect, we randomly deployed 12 arenas (four for each treatment) separated by 10 m. We had a total of 216 arenas (forest $n = 108$, pastures $n = 108$) in 18 transects across two habitat types in six sites (**Figure 1**). Dung removal was measured after 10 days. Only samples with termite or dung beetle activity (presence of these insects, termite galleries, dung beetle tunnels) were considered in the analyses. At the start of the experiment, we estimated the moisture content of twenty dung subsamples by determining their fresh and dry weight and used this value to calculate the initial dry biomass and the relative weight loss of each sample, following to Sitters et al. (2014). Dry weights were obtained oven drying fresh dung samples at 60°C for 24 h. Dung removal was calculated as a percentage of dry weight loss after 10 days for each experimental treatment. Termites and dung beetles were carefully removed from dung samples in the bags and identified to the lowest possible taxon using taxonomic keys (Vaz-De-Mello et al., 2011; Rocha et al., 2017) and comparison with a reference collection from the Museu de Zoologia da Universidade de São Paulo (MZUSP) and the Invertebrate Ecology and Conservation Laboratory, at the Universidade Federal de Lavras (UFLA), Brazil, respectively.

TABLE 1 Richness and overall abundance of dung beetles and termites for the different treatments and habitat types.

Accessibility treatments	Dung bag mesh size	Symbol	Habitat	Richness (abundance)	
				Dung beetles	Termites
Macrodetritivore inaccessible	1 mm		Forest	0	0
			Pasture	0	0
Only termite accessible	3 mm		Forest	0	6 (213)
			Pasture	0	4 (36)
Macrodetritivore accessible	20 mm		Forest	26 (291)	1 (10)
			Pasture	32 (664)	2 (7)

Statistical analyses

All the analysis and graphics were generated using R version 3.4.4 (R Core Team, 2019). We examined differences in richness between pastures and forest fragments through individual-based accumulation curves (Chao et al., 2014) using de iNEXT package (Hsieh et al., 2016). A permutational multivariate analysis of variance (PERMANOVA) was used to evaluate differences of termites and dung beetle community composition between habitats (Anderson, 2001). Singletons and doubletons were excluded from the analyses. We fitted beta regressions models with a logit link function using the *betareg* function (Cribari-Neto and Zeileis, 2010) to test for differences of the percentage of dung removal between habitat types and the experimental treatments. Explanatory variables to affect the outcome were the type of habitat and macrodetritivore accessibility. We carried out least square mean analyses for multiple comparisons to evaluated the effect of model predictors using the *emmeans* function of the lsmeans package (Lenth, 2016). The significant threshold was $P < 0.05$.

Results

Dung samples in the 3 mm mesh bags were colonized by termites and micro arthropods (mites and springtails), while samples in the 20 mm bags also attracted dung beetles. Dung samples in the 1 mm bags were colonized only by micro-arthropods. Simultaneous colonization by termites and dung beetles was observed in only 12 bags. We collected eight species of termites from the dung bags. *Ruptitermes reconditus* (Apicotermitinae) was found in both pastures and forest fragments; however, it was the most abundant species in forest fragments). Three species were found exclusively in forest fragments and one in the pastures. In the areas with cattle activity, we found seven species of termites in 36.6% of the dry dung pats, with a higher incidence of *R. reconditus* and *Procornitermes araujoi* (Syntermitinae) (**Supplementary Table 3**). Fresh wet dung pats were not colonized by termites. On the other hand, 40 dung beetle species were collected. *Uroxys* sp. (Ateuchini) and *Dichotomius bos* (Coprini) were the most abundant species in forest fragments and pastures, respectively (**Supplementary Table 2**).

Our results indicated that richness (**Figures 2A,B**) and species composition of termite and dung beetles were not affected by habitat loss (Termites: $F = 0.43$; $P = 0.50$; Dung-beetles: $F = 4.63$; $P = 0.10$; PERMANOVA), but the abundance of these insects on pastures decreased (**Figures 2C–F**). Dung removal has also been impacted by reduction of forest cover and macrodetritivore suppression. For instance, exclusion of dung beetles significantly reduced the percentage of dung removal within forest fragments ($Z = -17.78$; $P < 0.001$) but not on pastures ($Z = -0.89$; $P = 0.375$). On the other hand, termites removed more dung from pastures than from forest fragments ($Z = 6.33$; $P < 0.001$). Finally, dung samples in the macrodetritivore-inaccessible bags showed a non-significant weight reduction ($Z = 1.29$; $P = 0.198$) (**Supplementary Tables 4, 5** and **Figure 3**).

Discussion

In this study, we demonstrate that termites play an important role in dung removal, particularly in pasture areas, where these insects were the main dung removals. Savannah termites are more tolerant to habitat perturbation due to their cryptic lifestyle, their thermoregulated nests or by their habit of foraging at night when temperature and humidity are suitable for these insects (Coles De Negret and Redford, 1982; Korb, 2003). In this study, the absence of tree cover excluded forest-restricted dung beetle species as found in other ecosystems (Halffter and Arellano, 2002). Although the experimental setup did not allow us to quantify the dung removal by dung beetles alone, the exclusion of these insects significantly reduced dung removal within the forest fragments, but had no effect on pastures. It is possible that as dung beetle abundance decreases, termites assume a similar role in the ecosystem, thereby mitigating the effects of habitat loss on dung beetle abundance. These results support that the functional role of termites in removal of mammalian dung is widely

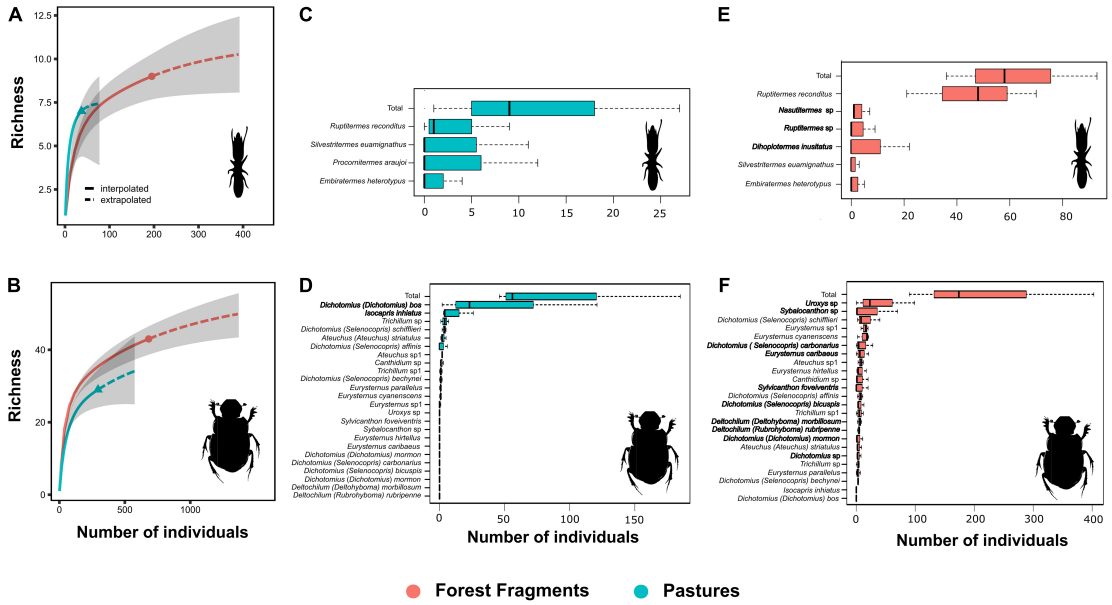

FIGURE 2
(A,B) Individual-based species accumulation curves across forest fragments and pastures. Curves were plotted based on data grouped across all sites. The solid line shows predictions based on interpolation and the dashed part shows predictions based on extrapolation. Ninety-five percent confidence intervals are shown as shaded areas. **(C–F)** Species abundance for pastures (blue boxes) and forest fragments (red boxes). Boxplots report median, upper and lower quartiles, and maximum and minimum values. Habitat specialists are marked in bold.

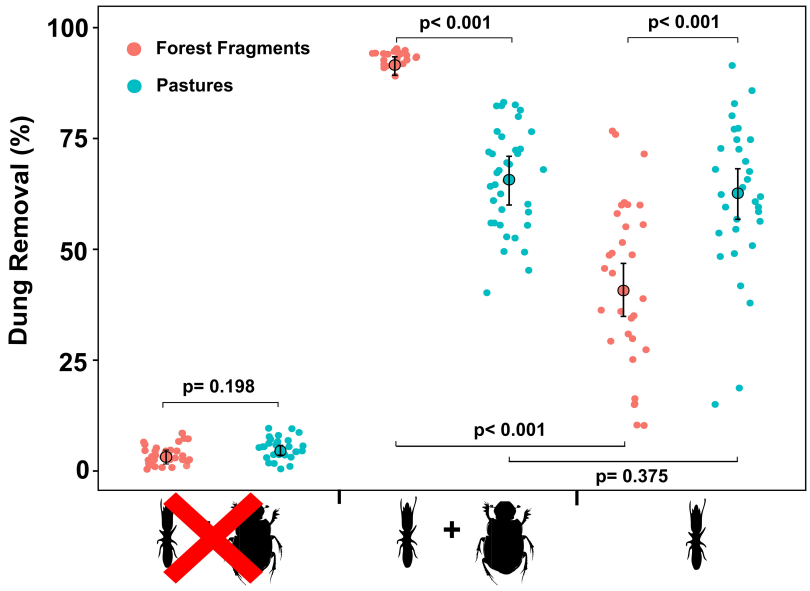

FIGURE 3
Percentage of dung removal among different experimental treatments (see **Table 1** for description) in the two habitat types. The plot shows de modeled mean (GLMM) represented by large circles, the modeled 95% confidence intervals of each mean (error bars), and the model-adjusted individual response values, represented by the small dots and the *P*-values of the least square mean analyses for pairwise comparisons.

underestimated (Freymann et al., 2008), especially in disturbed habitats.

Savannas and pastures in Brazil are populated by a wide variety of termite feeding guilds (Carrijo et al., 2009) and our results showed that termite dung removal was higher on the pastures than in forest fragments, despite the decreasing abundance of these insects in the dung bags in this habitat in comparison with forest fragments. Due to the high temperatures

and low humidity, termites from savannas and pastures forage mainly at night to avoid dehydration. Dung bags were collected during daylight hours, which may explain the lower abundance of termite foragers observed in the pastures. Termites within forest fragments, on the other hand, are naturally protected against desiccation. Finally, in the absence of dung beetles and termites, physical weathering, the activity of micro arthropods and microbes account for only 5% of dung removal.

In the pastures with cattle activity, termites were only found in dry dung pats. This can be explained by the following factors. First, most adult dung beetles feed on fresh dung (Holter and Scholtz, 2007) and termite behavior may change in response to competition from dung beetles. The second possible explanation is the tolerance of termites to climatic conditions in the pastures, which could also allow these insects to use dry dung pats for a longer period. In addition, dung beetles, due to their narrow thermal tolerances, cannot cope with the drastic temperature and humidity changes characteristic of deforested habitats (Barragán et al., 2014; Gómez-Cifuentes et al., 2017); a third possible explanation is fiber, the main component of dry cattle dung (Holter, 2016) that could act as an extra source of grass and litter and thus attract termites; and finally, dung may provide termites with nitrogen to supplement their low-nitrogen diet (Higashi et al., 1992). Although there is no information on the interaction between dung beetle and termites, the small number of samples colonized by both insects suggests that they may be mutually exclusive (Gould et al., 2001).

Although this work is constrained by a relatively short duration and low number of sites, this is the first study to show the relative contribution of termites to dung removal under the influence of habitat degradation. A better understanding of termites' role in dung removal could be achieved by collecting samples at night when termites forage, and more species and individuals could be registered. To further disentangle climatic effects of season, additional experiments of dung removal with a longer duration could be established. Our results support the utility of termites as focal taxa in cattle pastures. The expansion of livestock in Brazil is expected to continue growing over the next decade (OECD and FAO, 2021) leading to increased deforestation and cattle-dung accumulation. Although termites from pastures in Brazil are often considered pests and their nests are removed by local farmers, dung removal by termites in livestock areas could reduce harmful emissions of ammonia and greenhouse gasses. Termite benefits are also linked to nutrient cycling and pedoturbation that help preserve soils (Herrick and Lal, 1996) and conservation of these insects should be a priority.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

AA designed the study. AA and FA performed the experiments. AA, FA, and DA-O analyzed the data. AA, MR, and DA-O wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.982602/full#supplementary-material

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Daniel Aguilera-Olivares,
Universidad de Concepción,
Chile

REVIEWED BY
Juan José Morrone,
National Autonomous University of Mexico,
Mexico
Evandro Marsola Moraes,
Federal University of Sao Carlos, Brazil

*CORRESPONDENCE
Tiago F. Carrijo
tiagocarrijo@gmail.com

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Evolutionary history of *Nasutitermes kemneri* (Termitidae, Nasutitermitinae), a termite from the South American diagonal of open formations

Karolina S. Kohari[1], Valeria Palma-Onetto[2], Rudolf H. Scheffrahn[3], Alexandre Vasconcellos[4], Eliana M. Cancello[5], Rafaella G. Santos[5] and Tiago F. Carrijo[1]*

[1]Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, SP, Brazil, [2]Laboratory of Chemical Ecology, Departamento de Química Ambiental, Facultad de Ciencias, Universidad Católica de la Santísima Concepción, Concepción, Chile, [3]Fort Lauderdale Research and Education Center, University of Florida, Institute of Food and Agricultural Sciences, Davie, FL, United States, [4]Laboratório de Termitologia, Departamento de Sistemática e Ecologia, Universidade Federal da Paraíba, João Pessoa, PB, Brazil, [5]Museu de Zoologia da Universidade de São Paulo, São Paulo, SP, Brazil

Little is known about the phylogeography of termites in the Neotropical region. Here, we explored the genetic patterns and phylogeographical processes in the evolutionary history of *Nasutitermes kemneri,* an endemic termite of the South American diagonal of open formations (DOF) formed by the Chaco, Cerrado, and Caatinga phytogeographic domains. We sampled 60 individuals across the three domains of the DOF, and using the mitochondrial genes 16S, COI, and COII, as well as the nuclear gene ITS, evaluated the genetic diversity and divergence time of the populations, along with their genetic structure. The results show a strong genetic and spatial structure within the samples, evidencing the existence of two well-differentiated genetic groups: the Northeastern and the Southwestern populations, which diverged about 2.5 Mya, during the Pliocene-Pleistocene boundary. The Northeastern population, which encompasses Caatinga and northern portions of Cerrado, has an intricate structure and seems to have suffered repetitive retraction-expansion events due to climactic fluctuations during the Quaternary. The Southwestern population, which ranges from central-south Cerrado to the northeast peripherical portions of the Chaco, displays a star-shaped haplotype structure, indicating that this region may have acted as a refugia during interglacial periods.

KEYWORDS

Isoptera, phylogeography, molecular phylogeny, biogeography, Chaco, Cerrado, Caatinga

1. Introduction

There are more than 3,000 described species of termites (Blattaria: Isoptera) (Krishna et al., 2013), and they are one the most successful terrestrial organisms on Earth. This success is due to a sum of ecologically relevant characteristics. Termites may be poor land dispersers and weak flyers (< 2 km; Hu et al., 2007; Tonini et al., 2013; Mullins et al., 2015), however, transoceanic dispersal events mostly *via* flotsam (Bourguignon et al., 2017) facilitated their near world-wide distribution. Coupled with their ability to decompose cellulosic compounds (Jouquet et al., 2016), these eusocial insects thrived in new ecological opportunities and became keystone species and ecosystem engineers with an exceptional biomass in tropical ecosystems (Tuma et al., 2020).

Despite the extension of insects across the world and being the largest and most diverse group of organisms on Earth (Chapman, 2009), their phylogenetic relationships have only recently become evident due to advances in next generation technology (Trautwein et al., 2012; Yeates et al., 2016). Not much is known about the evolutionary history of species and their populations in South America, which is poorly studied in comparison to North America and Europe (see Tembrock et al., 2019), and what little is known mostly focuses on agricultural pests due to their economic relevance (i.e., Tembrock et al., 2019; Raszick et al., 2021; Vilardi et al., 2021). Phylogeographic studies would help to clarify the evolutionary history of species and their populations through the use of several resources such as population genetics and phylogenetics to understand the geological and climatic processes that determined the current geographic distribution of genealogical lineages (Avise et al., 1987; Avise, 2000).

Little is known about termites' species evolutionary history, with most articles just focusing on species of the Rhinotermitidae family (e.g., Kutnik et al., 2004; Szalanski et al., 2004; Park et al., 2006; Tripodi et al., 2006; Jenkins et al., 2007; Luchetti et al., 2007; Austin et al., 2008; Lefebvre et al., 2008; Li et al., 2009; Scicchitano et al., 2018; Hyseni and Garrick, 2019). Although being the evolutionary most recent and most diverse family, the phylogeography of the Termitidae remains scarcely studied (Ozeki et al., 2007; de Faria Santos et al., 2017, 2022; Singham et al., 2017). Among these, there are only two phylogeographic studies for *Nasutitermes* species: *N. corniger* and *N. ephratae* (de Faria Santos et al., 2017, 2022), up to date. In both species, significant degrees of genetic structure were found across the different domains of the wide Neotropical region. However, another species of the genus, *N. kemneri* (Figure 1), seems to be restricted to a smaller area known as the South American "diagonal of open formations" (DOF) (Mathews, 1977).

The DOF consists of three phytogeographic domains (or biomes) crossing the South America from southwest to northeast: Caatinga, seasonally dry tropical forests of northeastern Brazil; Cerrado, the central Brazilian savanna; and Chaco, seasonally dry subtropical forests in northern Argentina, Paraguay, and Bolivia (Vanzolini, 1963; Zanella, 2010). All these domains are characterized by marked seasonality with severe droughts, nevertheless, they are different physionomically and in vegetation, harboring unique biotas well adapted to such fluctuations (Werneck, 2011).

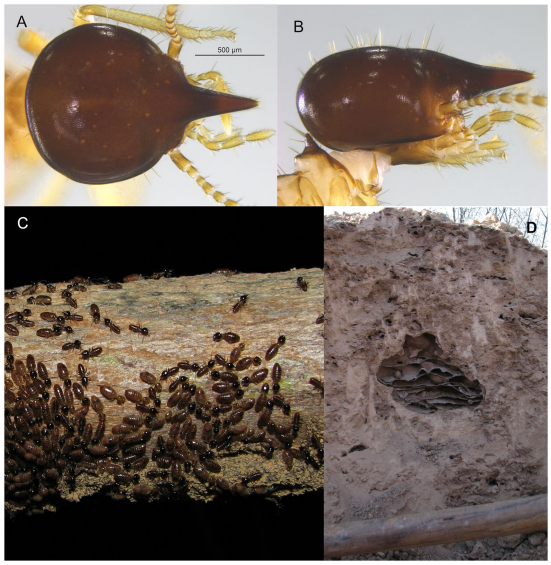

FIGURE 1
Nasutitermes kemneri. Soldier from Bolivia in dorsal **(A)** and lateral **(B)** views. Individuals foraging during twilight on a tree branch **(C)** and its nest inside what looks like an abandoned nest of *Syntermes* sp. **(D)** in the Caatinga phytogeographic domain, São Miguel do Tapuio, PI. As scale in **(D)**, the diameter of the pickaxe handle is approximately 5cm.

The current geographical arrangement of the DOF is the result of the Central Brazilian Plateau uplift since the Cenozoic (Martins-Ferreira et al., 2020), changing the extensions of the Caatinga, Cerrado, and Chaco domains, in addition to the periods of climatic fluctuations during the Quaternary, when the Earth experienced ice ages interspersed with warmer periods. During these fluctuations, dry vegetation would have expanded and contracted, respectively, during glacial maximums and minimums, while tropical forest regions would have followed the reverse pattern (Pinheiro and Monteiro, 2010; Zanella, 2010). Specifically, dry regions were connected during glacial periods, while when interglacial periods occurred, tropical forests expanded and disconnected dry regions (Pennington et al., 2004; Werneck et al., 2012). Consequently, these events not only shaped the current geomorphology of the region, but became important drivers of the diversification and distribution of biota across the DOF.

Nasutitermes kemneri Snyder and Emerson, 1949 is a termite species endemic to the DOF that feeds on wood in early stages of decomposition in a diversity of vegetation types (Mathews, 1977). Due to its well delimited area of distribution, this termite is a relevant study model to understand how species respond to the vegetational, elevational, and climatic shifts between the Caatinga–Cerrado–Chaco phytogeographic domains. Accordingly, this study aims to determine if there are divergences between the populations distributed across the DOF, and if these form a spatial

structure in genetic differentiation. Secondarily, through demographic and time estimation analyses, we tested if populations of *N. kemneri* contracted in size and were disconnected during interglacial periods but survived in climatic refugia. For this purpose, we used both nuclear (ITS) and mitochondrial molecular markers (COI, COII, and16S rRNA).

2. Materials and methods

2.1. Sampling, DNA extraction, and sequencing

A total of 60 individuals of *Nasutitermes kemneri* (Figure 1) were sampled, from Bolivia to Rio Grande do Norte state in Brazil, covering most of the South American DOF, and the known distribution of the species (Supplementary Table S1). Samples studied were from the Museum of Zoology of the University of São Paulo (MZUSP), the Federal University of Paraíba (UFPB), and University of Florida Termite Collection (UFTC).

Genomic DNA was extracted from workers' head and thorax using the DNeasy Blood & Tissue Kit—QIAGEN extraction kit following the manufacturer's instructions. Amplification was performed with the Master Mix (Prodimol) for PCR, where primers used for PCR are detailed in Supplementary Table S2, and cycle settings are specified in references therein. We amplified both mitochondrial and nuclear markers for subsequent analyses: the mitochondrial genes 16S rRNA, and cytochrome oxidase I and II (COI and COII), and the entire ribosomal internal transcribed spacer region (ITS1 + 5.8S + ITS2; Supplementary Tables S1, S2). Samples with confirmed amplification through agarose gel electrophoresis underwent DNA purification using ExoSap (GE Technology Infrastructure). Fragments were sequenced forward and reverse using the BigDye reagent kit (Perkin-Elmer) in an automatic sequencer ABI 3730 XL DNA Analyzer (Applied Biosystems), according to manufacturer's instructions.

We successfully amplified and sequenced 55 individuals, but not throughout all molecular markers, obtaining 55 sequences of 16S rRNA, 25 of COI, 53 of COII and 43 sequences of ITS. All sequences were deposited on GenBank database (access numbers in Supplementary Table S1).

2.2. Population genetics and phylogeography

To understand the genetic structure of *N. kemneri*, we performed various analyses with 16S rRNA, COII and ITS region sequences. We did not use the COI sequences to avoid incorporating many missing data to the matrix alignment.

Median-joining haplotype networks (Bandelt et al., 1999) were reconstructed in the PopART software (Leigh and Bryant, 2015), one for aligned and concatenated 16S rRNA and COII sequences through the MUSCLE algorithm in Geneious v9.1 (Biomatters Ltd., Auckland, New Zealand), and another for the ITS region sequences. Genetic diversity indexes (Hd, π) were estimated in DnaSP 5.0 (Librado and Rozas, 2009).

As ITS marker was highly conserved (only two parsimony informative sites), a Maximum Likelihood (ML) phylogenetic tree was inferred using a final alignment including only the 16S rRNA and COII molecular markers. Model optimization was performed with the R packages "ape" and "phangorn" (Schliep et al., 2018; Paradis and Schliep, 2019). The selected model for the entire alignment was GTR + I + G. The phylogeny was transformed into an ultrametric tree using the chronos function of the package "ape" (Paradis and Schliep, 2019) and plotted in the map using the package "phytools" (Revell, 2012).

To estimate the presence of spatial genetic structure within the sequences of *N. kemneri* we used Bayesian Analysis of Population Structure (BAPS) v6 (Corander et al., 2008) implementing the method of "spatial clustering of individuals," testing up to 8 clusters (populations; $K = 8$). For comparison, we conducted analyses in GENELAND 4.9.2 (Guillot et al., 2008, 2012) using both the uncorrelated and correlated allele frequency models (1.000.000 iterations, 100 thinning), given that the correlated frequency model may be more powerful at detecting subtle differentiations, but is more sensitive to departures from model assumptions (i.e., isolation-by-distance) than the uncorrelated frequency model. Furthermore, an Analysis of Molecular Variance (AMOVA) and Tajima's D neutrality test were performed with the package "pegas" (Paradis, 2010) to evaluate Fst magnitude and significance and detect signals of population expansion or retraction for the genetic groups of *N. kemneri*.

2.3. Dating analysis

To estimate the divergence time between the main populations of *N. kemneri* (Figure 2), we performed a phylogenetic analysis calibrated with fossils. For that, we used three mitochondrial markers (COI, COII, and 16SrRNA) from one individual of each main population, in addition to 32 sequences from GenBank (Supplementary Table S3). A GTR + G + I substitution model for the concatenated matrix was used.

The phylogenetic analysis was carried out under Bayesian inference with BEAST 1.8.0 (Drummond et al., 2012). A Yule speciation process (Gernhard et al., 2008), with a random starting tree, and an uncorrelated lognormal relaxed clock (Drummond et al., 2006), were used as tree priors.

The molecular clock was calibrated using six calibration nodes that were set as monophyletic (Supplementary Table S4). The fossils were used as minimum age constrains and implemented as exponential priors of node time (Supplementary Table S4). Times for most recent common ancestor priors were selected to have an exponential distribution (Ho, 2007), with mean and offset or standard deviation are showed in Supplementary Table S4.

The analysis was performed with Monte Carlo Markov Chain searches conducted for 40,000,000 generations. Convergence and

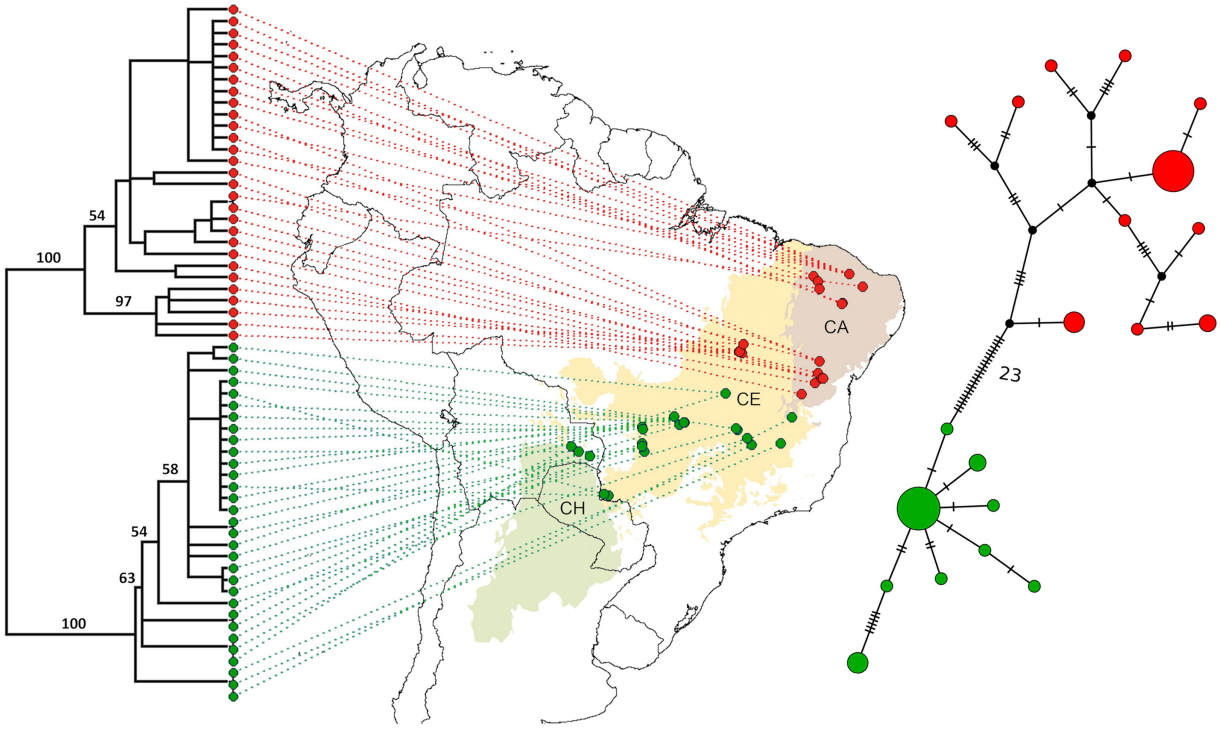

FIGURE 2
The two major groups of *Nasutitermes kemneri*: Northeastern (NE, in red) and Southwestern (SW, in green). In the left, a Maximum Likelihood (ML) phylogenetic tree with corresponding sampling sites, and in the right the haplotype network. Branch support of the phylogeny are bootstrap values; number of substitutions between the two major groups are included in the haplotype network. CH, Chaco; CE, Cerrado; and CA, Caatinga.

stationarity were assessed with Tracer 1.6 (Rambaut et al., 2018) and the first 1,000 trees (10%) were discarded as burn-in with TreeAnnotator 1.8.0 and visualized using FigTree 1.3.1.

3. Results

Sequencing efforts resulted in varying lengths: 321, 640, and 735 bp for 16 rRNA, COII, and ITS, respectively. ITS sequences were highly conserved in the 43 sampled individuals, with only 3 polymorphic sites. On the other hand, 14 polymorphic sites were present in the 55 16S rRNA sequences, 43 polymorphic sites were present in the 55 COII sequences, and the final alignment of 961 bp containing both 16S rRNA and COII genes revealed 53 polymorphic sites.

We used different approaches to estimate the degree of population differentiation. The genealogical relationships reconstructed by the ML phylogenetic tree evidenced high bootstrap support for the two mtDNA genetic groups of *N. kemneri* within the South American DOF, one at the Northeast (NE), and another one at the Southwest (SW; Figure 2).

The Median-Joining haplotype network of the two mitochondrial gene regions (16S rRNA + COII) also revealed the presence of these two groups, and that they are separated by 23 mutations, with no haplotypes in common (Figure 2). A total of 11 haplotypes were from the NE group, while 9 haplotypes were from the SW group. The ITS haplotype network illustrated one genetic group with only 4 closely related haplotypes (Supplementary Figure S1).

Furthermore, BAPS v6 and the correlated frequency model in GENELAND analyses used to infer population structure separated *N. kemneri* in the same two spatial clusters (Figure 3), further affirming the strong genetic differentiation found between these two genetic groups.

Notably, some genetic substructure in *N. kemneri* was hinted by the uncorrelated frequency model from GENELAND and BAPS non-spatial clustering of individuals. These results illustrated 4 clusters, distinguishing the Caatinga from northern Cerrado within the NE group, and separating the Cerrado from samples near the Paraná biogeographic dominion (Morrone et al., 2022; Supplementary Figure S2). Moreover, the phylogenetic tree shows high bootstrap values(= 97) which support the subtle divergence of the Caatinga and Cerrado within the Northeastern group (NE), while the easternmost individuals are the most divergent samples within the SW group (Figure 2). A 2-level AMOVA distinguishing both NE–SW and these four clusters further evidences the presence of sub-structure (Supplementary Table S5). However, since Cluster B ($N = 5$) and Cluster C ($N = 3$) are comprised of few individuals, further sampling is needed to firmly ascertain the presence of genetic sub-structure within *Nasutitermes kemneri*.

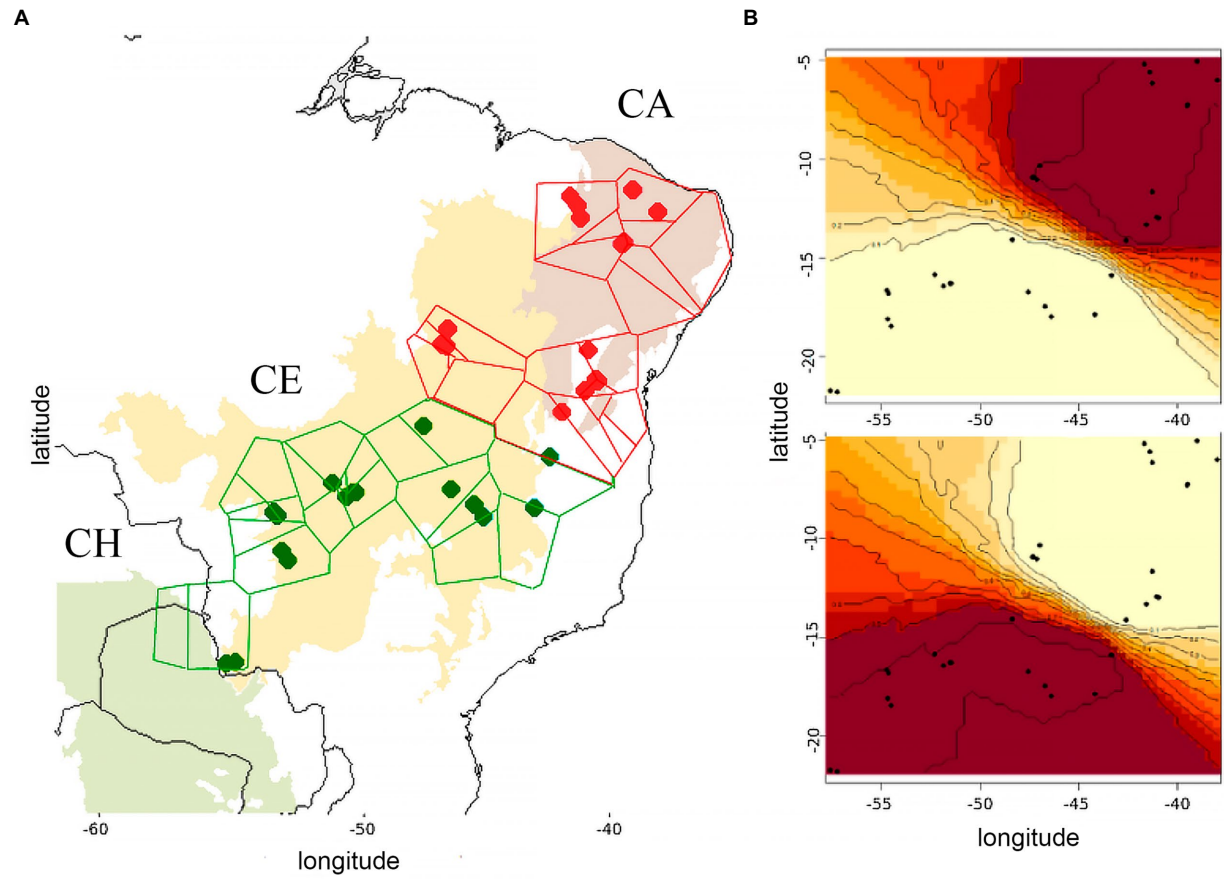

FIGURE 3
(A) Voronoi tessellation produced by the Bayesian spatial clustering analysis of 961bp (16S rRNA+COII) sequences from *Nasutitermes kemneri*, as implemented in BAPS v6. resulting in K =2, (ln(P)=−563.0329). **(B)** GENELAND map of posterior probabilities of cluster membership, correlated allele frequency model. CA, Caatinga; CE, Cerrado; and CH, Chaco.

Considering the highest level of differentiation broadly supported by previous analyses, the AMOVA partitioned in two groups (NE–SW) revealed a high variation among groups (82.74%) contrasting with a low variation within groups (17.26%; Table 1). The high Fst (0.827, $p < 0.0001$) confirms a strong genetic population structure as supported by the phylogenetic tree, haplotype network and Bayesian spatial clustering. Regarding the genetic diversity across the South American DOF, haplotype and nucleotide diversity was overall high (Hd = 0.867, $\pi = 0.00107$), and high but slightly lower within both genetic groups (NE Hd = 0.767, $\pi = 0.00734$, SW Hd = 0.723, $\pi = 0.00922$).

Demographic inferences through Tajima's D neutrality test showed no signal of population expansion or retraction for the entire species range ($D = 0.395$, $p = 0.69$) nor for each separate group (NE, $D = -1.496$, $p = 0.13$/SW, $D = -1.883$, $p = 0.059$). Finally, the dated phylogenetic analyses inferred that the divergence between NE and SW lineages occurred around 2.41 Mya (3.59–1.47, 95% height posterior density), during the Pliocene–Pleistocene boundary.

TABLE 1 Analysis of molecular variance results for Northeastern (NE) and Southwestern (SW) populations inferred by markers 16S rRNA and COII.

Source of variation	d.f.	Variation (%)	Fst	*p* value
Among populations	1	82.74	0.827	<0.001
Within populations	46	17.25	–	–

d.f. = degrees of freedom; Fst = fixation index.

4. Discussion

Results show a strong genetic and spatial structure within *N. kemneri* (Figure 2), evidenced by two highly differentiated groups, henceforth called populations. These populations are not restricted to the phytogeographic domain where they were sampled (Caatinga, Cerrado, and Chaco), instead, the NE population encompasses Caatinga and northern portions of Cerrado, while the Southwestern (SW) population ranges from central-south Cerrado to the northeast peripherical portions of

the Chaco (Figure 2). This Northeast–Southwest pattern of spatial differentiation is shared with other taxonomic groups distributed along the DOF such as squamates (Werneck et al., 2012b; Recorder et al., 2014; Fonseca et al., 2018), amphibians (Oliveira et al., 2018), flies (Moraes et al., 2009), bees (Miranda et al., 2016), marsupials (Carvalho et al., 2011), trees (Resende-Moreira et al., 2017), and, to some extent, birds (Rocha et al., 2020).

Estimates suggest that these populations diverged ~2.5 Mya, during the Pliocene-Pleistocene boundary. During this period, the Cerrado plateaus were already uplifted to their present-day elevations while other areas within the Cerrado subsided, forming peripheral depressions (Del'Arco and Bezerra, 1989). The depressions inhibited gene flow between east and west of the plateau, and promoted diversification of subsequently isolated populations of flora and fauna. As discussed by Bonatelli et al. (2021), while several species across the DOF experienced range shifts, there is no single demographic response to Pleistocene climatic fluctuations. Some species experimented demographic expansion, others exhibited the opposite response, and some, like *N. kemneri*, underwent different processes since the separation of both populations (i.e., 59), where each experienced different evolutionary histories, illustrated by the different structures displayed by the haplotype network.

The NE population network has an intricate structure, with many "missing" haplotypes, which could be due to repetitive extinction-expansion events. Demographic inferences do not offer statistical support for this hypothesis, however, the geoclimatic history of northeastern Brazil does. During the Quaternary, major paleoenvironmental changes occurred during wet periods throughout the Pleistocene (Auler et al., 2004). Increased rainfalls facilitated the expansion of semi-deciduous forest to the Caatinga (Auler et al., 2004; Wang et al., 2004; Werneck, 2011), which possibly provided new ecological niches for *N. kemneri* to exploit besides xeric shrublands and an increase in biomass. On the contrary, arid periods led to regional extinction of taxa adapted to wet forests such as herpetofauna, as discussed by Gehara et al. (2017), and the cyclic nature of these humid-drier events left its mark on the NE population, accounting for the high genetic diversity and complex haplotype network. Moreover, recent studies have shown that termites are resistant to temperature shifts (Woon et al., 2019; Janowiecki et al., 2020; Woon et al., 2022), but precipitation would be the main factor in determining termite distribution, abundance, and survival rate (Woon et al., 2019; Pozo-Santiago et al., 2020). Although we did not measure this, it seems that *N. kemneri* is more abundant in the most humid phytogeographic domain, the Cerrado, than in the semiarid ones, Caatinga and Chaco. In the same way, elevation plays important roles in both restrictive and non-restrictive effects of temperature and humidity, as well as in the interaction between these factors, and while the Caatinga is composed mostly by flattened surfaces between 300 and 500 m above sea level (Silva et al., 2017), the Cerrado has higher plateaus in central and northeastern regions (Werneck et al., 2012).

The SW population displays a star-shaped haplotype group, which is characteristic of refugia with later population expansion, yet demographic inferences lack statistical support. Nevertheless, during the Quaternary climatic and vegetation fluctuations, the general area currently occupied by Cerrado appears to have been drier (Collevatti et al., 2020; Oliveira et al., 2020), paleoclimatic model reconstructions predicted areas of historical climatic stability (Werneck et al., 2012). These stable areas (the higher plateaus in central and north-eastern Cerrado) were important refugia for taxa such as trees (Ramos et al., 2007; Buzatti et al., 2017; Resende-Moreira et al., 2017; Camps et al., 2018), and may, consequently, also serve as refugia for wood feeding termites.

Finally, while the spatially explicit Bayesian clustering programs BAPS and GENELAND reached a consensus in dividing *N. kemneri* in two populations, the uncorrelated frequency model in GENELAND and the non-spatial BAPS clustering are also worth noting and briefly discussed. These less robust approaches hint at subtle population substructure within both the NE and SW populations, wholly distinguishing Caatinga (Cluster A) and northeastern Cerrado (Cluster B) in NE, and separating central western Cerrado (Cluster C) from the rest of Cerrado (Cluster D) in SW. Tree species with a similar distribution across the DOF (and some that extent to the Parana dominion), show fairly recent (~800.000 years BP) fine-scale genetic structure within the Chaco-Cerrado-Atlantic Forest, from 3 to up to 7 haplogroups/lineages (Collevatti et al., 2009; Novaes et al., 2010, 2013). These lineages present a similar pattern of distribution to Clusters A, B, C, and D of *N. kemneri*, and the recent divergence time between the main populations (NE/SW) at the Pliocene-Pleistocene transition (with confidence intervals spanning from Late Miocene to Middle Pleistocene) may explain the low resolution of these analyses, where subtle climatic or geographic differences within these phytogeographic domain may be driving this incipient or shallower population sub-structuring. In fact, biogeographic districts based on tree species (Françoso et al., 2020) partially match the distribution of these clusters, citing different temperatures, seasonal variation, radiation and the degree of cover transformation as responsible for the floristic differences. However, further sampling is needed to determine the extent and significance of these patterns in *N. kemneri*.

This study provides important inferences about the general panorama of the evolutionary history of *N. kemneri* in the DOF, its endemic region, offering important contributions to the understanding of biogeographic and phylogeographic issues in the Neotropics. Our results support an ancient divergence between two well-defined lineages (NE and SW) for *N. kemneri*, which occurred during the Plio-Pleistocene transition, reinforcing the role of climatic and vegetation variation events in the diversification of the DOF biota (Del'Arco and Bezerra, 1989; Wang et al., 2004). Further climatic fluctuations during the Quaternary had pronounced effects on the Caatinga biota (Bonatelli et al., 2021) and could have facilitated the expansion and diversification of *N. kemneri* across the NE region of the

DOF, while refugia zones in the Southeastern region may account for the constricted diversity patterns within the Cerrado, and altogether explain the unique evolutionary history of these two separate populations. The findings presented in this work, however, came from a few mitochondrial markers, and the investigation using multi-locus nuclear data can reveal other relevant patterns in the population genetic structure in this species.

Most of the integrative phylogeographic and predictive studies in the DOF rely heavily on data from herpetofauna and plants, where insects are less than 15% of the species considered (Del'Arco and Bezerra, 1989). In this sense, data presented in this study not only aids the understanding of the evolutionary history of *N. kemneri* and add evidence to the response patterns described for this area (Del'Arco and Bezerra, 1989; Wang et al., 2004), but also partly accounts for our knowledge gap. Furthering our knowledge on biotic variables that influenced the demography and distribution of DOF endemic species is key, and especially insects (Wilson and Fox, 2021) for future decisions in conservation strategies of the DOF threatened phytogeographic domains and potential effects linked to climate warming.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

KK and RGS made the laboratory work. KK, VP-O, RGS, and TC did the analyses. AV, EC, and RHS provided the specimens. RHS provided photos of the *N. kemneri* soldier. KK, VP-O, and TC did the writing. RHS, AV, and EC edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1081114/full#supplementary-material

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EDITED BY
Daniel Aguilera-Olivares,
Universidad de Concepción,
Chile

REVIEWED BY
Gillian H. Gile,
Arizona State University,
United States
Peter Vdacny,
Comenius University,
Slovakia

*CORRESPONDENCE
Renate Radek
✉ renate.radek@fu-berlin.de
Andreas Brune
✉ brune@mpi-marburg.mpg.de

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New insights into the coevolutionary history of termites and their gut flagellates: Description of *Retractinympha glossotermitis* gen. nov. sp. nov. (Retractinymphidae fam. nov.)

Renate Radek[1]*, Katja Platt[2], Deniz Öztas[1], Jan Šobotník[3], David Sillam-Dussès[4], Robert Hanus[5] and Andreas Brune[2]*

[1]Evolutionary Biology, Institute for Biology, Freie Universität Berlin, Berlin, Germany, [2]Research Group Insect Gut Microbiology and Symbiosis, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany, [3]Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Czechia, [4]Laboratory of Experimental and Comparative Ethology, UR 4443, University Sorbonne Paris Nord, Villetaneuse, France, [5]Research Group Chemistry of Social Insects, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czechia

Lower termites harbor diverse consortia of symbiotic gut flagellates. Despite numerous evidence for co-cladogenesis, the evolutionary history of these associations remains unclear. Here, we present Retractinymphidae fam. nov., a monogeneric lineage of Trichonymphida from Serritermitidae. Although *Retractinympha glossotermitis* gen. nov. sp. nov. morphologically resembles members of the genus *Pseudotrichonympha*, phylogenetic analysis identified it as sister group of the Teranymphidae. We compared morphology and ultrastructure of *R. glossotermitis* to that of *Pseudotrichonympha* and other Teranymphidae, including the so-far undescribed *Pseudotrichonympha solitaria* sp. nov. from *Termitogeton planus* (Rhinotermitidae). Like all Teranymphidae, *R. glossotermitis* is a large, elongated flagellate with a bilaterally symmetric rostrum, an anterior, flagella-free operculum, and an internal rostral tube. However, it is readily distinguished by the length of its rostral flagella, which never exceeds that of the postrostral flagella, and its retractable anterior end. Inclusion of the hitherto unstudied *Stylotermes halumicus* (Stylotermitidae) in our survey of trichonymphid flagellates in Neoisoptera confirmed that the combined presence of *Heliconympha* and *Retractinympha* and absence of *Pseudotrichonympha* is unique to Serritermitidae. The close phylogenetic relatedness of *Heliconympha* in Serritermitidae to the spirotrichosomid flagellates in Stolotermitidae provides strong support for their acquisition by horizontal transmission.

KEYWORDS

coevolution, diversity, Neoisoptera, Parabasalia, phylogeny, *Pseudotrichonympha*, transfaunation, ultrastructure

Introduction

Symbiotic flagellates play an essential role in the digestion of lignocellulose in the hindguts of lower termites and their phylogenetic sister group, wood-feeding cockroaches of the genus *Cryptocercus* (Cryptocercidae; Hongoh, 2011; Brune, 2014). Each host family harbors a unique assemblage of flagellate symbionts that is specific for the respective host and typically similar in composition among

members of the same termite family [see reviews by Inoue et al. (2000), Kitade (2004), and Ohkuma and Brune (2011)]. This led to the hypothesis that flagellates were present already in the common ancestor of Xylophagoidea [i.e., termites (Isoptera) and Cryptocercidae; Engel, 2011] and have been passed on from parent to offspring (Lo and Eggleton, 2011). This vertical transmission is driven by proctodeal trophallaxis, a behavioral trait that is a synapomorphy of Xylophagoidea (Nalepa, 1991, 2015) and has favored co-cladogenesis between different flagellate lineages and their termite hosts (e.g., Noda et al., 2007, 2018; Ikeda-Ohtsubo and Brune, 2009; Ohkuma et al., 2009; Jasso-Selles et al., 2017; Radek et al., 2018). For termite classification and taxonomic details, see Engel et al. (2009), Krishna et al. (2013), and Wang et al. (2022); their gut flagellates have been covered by Čepička et al. (2016) and Hampl (2016).

While Cryptocercidae and the basal termite families (Mastotermitidae, Teletisoptera, and Kalotermitidae) harbor numerous (typically 10–20) flagellate species from the phyla Parabasalia and Preaxostyla (order Oxymonadida), the diversity of the flagellate communities in the crown families of termites (Neoisoptera) is substantially reduced (e.g., Yamin, 1979; Kitade and Matsumoto, 1993; Inoue et al., 2000; Brugerolle and Bordereau, 2004; Kitade et al., 2012). Termitidae have lost all flagellates, and most Rhinotermitidae have retained only a few lineages of parabasalids (Kitade and Matsumoto, 1998; Jasso-Selles et al., 2017), with the genus *Reticulitermes* forming a notable exception (see below). A study of the flagellate genus *Pseudotrichonympha*, a large cellulolytic member of the Teranymphidae (order Trichonymphida), documented co-cladogenesis with Rhinotermitidae, without any obvious host switches (Noda et al., 2007). However, many members of the genus *Pseudotrichonympha* have been characterized only on a morphological basis, and only few representatives with SSU rRNA gene sequences have been formally described (Noda et al., 2007; Saldarriaga et al., 2011; Jasso-Selles et al., 2017).

The evolutionary history of Trichonymphida in Neoisoptera has been obscured by unresolved relationships among particular host lineages and a lack of information on their flagellate microbiota. However, comparative analyses of mitochondrial genome sequences have provided increasingly robust host phylogenies that have improved our understanding of termite evolution and diversification (Chouvenc et al., 2021). Rhinotermitidae have been shown to be paraphyletic to both Serritermitidae and Termitidae (Bourguignon et al., 2015; Bucek et al., 2019), with Stylotermitidae in a basal position (Wu et al., 2018).

It is well documented that the flagellate assemblages in the genus *Reticulitermes* (Rhinotermitidae) differ fundamentally from those of other rhinotermitids but resemble those of the genus *Hodotermopsis* (Hodotermopsidae; Yamin, 1979; Kitade and Matsumoto, 1998). This scenario has been explained by an ancestral horizontal transfer of flagellates (also referred to as "transfaunation") from a hodotermopsid to a rhinotermitid host (Kitade, 2004; Lo and Eggleton, 2011) – a widely accepted hypothesis that is backed by the close relatedness of the corresponding taxa in molecular phylogenies (Ikeda-Ohtsubo and Brune, 2009; Ohkuma et al., 2009; James et al., 2013; Gile et al., 2018, 2021).

Likewise, also the flagellate communities of Serritermitidae differ fundamentally from those of their rhinotermitid relatives (Radek et al., 2018). Based on morphological and phylogenetic evidence, it has been proposed that the flagellates of the genus *Heliconympha*, which are exclusively present in Serritermitidae, were acquired by horizontal transfer, presumably from a stolotermitid host (Radek et al., 2018). Since the first molecular data on Spirotrichosomidae from *Stolotermes* (Izawa et al., 2017) became available only after the study on *Heliconympha* had been submitted, a test of this hypothesis is still lacking. Also, a description of the second, *Pseudotrichonympha*-like lineage of flagellates from Serritermitidae, which show a superficial resemblance to members of the genus *Pseudotrichonympha* but are only distantly related to Teranymphidae (Radek et al., 2018), is still pending. Moreover, there is absolutely no information on the composition of the flagellate assemblages in the Stylotermitidae, the most basal of the extant neoisopteran families (Wu et al., 2018; Barden and Engel, 2020).

Here, we characterize the *Pseudotrichonympha*-like flagellate from *Glossotermes oculatus* and *Serritermes serrifer* (Serritermitidae) and propose a new genus and family for this lineage. Moreover, we describe a new *Pseudotrichonympha* species from the termite genus *Termitogeton*, a basal lineage of Rhinotermitidae, and investigate the diversity of flagellates in *Stylotermes halumicus* and their relationship to the flagellates of other Neoisoptera.

Materials and methods

Termites

Glossotermes oculatus was collected in French Guiana in 2013 and live specimens were processed as described (Radek et al., 2018). *Termitogeton planus* was collected in West Papua, Indonesia, in 2011 (Dolejšová et al., 2014); specimens were preserved in 96% ethanol. Species identification was verified by sequencing the mitochondrial cytochrome *c* oxidase subunit II (COII) genes (Inward et al., 2007); the GenBank accession numbers are KY750729 and MN528021. *Stylotermes halumicus* was collected in China in 2015 (collection ID CHI15-156); specimens were preserved in RNAlater (Invitrogen). Their COII gene sequence was identical to that encoded in the mitochondrial genome previously reported for the same material (KY449049; Wu et al., 2018).

Light microscopy

The hindgut paunch of worker termites was ruptured with fine-tipped forceps, and the content was released in a drop of 0.6% NaCl (for the direct observation of living flagellates) or fixed in a drop of 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.0). For the visualization of nucleus, flagella, basal bodies, axostyle, and dictyosomes (parabasal bodies), fixed cells were stained with protargol (silver proteinate) according to procedure A of Foissner (2014). Nuclei were visualized also by fluorescence microscopy after immersing the samples in a solution of 2 ng/ml 4,6-diamidino-2-phenylindole (DAPI; Serva, Heidelberg, Germany) for 10 min.

The slide mounts were observed with an Axiophot light microscope (Zeiss) equipped with differential interference contrast and epifluorescence illumination. Images were recorded with a MicroLive digital camera (Linkenheld, Oppenau, Germany).[1] All measurements were taken from protargol-stained slides.

Electron microscopy

For scanning electron microscopy (SEM), hindgut content was fixed in 2.5% glutaraldehyde. Samples were postfixed in 1% OsO_4, critical

1 http://www.mikroskopie.de/

point-dried, sputtered with gold, and inspected with an environmental scanning electron microscope (FEI Quanta 200). For details, see Radek et al. (2018).

For transmission electron microscopy (TEM), the same fixation procedure was used, but ruthenium red was added to both fixation solutions to enhance the contrast of the glycocalyx. Samples were embedded in Spurr's resin. Ultrathin sections were stained with saturated uranyl acetate and lead citrate and inspected with a Philips EM 208 electron microscope. For details, see Radek et al. (2018).

SSU rRNA gene sequencing and phylogenetic analysis

Termite hindguts were homogenized, DNA was extracted, and SSU rRNA genes were amplified, cloned, and sequenced following the procedure described by Radek et al. (2019), with the following exceptions: For the sample of *Stylotermes halumicus*, we used the Parabasalia-specific primer pair Para19-36f (5′-CTG CCA AGG AAG YAY AC-3′) and Fla1484-1501r (5′-GTT ACG ACT TCT CCT TCC-3′) at an association temperature (T_a) of 52°C. For the sample of *Termitogeton planus*, which was strongly degraded, we amplified the SSU rRNA gene of *Pseudotrichonympha* sp. by nested PCR, using the flagellate-specific primer pair EUK19f (5′-AYY TGG TTG ATY CTG CCA-3′) and EUK1772r (5′-CBG CAG GTT CAC CTA C-3′; Ohkuma et al., 1998) at a T_a of 50°C for the first PCR, and the *Pseudotrichonympha*-specific primer pair PsTrn41f (5′-GGT CAT AGA TTA AGC CAT GC-3′) and Fla1484r (5′-CTT GTT ACG ACT TCT CCT TCC-3′, Radek et al., 2019) at a T_a of 59°C for the second PCR. Amplified DNA was purified and sequenced directly with the same primers. To confirm the absence of other parabasalids, we also used the Parabasalia-specific primer pair Para936f (5′-GAA TTG ACG GAA GGG CAC A-3′) and Para1201r (5′-GCA TCT RAA GGR CAT CAC G-3′) at a T_a of 57°C for the second PCR. The sequences were deposited at GenBank under accession numbers MT936308–26 and MN523346.

New sequences and parabasalid sequences from public databases that were not yet included in the *Silva* SSURef database (Quast et al., 2013; version 106)[2] were imported using the *ARB* software package (Ludwig et al., 2004; version 7.0) and aligned with the reference sequences using the *Silva Incremental Aligner* (*SINA* version 1.2.11; Pruesse et al., 2012). The alignment was manually refined considering the secondary structure of the rRNA, and ambiguously aligned positions were removed. The final dataset consisted of 1,502 sites, of which 595 were invariant and 752 were parsimony-informative sites.

Phylogenetic trees were reconstructed by maximum-likelihood analysis with *IQ-TREE* 1.6.12 (Nguyen et al., 2015) using the best-fit evolutionary model (GTR + F + I + G4) suggested by *ModelFinder* (Kalyaanamoorthy et al., 2017) under the Bayesian information criterion. Tree topology was tested with *PhyML* v3.0 (Guindon et al., 2010) and by Bayesian inference analysis (*MrBayes*, Ronquist et al., 2012; 4 chains, 1,000,000 generations, burn-in 0.25). Node support was assessed with the Shimodaira-Hasegawa approximate likelihood ratio test (*SH-aLRT*, Guindon et al., 2010) and by ultrafast bootstrap analysis (*UFBoot*, 1,000 replicates, Hoang et al., 2018).

2 https://www.arb-silva.de/

Results

Morphology of the *Pseudotrichonympha*-like flagellate from *Glossotermes oculatus*

The largest flagellate in *G. oculatus* with its straight bands of flagella is easily distinguished from the medium-sized cells of *Heliconympha glossotermitis* with their spiraled bands of flagella and from the small *Hexamastix*-like cells with their bundle of six flagella (Radek et al., 2018). It resembles members of the genus *Pseudotrichonympha* in its fusiform shape, the presence of a rostrum at the anterior cell pole, and an almost complete flagellation of the cell surface (Figure 1A). Protargol-stained cells measured 107–260 (mean 163) µm in length and 60–92 (mean 70) µm in width ($n = 40$; Figures 1B,C). The pointed rostrum is capped by a hemispherical operculum (diameter *ca.* 8.5 µm) and surrounded by *ca.* 60 to 75 flagella (Figures 1A,D,F–H). The border of the operculum is circular (Figures 1G,H). In fixed preparations, and also during live observations, the anterior cell pole was frequently retracted, creating a cup-like invagination of varying depth that completely engulfs the rostrum like a high collar (Figures 1B,C). In such cases, we added the estimated length of the retracted anterior pole to the total cell length to achieve consistent length measurements. The posterior cell pole is moderately pointed in extended cells (Figures 1A,B) and more rounded in strongly contracted cells (Figure 1C).

Numerous flagella cover the entire cell surface except the operculum (Figure 1A–D,F–H). They are arranged in longitudinal rows. The rostral flagella adjacent to the operculum (series-1 flagella) are short (5.4–8.7 µm, mean 7.3 µm; $n = 5$; Figure 1G), while the flagella at the base of the rostrum (series-2 flagella) have the same length as the postrostral flagella (22–28 µm, mean 25 µm; $n = 12$). Rostral and postrostral flagella move independently of each other. Prokaryotic cells with a helical morphology are attached between the rostral flagella (Figure 1G). Protargol-stained cells show a rostral tube with darkly contrasted borders (Figures 1C,D inset), which measures 12–15.5 µm in length (mean 14.3 µm) and 4.6–6.8 µm in outer diameter (mean 5 µm, $n = 10$).

The single, drop-shaped nucleus is located in the anterior part of the post-rostral region, typically in a lateral position (Figure 1A,C–E). Its length is 26.5–66.4 µm (mean 45.6 µm) and the width is 14.5–42.1 µm (mean 25.6 µm; $n = 9$). In expanded cells, the tip of the nucleus points toward the rostrum (Figures 1D,E), but in contracted cells, it is turned sideways (Figure 1C). The obtuse end of the nucleus contains the condensed chromosomes, whereas the pointed end contains hyaline nucleoplasm (Figure 1E). DAPI, a DNA-specific fluorescent dye, strongly stained only the periphery of the nucleus but not the chromatin at the center (Figure 1D). The cytoplasm contains numerous ingested wood fragments that obscured other cell organelles (Figures 1B,C). Axostyles and parabasal bodies were not visible by light microscopy.

Ultrastructure of the *Pseudotrichonympha*-like flagellate from *Glossotermes oculatus*

Ultra-thin sections revealed more details of the *Pseudotrichonympha*-like cells (Figures 1I–L, 2). Oblique transverse sections of the anterior cell pole show the compact and regular inner structure of the rostrum, and the longitudinal rows of rostral and post-rostral flagella (Figures 1I–K). The outer cytoplasmic layer of the rostrum contains

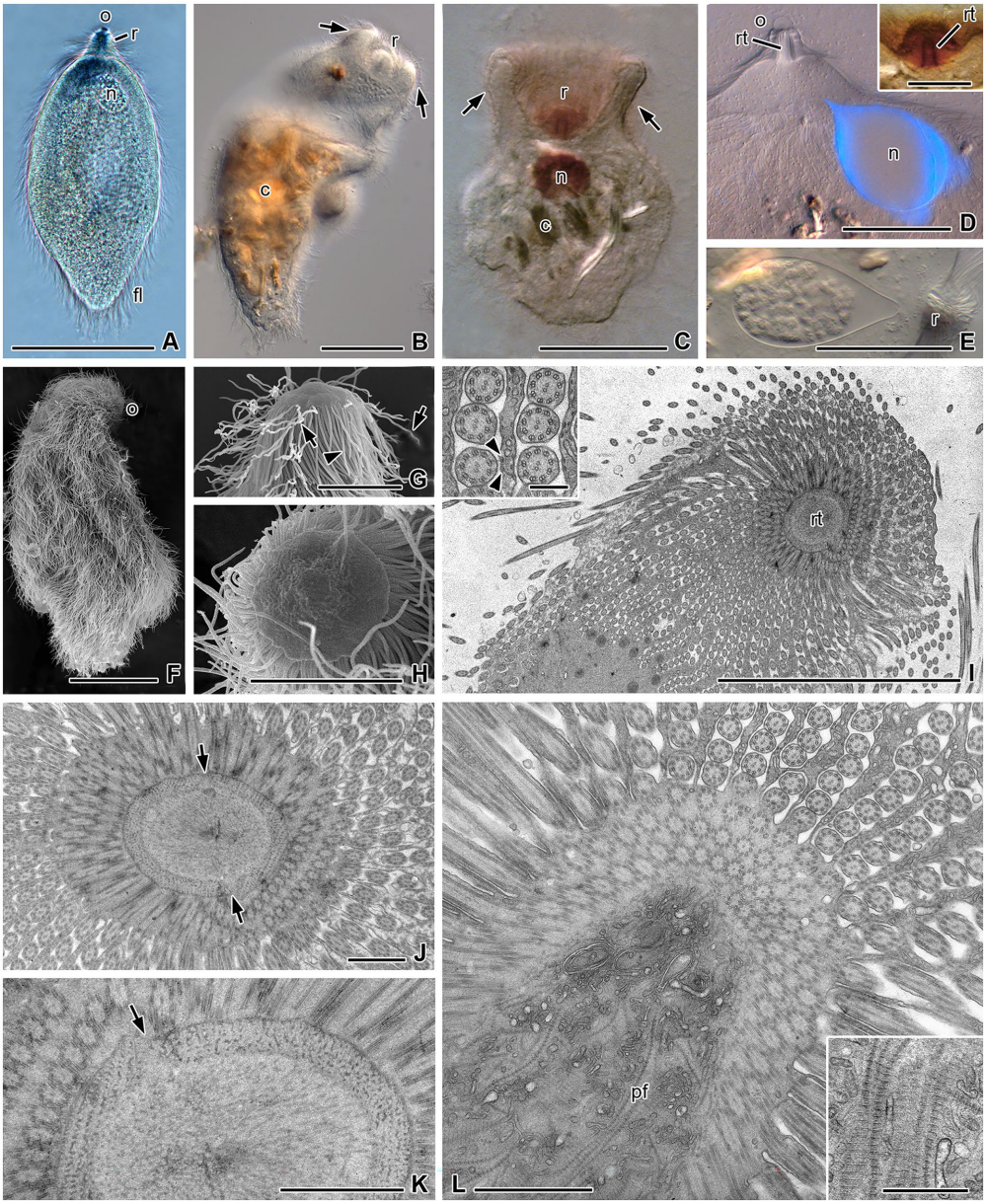

FIGURE 1
Morphology of *Retractinympha glossotermitis* and ultrastructural details of its anterior cell pole. **(A)** Lemon-shaped living cell with a cap-like operculum (o) at the tip of the rostrum (r), a granular nucleus (n), and complete cover with flagella (fl). **(B,C)** Cells with rostrum retracted in a bowl-like indentation and lignocellulose particles (c). **(D)** Anterior half of a cell with DAPI-stained, pear-shaped nucleus, hemispherical operculum, and rostral tube (rt). Inset: rostrum with rostral tube. **(E)** Pear-shaped nucleus with condensed chromosomes and anterior hyaline region. **(F)** A cell completely covered with flagella, except at the operculum. **(G)** Dome-shaped operculum, lateral view; helical spirochetes (arrows) are attached between the short flagella (arrowhead). **(H)** Circular operculum, top view. **(I)** Oblique section through anterior cell pole with rostral tube and longitudinal rows of flagella flanked by thin cytoplasmic ridges. Inset: Flagella attached to ridges by electron-dense cell contacts (arrowheads). **(J,K)** Cross-sections through anterior part of rostrum; rostral tube bordered by a dense layer of parabasal filaments and loosely arranged filaments inside tube; arrows indicate disjuncture of rostral tube wall. **(L)** Oblique section through posterior part of rostrum; interior filled with cross-striated parabasal filaments and vesicles. Inset: Parabasal filaments in higher magnification. **(A–E)** Light microscopy. **(A)** Bright field, **(B–E)** differential interference contrast, **(D)** DAPI staining , and (inset **D**) protargol staining. **(F–H)** Scanning electron microscopy. **(I–L)** Transmission electron microscopy. Scale bars **(A–F)** 50 µm, **(G–I)** 10 µm, (inset **I**) 0.2 µm, **(J–L)** 1 µm.

about 35 to 45 longitudinal rows of basal bodies that surround the rostral tube. Rostral basal bodies measure roughly 1 µm in length (950 nm; $n = 6$). The cross-sectioned rostral tube is bounded by a dense ring of regularly spaced parabasal filaments and contains loosely arranged filaments in its center (Figures 1I–K). The ring of parabasal filaments seems to consist of two symmetric plates that face each other (Figures 1J,K). The rows of post-rostral flagella arise from long longitudinal grooves that are separated by ectoplasmic ridges of about 140 nm ($n = 10$) thickness (Figure 1 inset I, L). The flagella are attached to the ridges by electron-dense material supporting the membranes (Figure 1I inset, Figures 2A,C). At their very proximal end, the flagella sit singly in little pits. Here, electron-dense structures arising at the peripheral side of the microtubular duplets pass the gap between the membrane of the flagellum and the membrane of the pit and end in electron-dense bodies underneath the pit membrane (Figure 2A, arrows). The number of these contact bridges is variable. Apart from the

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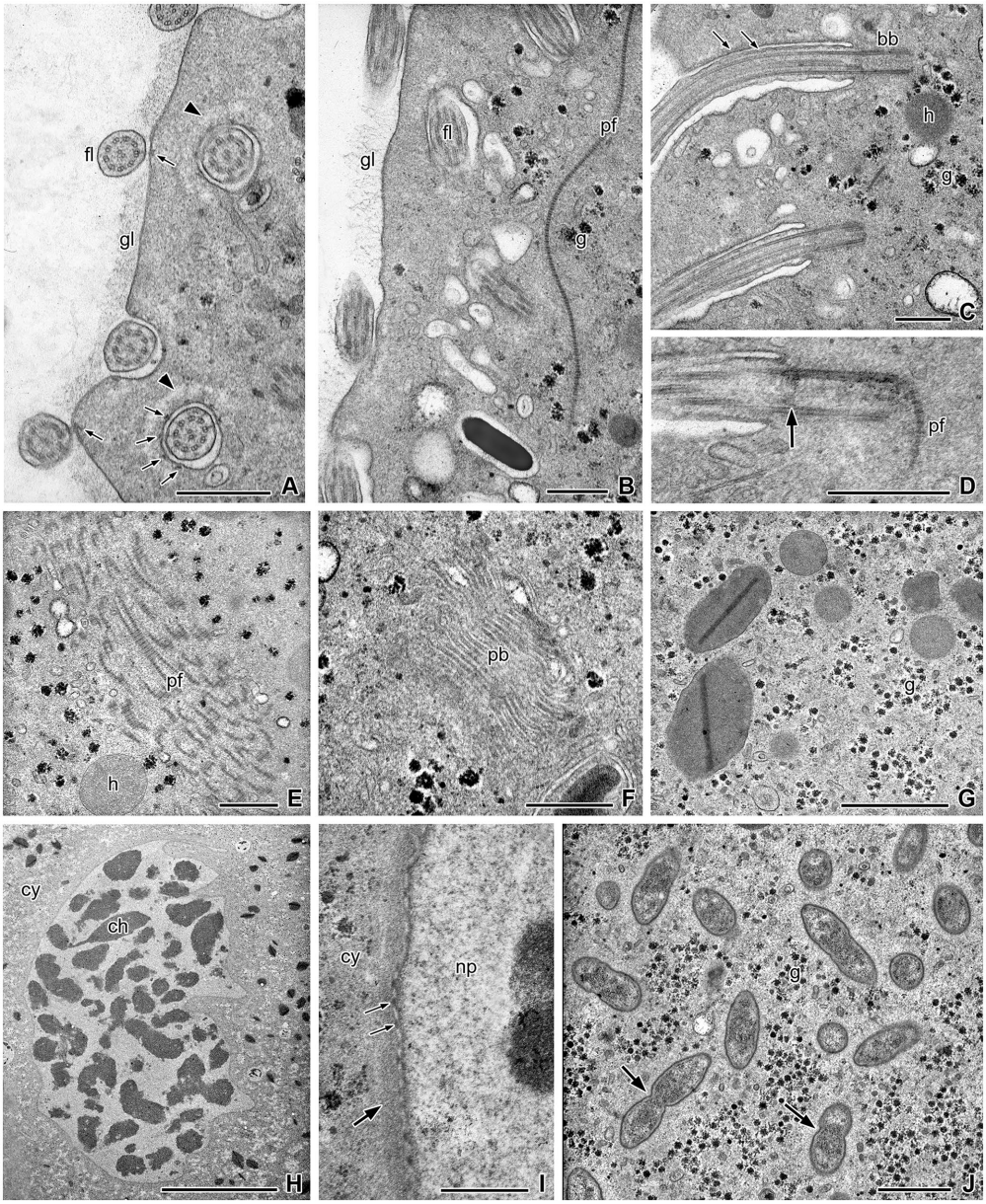

FIGURE 2
Ultrastructural details of the cell body of *Retractinympha glossotermitis*. **(A)** Body surface covered by thick layer of glycocalyx (gl); cross-sectioned flagella (fl) with contact sites to plasma membrane (thin arrows), and basal part of flagella in narrow depressions surrounded by bright zones of cytoplasm (arrowheads). **(B)** A cross-striated parabasal filament (pf) close to the flagellar region; glycogen (g). **(C)** Longitudinal sections of flagella and basal bodies (bb), arrows point to attachment sites; hydrogenosome (h). **(D)** Longitudinal section of basal body with transition plate to flagellum (arrow) and associated parabasal filament. **(E)** Strand of parabasal filaments. **(F)** Parabasal body (pb). **(G)** Small rounded hydrogenosomes and larger hydrogenosomes with interior stained plate. **(H)** Nucleus with condensed chromatin (ch); cytoplasm (cy). **(I)** Nuclear envelope with surrounding layer of dense cytoplasm (thick arrow) and nuclear pores (thin arrows); nucleoplasm (np). **(J)** Endobiotic bacteria (arrows) in direct contact to the cytoplasm. Transmission electron microscopy. Scale bars **(A–G,I)** 0.5 µm, **(H)** 10 µm, **(J)** 1 µm.

electron dense material of the contact sites, the pits are surrounded by a layer of electron lucent cytoplasm. The basal bodies of the post-rostral flagella are about half as long (540 nm; $n = 7$) as those of the rostral flagella (Figures 2C,D). An electron-dense plate indicates the transition point from basal bodies to flagella (Figure 2D). In rare cases, a connected cross-striated parabasal filament was observed at the basis of the post-rostral basal bodies (Figure 2D).

The parabasal filaments that arise in the rostral tube continue as a bundle of cross-striated bands with numerous intervening vesicular structures into the foremost part of the post-rostral region (Figure 1L and inset). Another bundle of filaments was observed deeper in the cell body (Figure 2E). Thin single parabasal filaments run parallel to the plasma membrane close to the basal bodies (Figure 2E). Parabasal bodies (dictyosomes) are about 1.2 µm in diameter and possess about 14 to 17 layers of cisterns and peripheral vesicles ($n = 4$). They occur close to the cell surface but are not regularly associated with the rows of basal bodies (Figure 2F). Connections between parabasal filaments and the cisterns of the dictyosomes were not observed.

The cell surface is covered by a conspicuous glycocalyx (thickness *ca.* 12 nm; $n = 10$) that contains fibrils and granules and is reduced in the

regions where flagella adhere to the surface (Figures 2A,B). The cytoplasm of the cells consists of an outer, finely granular ectoplasm, in which the flagella are anchored, and an endoplasm that contains the nucleus, numerous hydrogenosomes, glycogen granules, vesicles, endobacteria, and food particles (Figures 2A–J). The nucleus has an elongated but irregular form with folds and contains electron-dense chromatin aggregations in an electron-light nucleoplasm (Figure 2H). The nuclear envelope possesses numerous pores and is surrounded by an electron-dense cytoplasmic layer of about 100 nm thickness (Figure 2I). Hydrogenosomes appear in two different morphological variations. They are either small and rounded, with a homogenous interior, or larger and elongated, with a densely stained interior plate (Figure 2G). Many, but not all cells contain numerous endobacteria that are distributed in the cytoplasm (Figure 2J). The bacteria have tapered ends and are not enclosed in vacuoles; some are in the process of cell division.

Morphology of the *Pseudotrichonympha* sp. from *Termitogeton planus*

The hindgut of *T. planus* harbors only a single morphotype of flagellates. Like other members of the genus *Pseudotrichonympha*, the cells are long and slender with tapered ends (Figures 3A,B,E,H). They measure 140–235 µm in length (mean 194 µm) and at their greatest diameter 14–30 µm in width (mean 23 µm; $n = 20$). Contractions of the flexible cells may cause temporary thickening of the body, and swimming cells are often flat and twisted into a wide spiral (Figure 3B). The entire cell surface is covered with flagella except for the dome-shaped operculum and (in some cells), the very posterior end. In the light microscope, two zones with different lengths of flagella are easily observed. A ring of long flagella (mean length 28 µm, $n = 10$) is at the base of the rostrum (Figures 3A,C,E,G,H). These flagella, which move independently of the postrostral flagella and can flap far to the anterior end (Figure 3C), have been defined as series-2 flagella (De Mello, 1927). The postrostral flagella (series-3 flagella) are oriented backward and are comparably short, measuring only about 13 µm ($n = 10$). They arise in parallel rows of basal bodies that run from the anterior to the posterior cell pole and are often somewhat oblique to the longitudinal cell axis (Figures 3E,F). In the higher resolution of a scanning electron microscope, a third series of tiny flagella (series-1 flagella) can be observed at the foremost part of the rostrum (Figure 3I). They are partly hidden by a ring of slender, flattened lappets of about 4 µm length arising from the operculum. In top view, the circular operculum shows a central smooth part that tends to collapse in the SEM samples (Figure 3H inset). The smooth center is surrounded by a ring of bulging membrane folds, whose interspaces prolong to the flattened lappets (Figures 3H,I inset). Phase-contrast light microscopy occasionally revealed a structure composed of lappets plus series-1 flagella (Figure 3D).

The rostral region is 21 µm (18–25 µm) long and 15.5 µm (12.5–19.5 µm) wide ($n = 10$). Protargol-staining reveals a rostral tube of about 17 µm length ($n = 10$; Figure 3G), and the rows of basal bodies in the periphery of the rostrum are more numerous than in the adjacent cell body (Figure 3F, upper inset). Parabasal filaments appear as thin lines running parallel to the postrostral rows of basal bodies (Figure 3F, lower inset). The nucleus generally lies in the anterior third of the body, about 50 µm ($n = 20$) behind the anterior cell pole (Figures 3D,E), and rarely in the middle or even posterior region. It has an oval shape, measuring 9.3–13.7 µm (mean 11.8 µm) in length and 5.3–9.4 µm (mean 7.2 µm) in width ($n = 20$). The endoplasm of the cells contains wood fragments (Figures 3A,J). In protargol-stained specimens, dictyosomes are visible as dark spots or rings (Figure 3F). Their position is not related to the paths of the basal bodies. There are numerous dictyosomes in the cytoplasm anterior to the nucleus (Figure 3F).

Ultrastructure of the *Pseudotrichonympha* sp. from *Termitogeton planus*

The ultrastructure of the specimens from *T. planus* was almost identical to the detailed descriptions of Grimstone and Gibbons (1966) and Hollande and Carruette-Valentin (1971) for other *Pseudotrichonympha* species. In the following, we comment only on structural details that are either noteworthy or not mentioned in these studies. The postrostral flagella arise from long (*ca.* 3 µm) basal bodies. Their proximal part is embedded in short pouches (Figure 3J), in which they are attached to the plasma membrane at several contact sites (Figure 3L). The contact sites are supported by electron-dense material located under both plasma and flagellar membranes. Also outside the pouches, the proximal portion of the flagella remains attached to the cell body at one contact site (Figure 3K). In oblique cross-sections of basal body rows, a part of a sinus-like parabasal filament appears when the level of the section is directly underneath a basal body (Figure 3L and inset). The cytoplasm of the cells regularly contains endobiotic bacteria that are not enclosed in vacuoles, food vacuoles with wood particles, and globular hydrogenosomes (Figures 3J,K).

Phylogenetic analysis of Trichonymphida

Our phylogenetic analyses of all SSU rRNA gene sequences of Trichonymphida available to date (Figure 4) confirmed that the *Pseudotrichonympha*-like flagellate from *G. oculatus* and the corresponding phylotypes from *Serritermes serrifer* form a tight and highly supported clade (Cluster I in Radek et al., 2018; Retractinymphidae in Figure 4). Its deep-branching sister position to the Teranymphidae, however, was only weakly supported, and became inconsistent when rapidly evolving positions (up to 124 sites below the 50% identity threshold) were removed from the alignment (details not shown).

The internal topology of the Teranymphidae clade confirms the paraphyly of the genus *Eucomonympha* (Carpenter and Keeling, 2007; Ohkuma et al., 2009) and the previously reported sister position of the genus *Pseudotrichonympha* to the *Eucomonympha/Teranympha* clade (Ohkuma et al., 2005). The internal topology of the *Pseudotrichonympha* clade confirms the cospeciation of *Pseudotrichonympha* spp. with their rhinotermitid hosts documented already by Noda et al. (2007). The cospeciation hypothesis agrees with the sister position of the *Pseudotrichonympha* phylotypes from the two closely related phylotypes of *Termitogeton planus* (Noda et al., 2007; this study). As in previous SSU rRNA-based studies, the exact relationships between the symbionts of *Termitogeton*, *Psammotermes*, and *Prorhinotermes* spp. remain unresolved (Noda et al., 2007; del Campo et al., 2017). The same applies also to the position of the *Pseudotrichonympha* phylotype from *Stylotermes halumicus*.

The *Leptospironympha*-like flagellates from Serritermitidae (Cluster II in Radek et al., 2018), which had been classified in the genus *Heliconympha* (Radek et al., 2018), form a well-supported sister group to the spirotrichosomid flagellates from *Stolotermes victoriensis* (Izawa et al., 2017), whose sequences had not yet been

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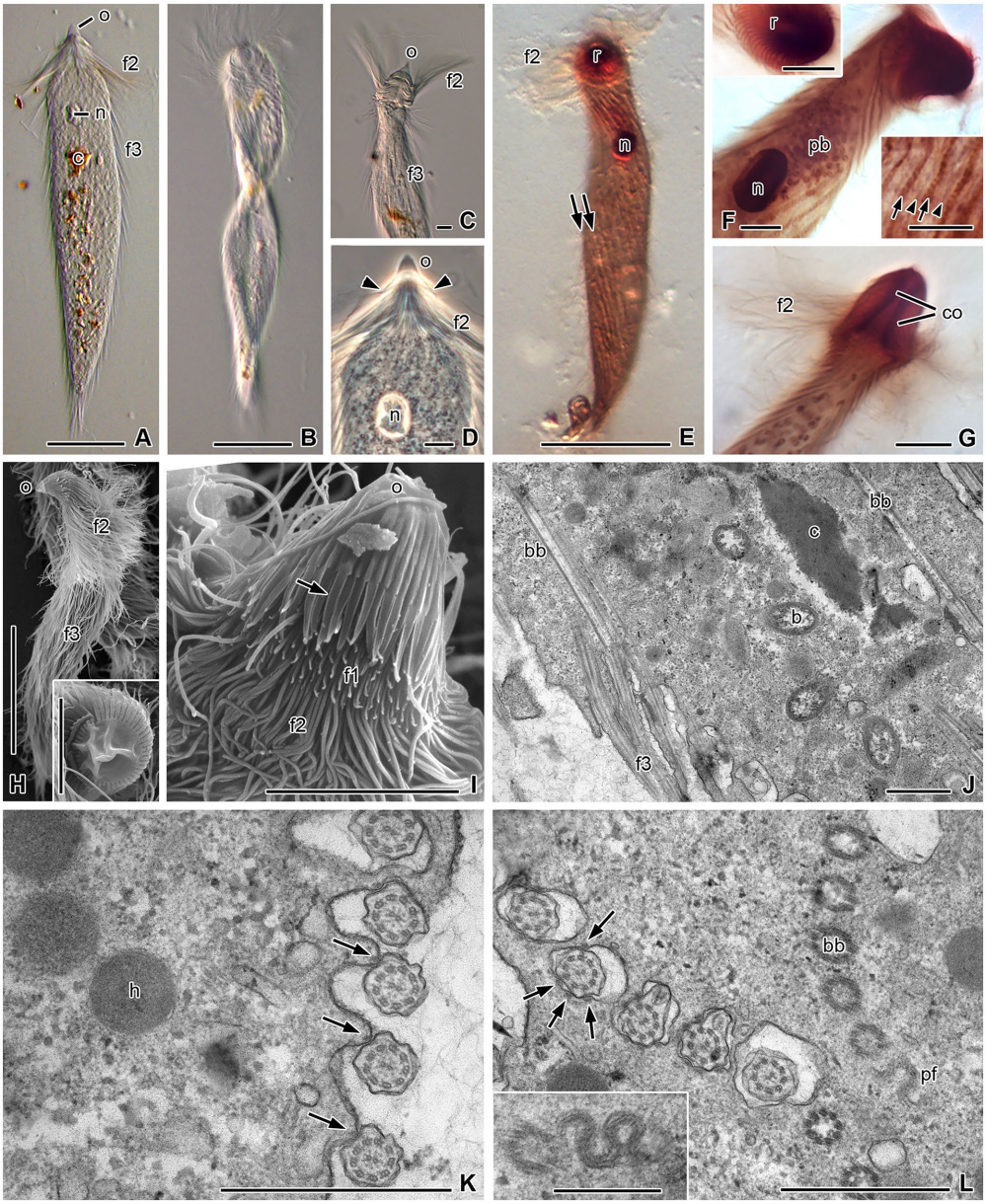

FIGURE 3
Morphology and ultrastructural details of *Pseudotrichonympha solitaria*. **(A)** Typical elongated cell with anterior operculum (o), a series of long flagella at the base of the rostrum (f2), and shorter postrostral flagella (f3). Nucleus (n) in anterior third of cell, ingested lignocellulose particles (c) in cytoplasm. **(B)** Twisted cell in motion. **(C)** Long rostral flagella struck forward. **(D)** Anterior third of body; extensions of operculum (arrowheads) are shorter than f3. **(E–G)** Protargol-staining contrasts nucleus, rows of basal bodies (arrows), parabasal filaments (arrowheads), parabasal bodies (pb), and columella (co) inside rostrum (r). **(H)** A total cell with pointed operculum and flagella series f2 and f3. Inset: Operculum in top view. **(I)** Rostrum in side view; apical operculum with numerous slender, leaf-like lappets posteriorly (arrow), which partially cover short series-1 flagella (f1). **(J)** Longitudinal section through cell periphery; long basal bodies (bb) of f3, cytoplasmic bacteria (b), and ingested lignocellulose particles. **(K)** Cell periphery in cross-section; flagella attached to cell surface, with electron-dense structures (arrows) supporting attachment sites; hydrogenosomes (h). **(L)** Basal part of flagella in pouches of the cell surface, with several contact sites to plasma membrane (arrows); inset: sinus-like parabasal filament (pf) under obliquely cross-sectioned rows of basal bodies. **(A–G)** Light microscopy: **(A–C)** differential interference contrast, **(D)** phase contrast, **(E–G)** protargol staining. **(H,I)** Scanning electron microscopy. **(J–L)** Transmission electron microscopy. Scale bars **(A,B,E,H)** 50 µm, (**C,D,F,G**, insets **F,H,I**) 10 µm, **(J–L)** 1 µm, (inset **L**) 0.5 µm.

analyzed in this context. The results of the present analysis (Figure 4) fully agree with the morphology and ultrastructure of *Heliconympha glossotermitis* and the classification of the genus *Heliconympha* in the family Spirotrichosomidae (Radek et al., 2018). Notably, the single *Leptospironympha* sequence from *Cryptocercus* occupies a moderately supported position basal to Spirotrichosomidae from termites. The paraphyletic status of the family Hoplonymphidae agrees with previous results (e.g., Carpenter et al., 2010; Mee et al., 2019).

Flagellate phylotypes from *Stylotermes halumicus* and *Termitogeton planus*

For lack of fresh material, we could not obtain any morphological data for the flagellates of *Stylotermes halumicus*. Amplification of the SSU rRNA genes with flagellate-specific primers yielded a clone library (19 clones) that consisted exclusively of homologs from parabasalids and comprised three phylotypes (>99.5% sequence similarity). One of the

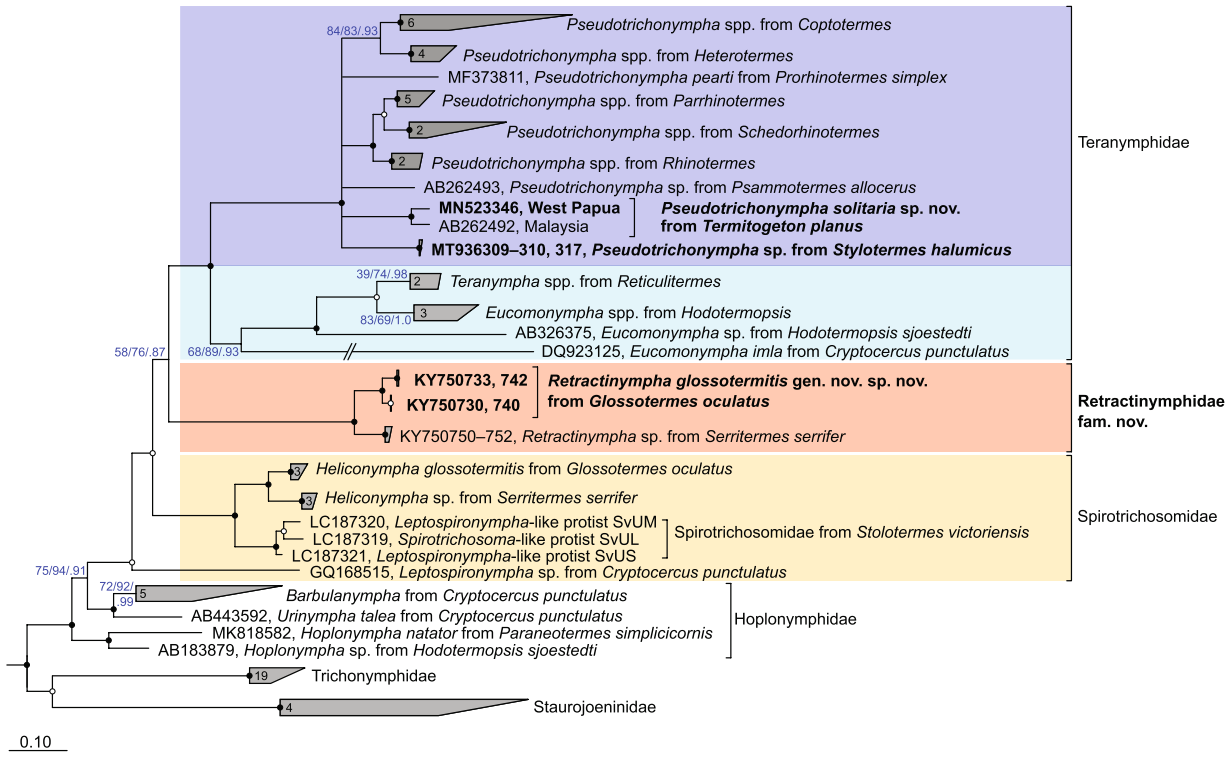

FIGURE 4
Phylogenetic analysis of the SSU rRNA genes of Trichonymphida. The position of *Retractinympha glossotermitis* and the *Pseudotrichonympha* spp. obtained in this study are shown in bold. The maximum-likelihood tree is a consensus tree from 1,000 bootstrap trees reconstructed with IQ-TREE, using *Lophomonas striata* and members of Trichomonadea as outgroup. Tree topology was tested with PhyML and Bayesian analysis (Ba); conflicting nodes are shown as multifurcation. Bullets indicate high node support in all analyses: SH-aLRT/ultrafast bootstrap/Ba posterior probabilities ≥96/99/1.00 (•), ≥ 80/95/0.98 (•); in other cases, individual values are shown. Collapsed clades are labeled with the number of sequences included. For more details, including accession numbers of all sequences, see Supplementary Figure S1.

phylotypes (11 clones) fell into the radiation of *Pseudotrichonympha* spp. from Rhinotermitidae (Figure 4). The two other phylotypes (5 and 3 clones, respectively) were highly similar (< 3.5% sequence divergence) and most closely related to a flagellate from *Heterotermes tenuis* (Rhinotermitidae) that was recently assigned to the genus *Cthulhu* (De Martini et al., 2021). They form a well-supported clade (<10% sequence divergence) with *Cthulhu macrofasciculumque* from *Prorhinotermes simplex* (Rhinotermitidae; James et al., 2013) and unclassified *Hexamastix*-like flagellates from *Glossotermes* and *Serritermes* spp. (Serritermitidae; Radek et al., 2018) in the Honigbergiellida (Figure 5). No PCR products were obtained with Oxymonadida-specific primers (Radek et al., 2019).

The COII gene sequence of *Termitogeton planus* was identical to that reported for a specimen previously collected in almost the same location in West Papua (KP026298; Bourguignon et al., 2015) but differed significantly from those of specimens collected in other countries, including those from Malaysia (Supplementary Figure S1), where the type of *Termitogeton planus* was collected (Bourguignon and Roisin, 2011). This agrees with the notion that specimens from West Papua may represent a separate species (Parmentier and Roisin, 2003). The DNA in the ethanol-fixed samples was strongly degraded, but the SSU rRNA genes were successfully amplified using nested PCR. Direct sequencing yielded a clean sequence read along its entire length, which agrees with the observation that this termite harbors only a single morphotype of gut flagellates (see above). It was most similar (3.5% sequence divergence) to the sequence previously obtained from a suspension of *Pseudotrichonympha* sp. (AB262492) from specimens of *Termitogeton planus* collected in Malaysia (AF262598; Noda et al., 2007; Figure 4). A Parabasalia-specific internal primer set yielded the same phylotype, which is consistent with the absence of other gut flagellates.

Discussion

Coevolutionary history of Trichonymphida and Neoisoptera

The exclusive presence of flagellates of the order Trichonymphida in termites and Cryptocercidae suggests that the common ancestor of these groups was already colonized by ancestral lineages of these flagellates (Carpenter et al., 2009; Ohkuma et al., 2009). The basal position of *Cryptocercus* symbionts in several families of Trichonymphida (Trichonymphidae, Hoplonymphidae, Spirotrichosomidae, and Teranymphidae) strongly suggests that the common ancestor of Cryptocercidae and termites already harbored multiple lineages of Trichonymphida that had diversified before the split of the two lineages and were subsequently lost multiple times during termite evolution. Although the relationships between flagellates and their respective hosts are not always fully resolved, the results of the present study provide new insights into the coevolutionary history of Trichonymphida and their neoisopteran host families (Stylotermitidae, Serritermitidae, and Rhinotermitidae).

The identification of a *Pseudotrichonympha* phylotype in *Stylotermes* extends the presence of this flagellate genus to Stylotermitidae, the most

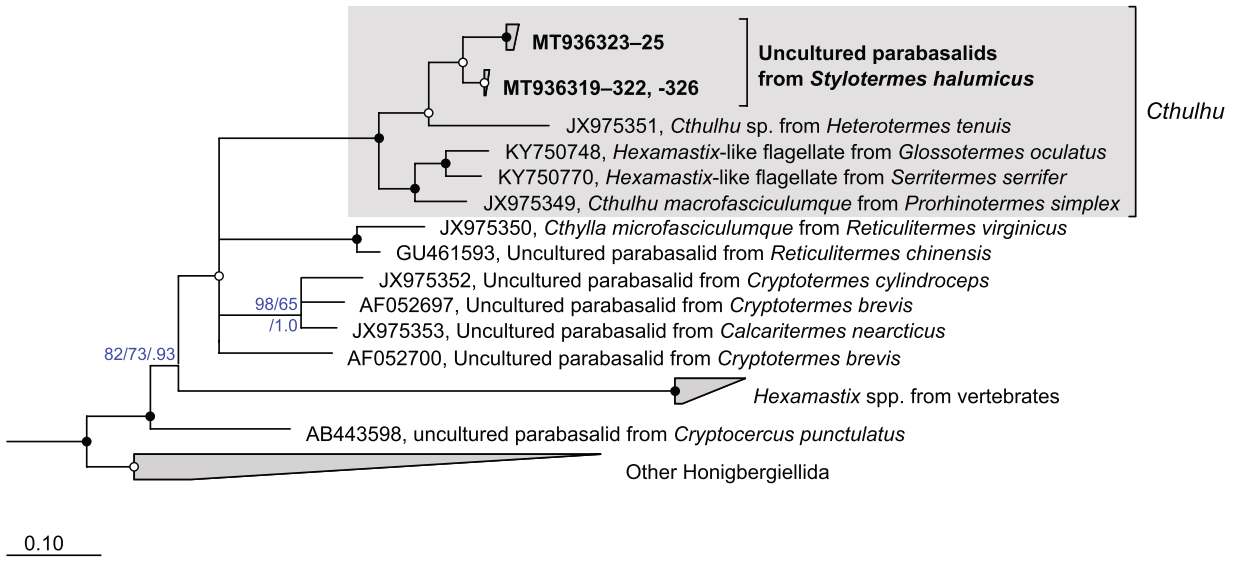

FIGURE 5
Phylogenetic analysis of the SSU rRNA genes of Honigbergiellida. The position of the uncultured parabasalids from *Stylotermes halumicus* is shown in bold. The maximum-likelihood tree was reconstructed with IQ-TREE using members of Trichomonadida as outgroup. Tree topology was tested with PhyML and Bayesian analysis (Ba); conflicting nodes are shown as multifurcation. Bullets indicate high node support in all analyses: SH-aLRT/ultrafast bootstrap/posterior probabilities ≥96/99/1.00 (•), ≥ 80/95/0.98 (•); in other cases, individual values are shown. Collapsed clades are labeled with the number of sequences included.

basal family of Neoisoptera. Although not all positions were fully resolved, it is likely that an ancestral member of the genus *Pseudotrichonympha* was present already before the radiation of Neoisoptera and subsequently cospeciated with its host (Noda et al., 2007; Figure 6). The *Pseudotrichonympha* lineage was lost at least three times, once in a common ancestor of the genus *Reticulitermes*, once in a common ancestor of Serritermitidae, and once in a common ancestor of Termitidae. Each of these losses was accompanied by the loss of some or – in the case of Termitidae – all other gut flagellates.

The loss of *Pseudotrichonympha* in *Reticulitermes* coincides with the appearance of several flagellate lineages that are not represented in other Neoisoptera but were apparently acquired by horizontal flagellate transfer from other, more basal termite families (Teletisoptera; Figure 6). The transfer of *Trichonympha* and *Teranympha* (Trichonymphida), *Spirotrichonympha* (Spirotrichonymphida), and several members of Pyrsonymphidae (Oxymonadida) from Hodotermopsidae to an ancestral member of *Reticulitermes*, which had been proposed already by Kitade (2004), is strongly supported by the close phylogenetic relatedness of the respective species (James et al., 2013; Gile et al., 2018, 2021; Radek et al., 2019; Figure 4). A similar scenario has been employed to explain the unique presence of *Heliconympha* in Serritermitidae (Radek et al., 2018). Here, the horizontal transfer of flagellates from an ancestral *Stolotermes* species (Figure 6) is substantiated by the close phylogenetic relatedness of the Spirotrichosomidae from *Stolotermes victoriensis* to members of the genus *Heliconympha* (Figure 4).

The origin of *Retractinympha* (see below), the second trichonymphid in Serritermitidae, however, remains unclear. Members of this genus represent a novel family-level lineage that has no representatives in any other termites investigated, and their presence in Serritermitidae could be explained by different scenarios. One would involve the presence of a separate lineage of trichonymphids in the neoisopteran ancestor that was vertically inherited but subsequently lost in all lineages but Serritermitidae. An alternative explanation would be the horizontal acquisition from a host outside of the Neoisoptera. A plausible opportunity would be the same transfer event that led to the acquisition of *Heliconympha* from Stolotermitidae. Since data on the molecular diversity of the flagellate communities in Stolotermitidae are available only for a single host species (Izawa et al., 2017), it is possible that relatives of *Retractinympha* will be discovered once other species of this termite family have been studied.

The genus *Retractinympha* (Retractinymphidae fam. nov.)

The superficial resemblance of the *Pseudotrichonympha*-like flagellates from *G. oculatus* and *Serritermes serrifer* to members of the genus *Pseudotrichonympha* stands in stark contrast to the results of the phylogenetic analysis, which identifies them as a deep-branching and only weakly supported sister group of Teranymphidae, with Spirotrichosomidae in a basal position (Radek et al., 2018; Figure 4). Based on phylogenetic evidence and the morphological differences discussed below, we propose to classify these flagellates in the new genus *Retractinympha*, with *Retractinympha glossotermitis* from *G. oculatus* as type species.

Retractinympha glossotermitis shows the general traits of Trichonymphida: (i) a bilaterally symmetric rostrum covered with numerous flagella, except at the operculum, (ii) numerous parabasal filaments that originate from two (or four) parabasal plates and may form a rostral tube, and (iii) the absence of a protruding axostyle (Čepička et al., 2010, 2016). Their fusiform shape and an almost complete flagellation with two series of rostral flagella, is typical also for members of the genus *Pseudotrichonympha*. However, there are several traits that allow the genus *Retractinympha* to be distinguished from Teranymphidae also on a morphological basis and to justify its classification in a new family, Retractinymphidae.

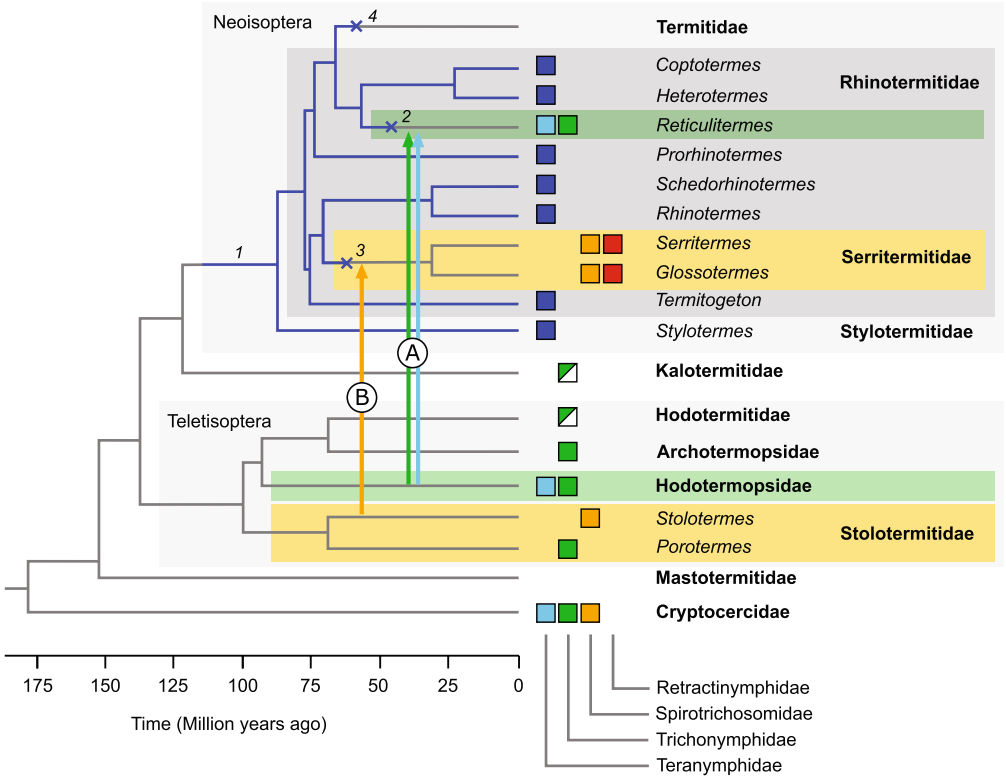

FIGURE 6
Evolutionary history of Trichonymphida in Neoisoptera. Boxes indicate the occurrence of a particular flagellate family in a termite genus (same colors as in Figure 4). We propose that a member of the genus *Pseudotrichonympha* was present already in the common ancestor of Neoisoptera (1) and subsequently cospeciated with its host (blue edges). The lineage was lost (×) several times during host evolution (2–4). Arrows indicate horizontal transfer events that led to the acquisition of Trichonymphidae and Teranymphidae in *Reticulitermes* **(A)** and of Spirotrichosomidae in Serritermitidae **(B)**. The origin of Retractinymphidae is unclear. The host phylogeny (schematic) is based on Bucek et al. (2019) and Wang et al. (2022).

The traits distinguishing Retractinymphidae from Teranymphidae are the length ratio of rostral to postrostral flagella, the presence of an axostyle, the shape of the nucleus, and the apparent absence of parabasal bodies. The rostral flagella of Teranymphidae are always longer than the postrostral flagella, both sets are of the same length in Retractinymphidae. The scattered axostyle fibers of Teranymphidae are absent from Retractinymphidae. While the nucleus of Teranymphidae is always rounded or oval, the drop-shaped nucleus of *R. glossotermitis* with its sharp end oriented toward the anterior cell pole is unique among members of the order Trichonymphida. While Teranymphidae possess numerous small, rounded parabasal bodies that are not in a specific relation to the nucleus, such structures are hardly visible in protargol-stained preparations of *R. glossotermitis*.

In addition to the family-specific traits, there are other traits that distinguish the genus *Retractinympha* from individual genera of Teranymphidae (*Teranympha*, *Eucomonympha*, and *Pseudotrichonympha*). While the parabasal filaments of *Pseudotrichonympha* appear as broad structures that run parallel to the basal body rows (Grimstone and Gibbons, 1966; Hollande and Carruette-Valentin, 1971), the parabasal filaments of *Retractinympha* are very fine fibers and rarely associated with the basal bodies. The rostral flagella are of uniform length in *Eucomonympha* and *Teranympha* but unequal (series 1 and 2) in *Retractinympha*. While the postrostral flagella of *Teranympha* are organized in multiple transverse rows that are separated by cytoplasmic bands (Koidzumi, 1921), *Retractinympha* shows the arrangement in tight longitudinal rows typically found also in other Trichonymphida. In addition, members of the genus *Teranympha* possess long axostylar bundles (Cleveland, 1938; Hollande and Carruette-Valentin, 1971; Carpenter and Keeling, 2007), whereas *Retractinympha* has no obvious axostyle.

The family Spirotrichosomidae

So far, members of Spirotrichosomidae (Hollande and Carruette-Valentin, 1971) had been detected exclusively in stolotermitids and in the genus *Cryptocercus*, which adds further support to their ancestral transfer from a stolotermitid to a serritermitid host (see above). The sister position of the genus *Heliconympha* to the spirotrichosomid flagellates of *Stolotermes victoriensis* (Figure 4) agrees with the morphological features shared by members of this family (Radek et al., 2018). Therefore, we propose to include the genus *Heliconympha* in the Spirotrichosomidae, which requires to emend the family description (see below).

Cryptocercus punctulatus harbors four species of spirotrichosomids (*Leptospironympha eupora*, *Leptospironympha rudis*, *Leptospironympha wachula*, and *Macrosporonympha xylopletha*, all described by Cleveland et al., 1934), but only a single rRNA gene sequence for an unspecified member of the genus *Leptospironympha* has been obtained (Carpenter et al., 2010). The four spirotrichosomids in *Stolotermes victoriensis* are *Spirotrichosoma capitata* Sutherland 1933, *Leptospironympha* (*Spirotrichosoma*) *obtusa* (Sutherland 1933), *Leptospironympha minor*

Cleveland and Day 1958, and *Leptospironympha numida* Cleveland and Day 1958. However, the three phylotypes of spirotrichosomids obtained from *Stolotermes victoriensis* (Izawa et al., 2017), which include both a large *Spirotrichosoma*-like species (designated SvUL; presumably representing the type species, *S. capitata*) and two smaller *Leptospironympha*-like species (SvUM and SvUS), form a tight cluster (<3% sequence dissimilarity), suggesting that they belong to the same genus. This would agree with the original description of *L. obtusa* as *Spirotrichosoma obtusa* by Sutherland (1933). Notably, the transfer of *S. obtusa* to the genus *Leptospironympha* Cleveland et al. 1934, which had been created to accommodate the three species of *Leptospironympha* from *C. punctulatus* (Cleveland et al., 1934), was based entirely on Sutherland's description. Cleveland et al. (1934) had actually cautioned that their own observations, albeit made from termites preserved in alcohol and hence not very dependable, did not indicate so close a relationship.

Only very few representatives of Spirotrichosomidae have been sequenced to date, but phylogenetic analyses indicate that the family is paraphyletic (Radek et al., 2018; this study). This possibility was raised already by Carpenter et al. (2010) based on the morphological features of *Leptospironympha* spp. described in *Cryptocercus* and *Stolotermes*. If future studies of the morphologically diverse species of spirotrichosomids confirm that the *Leptospironympha* spp. from *Cryptocercus* (comprising the type species, *L. eupora*) are monophyletic and sister to all spirotrichosomids from *Stolotermes*, the genus *Leptospironympha*, which had been included in Spirotrichosomidae by Hollande and Carruette-Valentin (1971), should be elevated to family level, and the *Leptospironympha* species from *Stolotermes* should be reclassified.

While the flagellar bands of Retractinymphidae and Teranymphidae are organized in straight or slightly slanted bands of flagella with single rows of basal bodies, the more basal Spirotrichosomidae possess spiral bands of flagella that contain many short rows of basal bodies (Čepička et al., 2016; Radek et al., 2018). Based on their ancestral position, it is likely that the spiral organization of Spirotrichosomidae was lost in a common ancestor of Retractinymphidae and Teranymphidae. A loss of the post-rostral spirals and a retention of the longitudinal secondary flagellar bands during the transition from Spirotrichosomidae to *Eucomonympha* had been suggested already by Carpenter et al. (2010). Alternatively, it is possible that the spiral organization has evolved twice independently in the spirotrichosomids of Cryptocercidae and Stolotermitidae.

The genus *Pseudotrichonympha* (Teranymphidae)

The genus *Pseudotrichonympha* comprises 23 described species and subspecies, and numerous representatives that remain to be described (Supplementary Figure S2; Supplementary Table S1). Quite a few species reportedly occur in several hosts but considering that all termites investigated to date harbor a unique phylotype (Noda et al., 2007; Saldarriaga et al., 2011; del Campo et al., 2017; Supplementary Figure S1), it is reasonable to assume that these reports will not hold up to scrutiny if different termite genera are concerned. Even in closely related hosts, the corresponding flagellates are divergent, as illustrated by the case of *Termitogeton planus*.

The presence of a flagellate of the genus *Pseudotrichonympha* in the genus *Termitogeton* (*T. umbilicatus*) was first reported by Grassi (1919). Almost a century later, the assignment was confirmed in a molecular study of *Termitogeton planus* (Noda et al., 2007), which identified the only gut flagellate of this termite as a distinct species in the radiation of the genus *Pseudotrichonympha*. Our characterization of *Pseudotrichonympha solitaria* did not reveal any structures that are unique to this species. Rather, it is the specific combination of features like variation in body size and form, lengths of the three series of flagella, lengths of apical cap, rostral tube and campanula, and size and position of the nucleus that allows the members of this genus to be distinguished (Das, 1976).

Pseudotrichonympha solitaria possesses all morphological traits shared by other species in the genus (see Brugerolle and Lee, 2000). The cells are large and slender, and except for the dome-shaped operculum, almost completely covered with longitudinal or slightly oblique rows of flagella. Also, other traits that are considered as genus-specific by some authors but not mentioned in most descriptions are present in *P. solitaria*. They include the instability of the thin-walled operculum during fixation or the stainable threads running along its sides (De Mello, 1954a,b). The latter probably correspond to the ring of slender lappets observed in the SEM images of *P. solitaria* but were absent from the few SEM images of other *Pseudotrichonympha* species (Saldarriaga et al., 2011).

Other fine structures, such as the sinus-like parabasal filaments underneath the postrostral rows of basal bodies and the lack of connections ("bandelettes cinétodesmales") between neighboring basal bodies match the detailed descriptions of other *Pseudotrichonympha* species (Grimstone and Gibbons, 1966; Hollande and Carruette-Valentin, 1971). The flexibility and torsion of the cell body observed in *P. solitaria* has been described for other species of the genus [e.g., *Pseudotrichonympha bachmani* (Calkins, 1936), *Pseudotrichonympha cardiformis* (Karandikar and Vittal, 1954)]. The ability to deeply retract the anterior cell pole, which is reflected in species epithet of *Pseudotrichonympha introflexibilis* (Dogiel, 1922), is present also in *Pseudotrichonympha leei* (del Campo et al., 2017) and in members of the eponymous genus *Retractinympha*. Such anterior retractions are considered to be elicited by abnormal conditions (Calkins, 1936), but although most pronounced in stained smears and older life preparations, they are common in fresh preparations of *R. glossotermitis*. The contractility itself may be a feature of the intracellular architecture. De Mello (1954b) considered so-called "myonemes," which he observed in stained specimens and described as single threads or dichotomously branched structures of whip-like bundles, to be responsible for the mobility of *Pseudotrichonympha*. However, we did not observe any myoneme-like structures in *R. glossotermitis* or *P. solitaria*.

Protologues

ZooBank number of publication: https://zoobank.org/urn:lsid:zoobank.org:pub:7EDA86B0-2C88-4D11-8391-602389366675.

Description of Retractinymphidae fam. nov. Radek and Brune

Taxonomy

Excavata, Parabasalia, Trichonymphea, Trichonymphida.

Etymology

N.L. fem. n. *Retractinympha*, a genus of flagellates. N.L. fem. n. *Retractinymphidae*, the family of *Retractinympha*.

Description

Cells completely covered by longitudinal rows of flagella. Rostral flagella not longer than postrostral flagella. First series of rostral flagella shorter than second series. Drop-shaped nucleus. Reduced axostyle. Parabasal bodies hardly visible.

Type genus

Retractinympha gen. nov.

ZooBank number:

https://zoobank.org/urn:lsid:zoobank.org:act:BD5481B7-9DA6-4B1A-A2A5-10CF3AE140A0.

Description of *Retractinympha* gen. nov. Radek and Brune

Taxonomy

Excavata, Parabasalia, Trichonymphea, Trichonymphida, Retractinymphidae.

Etymology

L. adj. *retractus*, perf. pass. part. of *retrahere*, to pull back, withdraw; L. fem. n. *nympha*, from Gr. *nýmphē* a beautiful maiden, nymph, common element of the genus names of hypermastigid flagellates in termite guts; N.L. fem. n. *Retractinympha*, a termite gut flagellate with a retractable rostrum.

Description

Large elongated cells with retractable rostrum. The genus is presently monospecific.

Type species

Retractinympha glossotermitis gen. nov. sp. nov.

ZooBank number:

https://zoobank.org/urn:lsid:zoobank.org:act:1231DFF9-D6D9-4ABA-A53A-94A78BD7650D.

Description of *Retractinympha glossotermitis* sp. nov. Radek and Brune

Taxonomy

Excavata, Parabasalia, Trichonymphea, Trichonymphida, Retractinymphidae, *Retractinympha*.

Etymology

N.L. gen. n. *glossotermitis*, referring to *Glossotermes*, the genus of termites colonized by this flagellate species.

Description

Spindle-shaped body measuring 107–260 (mean 163) µm in length and 60–92 (mean 70) µm in width. Short rostral flagella in series 1. Rostral flagella of series 2 and postrostral flagella have the same length of about 22–28 µm (mean 25 µm). The rostral tube measures 12–15.5 µm in length. Drop-shaped nucleus (mean 45.6 × 25.6 µm) located marginally in the upper part of the post-rostral body region; pointed end oriented toward the rostrum. Dictyosomes are neither associated with rows of basal bodies nor with parabasal filaments. Parabasal filaments are thin and rather straight. Axostyle not found.

Type host

The hindgut of *Glossotermes oculatus* Emerson 1950 (Serritermitidae).

Type host locality

The termites were collected near the Petit-Saut Dam south of Sinnamary (5.0662° N 53.0460° W) and in the Nouragues Natural Reserve (4.0717° N 52.7325° W) in French Guiana.

Hapantotype

Protargol-stained microscopy slide deposited at the Biology Centre of the Upper Austrian Museum, J.-W.-Klein-Strasse 73, 4040 Linz, Austria under type number 2019/66.

Gene sequences

SSU rRNA gene sequence accession numbers KY750730, KY750733.

ZooBank number:

https://zoobank.org/urn:lsid:zoobank.org:act:C170AEB2-19FE-405C-8615-851EADDAD8AF.

Description of *Pseudotrichonympha solitaria* sp. nov. Radek and Brune

Taxonomy

Excavata, Parabasalia, Trichonymphea, Trichonymphida, Teranymphidae, *Pseudotrichonympha*.

Etymology

L. fem. adj. *solitaria*, lonely, solitary; the only flagellate species in its termite host.

Description

Long, slender, completely flagellated cells (140–235 × 14–30 µm) with three series of flagella. Flagella at the tip of the rostrum (series 1) are very short and partially covered by leaf-like lappets of the operculum. Flagella at the base of the rostrum (series 2) are much longer (*ca*. 28 µm). Postrostral flagella (series 3) are *ca*. 13 µm long. Rostral tube of about 17 µm length. Oval nucleus (9.3–13.7 × 5.3–9.4 µm; mean 11.8 × 7.2 µm) in anterior third of body.

Type host

The hindgut of *Termitogeton planus* (Haviland 1898) (Rhinotermitidae), COII gene accession number MN528021.

Type host locality

West Papua, Indonesia, 30 km southeast of Nabire (3°29.213408′ S, 135°42.089227′ E).

Syntype

Protargol-stained microscopy slide deposited at the Biology Centre of the Upper Austrian Museum, J.-W.-Klein-Strasse 73, 4040 Linz, Austria under type number 2019/63.

Gene sequences

SSU rRNA gene accession numbers MN523346 (symbiont of type host; this study) and AB262492 (symbiont of *T. planus* from Malaysia; Noda et al., 2007).

ZooBank number:

http://zoobank.org/urn:lsid:zoobank.org:act:3567DB03-6C24-476A-AB52-F2FAEE010091.

Emended description of Spirotrichosomidae Hollande and Carruette-Valentin 1971

The description of the family is the same in the original description (Hollande and Carruette-Valentin, 1971), with the following addition:

Included genera

Apospironympha Cleveland and Day 1958; *Bispironympha* Bobyleva 1969; *Colospironympha* Cleveland and Day 1958; *Heliconympha* Radek et al. 2018; *Leptospironympha* Cleveland et al. 1934; *Macrospironympha* Cleveland et al. 1934; and *Spirotrichosoma* Sutherland 1933.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

RR and AB conceived the study and wrote the manuscript. JŠ, DS-D, and RH collected termites. RR and DÖ performed the structural analyses. KP and AB performed the molecular work and the phylogenetic analyses. All authors have read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1111484/full#supplementary-material

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EDITED BY
Alberto Arab,
Federal University of ABC, Brazil

REVIEWED BY
Fabiana E. Casarin,
Federal University of São Paulo, Brazil
Paulo Cristaldo,
Universidade Federal Rural de Pernambuco, Brazil

*CORRESPONDENCE
Sang-Bin Lee
lsb5162@ufl.edu

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Foraging proportion of the Formosan subterranean termite workers and soldiers in relation to soil type

Sang-Bin Lee * and Nan-Yao Su

Ft. Lauderdale Research and Education Center, Department of Entomology and Nematology, University of Florida, Fort Lauderdale, FL, United States

A colony of subterranean termites contains different castes such as workers and soldiers that perform various tasks. Foraging activity is vital among many tasks of a colony and ~20% of the worker population in a colony of *Coptotermes formosanus* engage in foraging. Although flexibility in task allocation plays a crucial role in maintaining colony function in fluctuating environments, such flexibility is barely understood in subterranean termites. Here, we investigated regulations of foraging task allocation in response to different soil types at the nest and the foraging site. At the nest and foraging site, either nitrogen-rich organic soil or nitrogen-poor sand, which differed in carbon and nitrogen ratio, was provided and the proportions of workers and soldiers at the foraging site were determined. Our results showed that the foraging worker proportion and per-capita wood consumption significantly differed depending on the soil type at the nest but not the soil type at the foraging site. When the colony had access to organic soil at the nest, the proportion of workers at the foraging site and per-capita wood consumption was much smaller than those with sand at the nest. Conversely, the proportion of soldiers at the foraging site remained the same regardless of the soil type at the nest and the foraging site. In brief, the current study showed flexible regulation of foraging task allocation in *C. formosanus* and demonstrated that perturbation of soil type alters the allocation of workers, but not soldiers, in the colony.

KEYWORDS

task allocation, *C. formosanus*, subterranean termite, flexibility, foraging

Introduction

Social insects are one of the most successful organisms in various terrestrial ecosystems and their success is often explained by an elaborate task division system in the colony (Hölldobler and Wilson, 2009). Division of labor in insect societies allows the colony members to perform several tasks simultaneously, such as reproduction, foraging, brood care, and defense with highly specialized individuals (Oster and Wilson, 1978; Porter and Tschinkel, 1985; Jeanne, 1986; Robinson, 1992; Beshers and Fewell, 2001). A total of two major factors are critical for individual task determination: age (age polyethism) and morphology (morphological polyethism) (Oster and Wilson, 1978; Robinson, 1992). Age polyethism is observed across various social insects (Free, 1964; Oster and Wilson, 1978; McMahan, 1979; Gerber et al., 1988; Moritz, 1988; Noirot, 1989; Hölldobler and Wilson, 1990; Seeley and Kolmes, 1991; Robinson, 1992; Beshers and Fewell, 2001), whereas morphological polyethism is not as common as age polyethism and is found only in a few

ant species and nearly all termites (Oster and Wilson, 1978; Wheeler, 1986; Noirot, 1989; Hölldobler and Wilson, 1990).

Foraging is an important task for survival and maintaining a colony, but it is a costly and risky task (Fewell, 1988; Korb and Linsenmair, 2002; Rytter and Shik, 2016; Pyke and Starr, 2021). Individuals are required to leave the safety of the nest in search of food resources, and they need to bring back food to provision the entire colony. In many social insects, foraging activities are performed by older individuals and only a small portion of colony members is often allocated to the foraging task (Golley and Gentry, 1964; Erickson, 1972; Rogers et al., 1972; Lewis et al., 1974; Bruin et al., 1977; Porter and Jorgensen, 1981; MacKay, 1983; Beekman et al., 2004; Du et al., 2017a; Su et al., 2017; Lee et al., 2022). If the environments are stable (i.e., no disturbances), the colony would maintain a certain proportion of individuals in the foraging task. By doing so, the colony may be able to achieve high efficiency in foraging with specialized individuals. However, the task allocation in the colony of social insects is known to be flexible, and the colony is capable to adjust the proportion of individuals in foraging task in response to changes in internal demand and external conditions (Robinson, 1992; Gordon, 1996, 2016; Beshers and Fewell, 2001; Loftus et al., 2021). Therefore, flexibility in task allocation plays a crucial role in the colony to maintain its function against such changes (Middleton and Latty, 2016).

Among many factors that could affect foraging task allocation, food quality has been widely explored in diverse social insects because optimal foraging theories predict that it would be advantageous if the colony consumed nutritious food while expending the same amount of energy in foraging (Kay, 2002; Pyke and Starr, 2021). When the food quality is not suitable or food becomes unavailable, foragers in a honeybee colony cease foraging activities (Von Frisch, 1967). On the other hands, several ant species increase the number of foraging individuals once they have access to food that has a high concentration of sucrose (Wilson, 1962; Szlep and Jacobi, 1967; Hangartner, 1969, 1970; Breed et al., 1987; De Biseau et al., 1991; Beckers et al., 1993), suggesting that food quality could play a vital role in regulating the number of foragers in ant colonies. Assuming the colony size does not change drastically in a short period of time, an increased or decreased number of foragers could indicate flexible foraging task allocation depending on food quality. In addition to food quality, the amount of food stored in the colony also affects foraging activities in honeybees. For instance, bee foragers make a decision whether to remain inside the hive or forage out based on the amount of nectar stored (Seeley, 1989; Seeley et al., 1991). However, it is elusive as to how the colony of subterranean termites adjusts the proportion of foraging workers in response to different food sources.

Contrary to other social insects, the impact of food quality on the proportion of foragers in termites is a challenging subject to study. This is largely because subterranean termites do not have many food options since they primarily feed on wood, either partially decayed by wood-decaying fungi (e.g., *Reticulitermes*) or living trees (e.g., *Coptotermes*) (Lenz, 1994). Wood is naturally very poor in terms of nutrition as the majority of nutrients in this diet are carbon with an extremely low amount of nitrogen (Matsumoto, 1976; La Fage and Nutting, 1978). However, it has been suggested that carbon and nitrogen balance is an important factor in determining colony size, and species with a better carbon–nitrogen balance tend to grow larger than those with a poor balance (Higashi et al., 1992). The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is known to form large colonies, with some mature colonies having millions of individuals (Su and Scheffrahn, 1988) and grow rapidly in a few years (Chouvenc and Su, 2014). The large colony size of this species implies that it has a better source of dietary nitrogen. Along with this speculation, a recent study on the dietary nitrogen of *C. formosanus* showed that colonies that have access to nitrogen-rich organic soil were able to grow eight times larger than colonies reared in nitrogen-poor sand (Mullins et al., 2021).

Subterranean termites such as *C. formosanus* build nests below ground or inside trees and forage for multiple food resources simultaneously over long distances through underground tunnels (King and Spink, 1969; Su and Scheffrahn, 1988). Mature colonies of *Coptotermes formosanus* maintain foraging abilities over decades even with the change in demographic compositions and breeding structures (Chouvenc et al., 2022). In *C. formosanus*, it has been reported that 10% to 20% of old workers in the colony perform foraging task and these foragers displayed task division (Du et al., 2017b; Lee et al., 2021, 2022). The foraging task allocation in the colony of *C. formosanus* showed resiliency against disturbances, and the colony maintained ∼20% of foraging workers despite the change in colony size and demographic composition due to the periodical removal of foragers (Lee et al., 2022).

In a termite colony, soldiers mainly perform defense regardless of evolutionary history from basal to derived, to protect nestmates from predators, and soldiers in termites have highly modified mandibles for defensive purposes (Tian and Zhou, 2014). While recent studies have shown soldiers in some termite species also act as scouts and actively participate in the foraging process (Casarin et al., 2008; Almeida et al., 2016; Sacramento et al., 2020). Different termite species have a specific soldier ratio in the colony which has evolved through the selection process and colonies maintain it through physiological constraints (Haverty, 1977). Workers are accompanied by soldiers when forage and ∼10% of individuals in a colony of *C. formosanus* are soldiers (Haverty, 1977). However, it is poorly understood how the colony of termites regulates the proportion of soldiers in a foraging group. If a high proportion of soldiers involves in foraging activity, it can be a burden to workers at the foraging site because soldiers must be fed by workers (Haverty, 1977, 1979; Haverty and Howard, 1981).

In this study, we investigated how colonies of *C. formosanus* regulate the proportion of workers and soldiers at the foraging site in response to different soil types at the nest and the foraging site. We prepared two distinct types of nest and foraging site, each filled with either nitrogen-rich organic soil or nitrogen-poor sand, yielding four different combinations. We hypothesized that (1) foraging worker proportion will be higher in colonies that have access to nitrogen-rich organic soil at the foraging site than those of colonies that have access to the sand foraging site. However, (2) soldier proportion at the foraging site will be stable regardless of soil type at the foraging site and the nest.

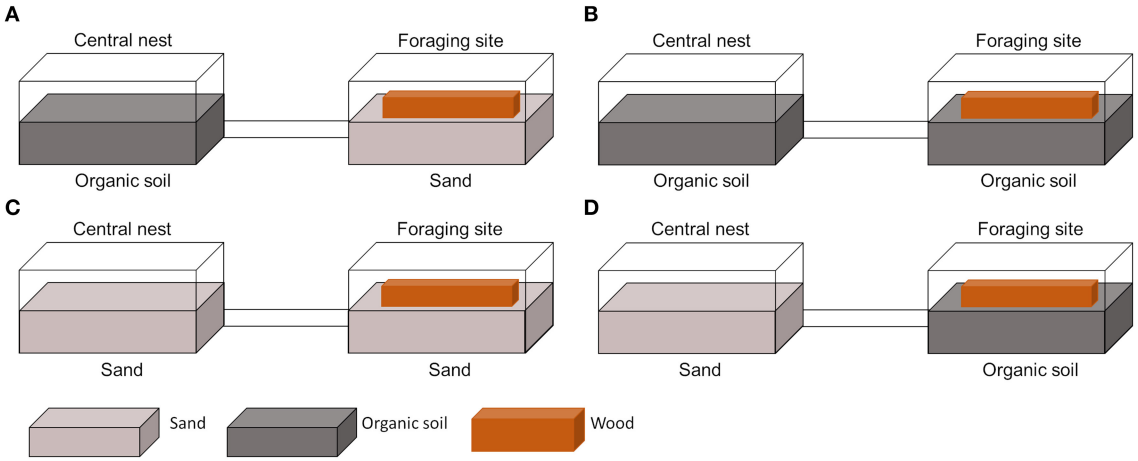

FIGURE 1
Schematic drawings of different nutrition availabilities at the nest and the foraging site. The left and right containers are defined as the nest and the foraging site, respectively. Light black and gray colors indicate nitrogen-rich organic soil (OS) and nitrogen-poor sand (S), respectively. The nest and foraging site were connected by a 3 m plastic tube, and a colony of *Coptotermes formosanus* was introduced into the nest. In total, four different conditions were examined, and 14 colonies were used as follows: **(A)** organic soil at both the nest and foraging site (OS–OS; three colonies), **(B)** organic soil at the nest and sand in the foraging site (OS–S; three colonies), **(C)** sand at the nest and organic soil in the foraging site (S–OS; four colonies), and **(D)** sand at both nest and foraging site (S–S; four colonies). As a food source, a piece of wood of the same size was provided for all experiments.

Materials and methods

Colony establishment

Several hundred winged images (i.e., alates) of *C. formosanus* were collected in Broward County, FL, USA, during the dispersal flight season (May 17th, 2016), using a light trap. Collected termites were immediately brought back to the laboratory to morphologically identify sexes. At the same time, hundreds of rearing units (8 × 2.5 cm, height × diameter, IntraPac, Plattsburgh, New York, United States) were prepared which contain 6 g of moistened organic soil at the bottom (3 cm high) and four pieces of wood (5 × 0.5 × 0.5 cm^3, *Picea* sp.) on top of the soil, and an agar solution (3%) was poured over the top of soil and wood pieces in each rearing unit. After 2 h, once agar solidified, a pair of reproductives was introduced into the vials and kept at 28 ± 1 °C. After 6–8 months, hundreds of surviving colonies were transferred to large vials (6.3 × 4.6 cm, height × diameter, IntraPac, Plattsburgh, New York, United States), containing the same components but in greater amounts.

One year after colony foundation, surviving colonies were further transferred to a container box (1.5 L, 17 × 12 × 7 cm, Pioneer Plastics, Dixon, Kentucky, United States), which contained moistened organic soil at the bottom (3–4 cm high) and a piece of wood (14.5 × 4 × 1 cm, *Picea* sp.), to accommodate colony growth. Prior to placing the vials in the box, each vial was equipped with a modified reproductive excluder as a lid, which allowed only workers and soldiers to pass through (Lee et al., 2019). The vial was, then, placed horizontally at the bottom corner of the container box. Weekly inspections were performed on colonies, and water and wood were replenished as needed. All colonies were maintained at 28 ± 1°C and 80 ± 2% humidity.

Bioassay to measure the proportion of termites

Prior to starting experiments (2 months before), the 2-year-old *C. formosanus* colonies were processed to count the number of termites in the colony, in order to choose similar-sized colonies and verify the presence of reproductives. We, then, prepared an artificial nest (hereafter: nest) using a container box (1.5 L, 17 × 12 × 7 cm, Pioneer Plastics, Dixon, Kentucky, United States) filled with either nitrogen-rich organic soil (Nature's Care, organic & natural potting mix, Scotts Miracle-Gro, Marysville, Ohio, United States) or nitrogen-poor sand. Both organic soil and sand were filled up to 5 cm deep and moistened with deionized water. A hole was drilled in the side of the container.

A piece of wood (14.5 × 4 × 1 cm, *Picea* sp.) was provided as food for 2 weeks prior to experiments. In the meantime, an artificial foraging site (hereafter: foraging site) was also prepared in the same manner as nests (described above). A piece of wood was dried in the oven at 60°C for 48 h and dry weight was measured. Then, it was soaked in the water for 3 days and placed at the foraging site on top of either organic soil or sand. After preparing the foraging site, the wood at the nest was removed, and two container boxes were connected by plastic tubes (3 m in length) (Figure 1). In the current study, a total of four different combinations were tested as follows: (1) organic soil nest and sand foraging site (OS–S) (Figure 1A), (2) organic soil nest and organic soil foraging site (OS–OS) (Figure 1B), (3) sand nest and sand foraging site (S–S) (Figure 1C), and (4) sand nest and organic soil foraging site (S–OS) (Figure 1D).

We observed that all colonies had reached the foraging site and started feeding on wood within a week. Then, the pieces of wood at the foraging site were removed and replaced with another piece of wood (14.5 × 4 × 1 cm, *Picea* sp.), prepared using the

same methods described above. All experimental units (in total: 14 colonies) units were covered with black plastic throughout the entire span of the experiment to prevent any disturbance, and temperature and humidity were kept at 28 ± 1°C and 80 ± 2%, respectively.

Data collection

The carbon and nitrogen ratio (C/N ratio) of sand and organic soil was measured using an elemental analyzer (LECO, CN628, MI, United States). Both sand and organic soil were dried in the oven at 60°C for a week, and a standard reference for the calibration was conducted using Ethylenediaminetetraacetic acid (EDTA) calibrator prior to the measurement and in between measurements of sand and organic soil.

The number of workers and soldiers at the foraging site was recorded every 3 days (i.e., 72 h interval). To count the number of termites at the foraging site, the connecting tubes between the nest and foraging site were clipped at the distal end of the foraging site. After counting, termites were returned to the foraging site. In total, we counted the number of termites at the foraging site five times. After the fifth count (i.e., 15 days), colonies were processed to determine the total number of termites in the colony and calculate the proportion of foraging workers and the proportion of soldiers at the foraging site. Larvae (the first and second instar) and pre-soldiers were counted as workers and soldiers, respectively. Wood at the foraging site was washed with water to remove debris and dried in the oven at 60°C for 48 h, and it was weighed to determine the amount of wood consumption.

The foraging worker proportion was calculated by the number of workers at the foraging site divided by the total number of workers in the colony (number of workers at the foraging site and the nest). Soldier proportion at the foraging site was determined in the same manner, with the number of soldiers at the foraging site divided by the total number of soldiers in the colony. Per-capita wood consumption (mg of wood consumed/number of termites) was also calculated. For the calculation of wood consumption, the amount of wood consumption (mg) was divided by the total number of termites in the colony.

Statistical analysis

In total, 14 colonies were examined [three colonies for OS–S (nest–foraging site), three colonies for OS–OS, four colonies for S–S, and four colonies for S–OS], and the number of termites in colonies in each combination was subjected to Kruskal–Wallis test ($P < 0.05$), to confirm no difference on colony size. In addition to the colony size, the carbon and nitrogen ratio of the sand ($n = 5$) and the organic soil ($n = 5$) was compared with a t-test ($P < 0.05$). Foraging worker proportion and soldier proportion at the foraging site were fitted to linear mixed models (LMMs), with the soil type (i.e., sand or organic soil) at the nest and foraging site treated as fixed effects, and both sampling date and colony as random effects to account for repeated measurements. In the LMMs, the fixed effect was treated as a simple main effect. Then, type II analysis of variance (ANOVA) using the Wald chi-square test was performed for foraging worker proportion and proportion of soldiers at the foraging site to determine the statistical significance of each explanatory variable. We also calculated the confidence interval of the foraging worker proportion and soldier proportion at the foraging site depending on the soil type at the nest and the foraging site. Per-capita wood consumption was subjected to a two-way analysis of variance (ANOVA) with soil type at the nest and foraging site as factors. All statistical analyses were performed in R 4.1.3 (Team, 2022).

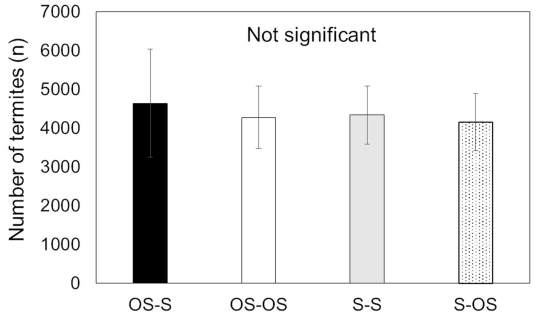

FIGURE 2
The average number of termites in colonies of *C. formosanus* used in this study. In total, four different conditions were tested using 14 different colonies. OS (organic soil) and S (sand) indicate the soil type of either the nest or the foraging site, from left (nest) to right (foraging site). The number of termites in each condition was compared with the Kruskal–Wallis test and showed no significant differences.

TABLE 1 Average percentages (mean ± sd) of carbon and nitrogen in organic soil and sand used in this study.

	Carbon (%) ($n = 5$)	Nitrogen (%) ($n = 5$)
Sand	0.0025 ± 0.0008 A	0.0000 ± 0.0000 A
Organic soil	16.9982 ± 2.2440 B	0.5991 ± 0.0725 B

Carbon and nitrogen ratio was measured using LECO CHN analyzer, and total five samples for organic soil and sand were used. Both sand and organic soil were dried in the oven (60°C) for 48 h before measurements. Different upper-case letters across column denote significant difference according to t-test ($P < 0.05$).

Results

In the current study, we used colonies with no statistical differences in colony size (i.e., number of termites in the colony) to exclude any effect from colony size ($W = 0.329$, $df = 3$, $P = 0.9546$, Figure 2), which allowed us to solely focus on the proportion of termites at the foraging site depending on soil type at the nest and foraging site. A comparison of carbon and nitrogen percentage showed that organic soil contained significantly more carbon and nitrogen than sand [Table 1; carbon (%): $t = -16.935$, $df = 10$, $P < 0.01$; nitrogen (%): $t = -18.482$, $df = 10$, $P < 0.01$].

Foraging worker proportions were significantly different between soil types at the nest ($\chi^2 = 14.217$, $df = 1$, $P < 0.01$; Figure 3A) but not at the foraging site ($\chi^2 = 0.003$, $df = 1$, $P = 0.954$; Figure 3B). When we a calculated 95% confidence interval (CI) based on the soil type at the nest, the CI of foraging worker

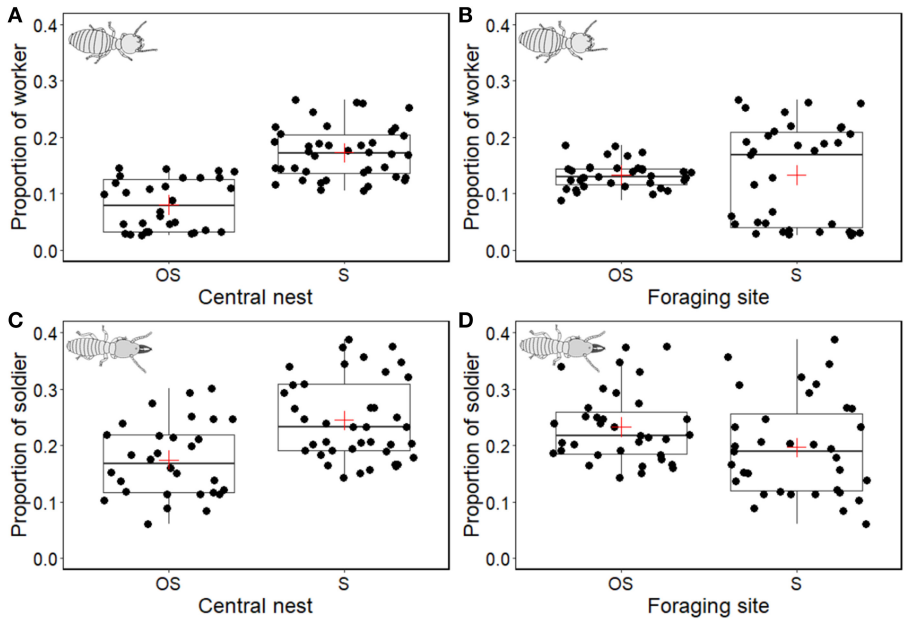

FIGURE 3
Box plots of the proportion of workers **(A, B)** and soldiers **(C, D)** at the foraging site in different nutrition availabilities. In total, 14 colonies of *C. formosanus* were tested [three colonies: OS (organic soil)–S (sand), from left to right, nest and foraging site, respectively; three colonies: OS–OS; four colonies: S–S; four colonies: S–OS]. The proportion of workers at the foraging site was calculated by the number of workers at the foraging site divided by the total number of workers in the colony. The proportion of soldiers at the foraging site was calculated in the same manner. Black dots indicate each collected data points from each sampling, and the red cross represent the mean and thick lines inside boxes indicate the median value of the data.

proportion at the organic soil nest was 0.08 ± 0.03 (mean ± CI) and at the sand nest was 0.17 ± 0.03 (mean ± CI). Based on the soil type at the foraging site, the CI of foraging worker proportion at the sand foraging site was 0.13 ± 0.06 (mean ± CI) and that at the organic soil foraging site was 0.13 ± 0.01 (mean ± CI). The foraging worker proportion was the greatest when both the nest and foraging site had sand (20.42 ± 2.60%, mean ± s.d), whereas the lowest foraging worker proportion was observed in the combination of the organic soil nest and the sand foraging site (3.94 ± 0.72%, mean ± s.d).

Contrary to foraging worker proportion, we found no significant fixed effects to explain soldier proportion at the foraging site according to the LMMs. It showed that the soil type at the nest ($\chi^2 = 3.505$, $df = 1$, $P = 0.061$; Figure 3C) and at the foraging site ($\chi^2 = 0.960$, $df = 1$, $P = 0.327$; Figure 3D) did not affect soldier proportion at the foraging site. The CI of soldier proportion at the foraging site overlapped, and it was 0.17 ± 0.05 (mean ± CI) at the organic soil nest and 0.24 ± 0.05 (mean ± CI) at the sand nest. When we calculated based on soil type at the foraging site, the CI of soldier proportion was 0.19 ± 0.06 at the sand foraging site and 0.25 ± 0.05 at the organic soil foraging site.

Similar to the proportion of workers at the foraging site, per-capita wood consumption showed that soil type at the nest was a significant factor ($F = 22.794$, $df = 1, 10$, $P < 0.01$), and the interaction of nest and foraging site was also significant ($F = 6.675$, $df = 1, 10$, $P < 0.05$). However, soil type at the foraging site did not affect per-capita wood consumption ($F = 1.843$, $df = 1, 10$, $P = 0.204$; Figure 4). Termites consumed the greatest amount of wood when both nest and foraging site contained sand, which was approximately three times greater than that consumed in organic soil nest and sand foraging site (Figure 4).

Discussion

Task allocation is an important mechanism in insect societies to maintain colony function in response to any change inside and outside the colonies, and flexible regulation of task allocation in the colony organized by individual's collective behaviors without hierarchical control plays an important role in the resiliency of the colony (Gordon, 1996; Middleton and Latty, 2016). Our current study found two important results about task allocation in the Formosan subterranean termite, *Coptotermes formosanus*. First, a colony of *C. formosanus* is capable of adjusting the proportion of foraging workers in response to the soil type at the nest. Contrary to the foraging worker proportion, the soldier proportion at the foraging site, however, was maintained at 10–20% regardless of nutrition availabilities at both the nest and foraging site. In this study, it was assumed that the food requirements of colonies remained the same, and colony demography is likely similar across colonies since we used colonies with no statistical difference in colony size and age (Figure 2).

When the colony had organic soil at the nest, the foraging worker proportion was significantly smaller than those of sand (Figure 3A). A recent study on colony growth in *C. formosanus* revealed a colony with access to organic soil was approximately three times larger in biomass and eight times larger in population size than colonies that were reared in the sand, suggesting that *C. formosanus* utilized organic matter from the organic soil (Mullins et al., 2021). Assuming similar-sized colonies require the same amount of food to provision all colony members (Patel et al., 2020), the low foraging worker proportion found in colonies with organic soil nest implies that these colonies

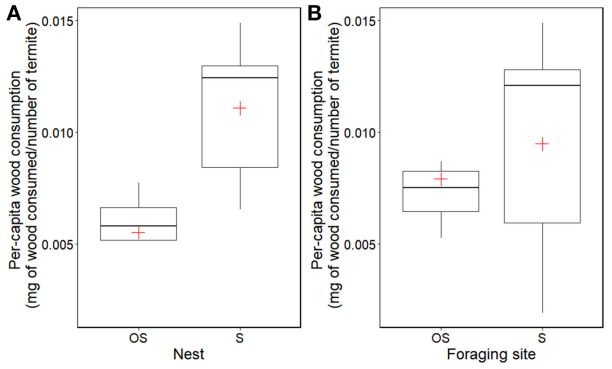

FIGURE 4
Per-capita wood consumption (mg of wood consumption/number of termites) of *C. formosanus* in different combinations during experiments. The amount of wood consumption was calculated by subtracting the weight of wood pre and post experiments: **(A)** nest, **(B)** foraging site. The number of termites was determined after the experiment by counting all individuals in each colony. Total of 14 colonies of *C. formosanus* were used. OS and S indicate organic soil and sand, respectively. Cross with red color and thick lines inside boxes represents the mean and median values of the data.

were able to provision colony members without sending out a large proportion of workers to forage for food in comparison to colonies with the sand nest (Figure 3). This result was further supported by our wood consumption analysis that the per-capita wood consumption of colonies with organic soil nest was significantly lower than those of colonies with sand nest (Figure 4). Allocating many workers to risky foraging task may be less advantageous if they have any available resources near the nest, as they could potentially encounter predators such as ants while foraging.

Contrary to the nest, the soil type at the foraging site did not significantly affect the proportion of foraging workers (Figure 3). This result suggested that once the colony started the foraging process due to the potential depletion of food resources near the nest, colonies will maintain a 10–20% proportion of foraging workers in the colony. It has been reported that colonies of *C. formosanus* could reduce the risk of worker loss caused by natural disturbances or anthropogenic events such as pesticide applications by allocating up to 20% of their workers to foraging task, while also achieving high foraging efficiency within their worker force (Lee et al., 2022). In addition, per-capita wood consumption found in the present study further showed no statistical differences when colonies had access to either organic or sand foraging site (Figure 4). In the case of sand foraging site and nest, the colony has to be entirely reliant on a piece of wood for food by sending out the maximum proportion of forging workers, whereas the colony with sand nest and organic soil foraging site might be able to utilize the organic matter from the foraging site. However, our results showed that both colonies did not differ in per-capita wood consumption (Figure 4), indicating colonies that provided either organic soil or sand at the foraging site consumed a similar amount of wood.

Because subterranean termites such as *Coptotermes* and *Reticulitermes* mainly feed on woody materials including living trees and decayed wood, the impact of food quality and quantity on foraging in a colony of subterranean termites is not well understood. However, a handful of termites in the family of Termitidae has shown that resource density and suitability affect their foraging (Araújo et al., 2011; Almeida et al., 2018). When *Nasutitermes* aff. *coxipoensis* colonies had access to low-resource density, termites constructed longer and more foraging trails, suggesting increased searching efforts (Almeida et al., 2018). On the other hand, when termites had a high density of food resource, shorter and less trails were observed and showed optimization of the tunnel in response to resource density (Almeida et al., 2018). Similarly, *Cornitermes cumulans* (Kollar) excavated tunnels intensively when resource suitability is low, whereas tunnel length, number, and tunneling speed decreased when the resource is abundant (Araújo et al., 2011). Although the impact of resource suitability and density on tunnel formation in subterranean termites is poorly understood, several optimizations on tunnel geometry and behaviors during tunnel constructions have been reported in *C. formosanus* (Lee et al., 2006, 2020; Michael et al., 2023).

This observation raises a question of why *C. formosanus* did not increase their foraging worker proportion when they have the sand nest and the organic soil foraging site to maximize cost and benefit in foraging. *Coptotermes formosanus* is known to construct underground nests, and they could inhabit multiple pieces of wood over long distance (King and Spink, 1969; Su and Scheffrahn, 1988). Therefore, *C. formosanus* continuously encounters soil near the nest and during underground tunnel excavations. Despite ubiquitous soil, it is possible that the acquisition of organic matters from soil may only occur near the nest, and foraging workers may focus on finding and feeding on pieces of wood at a remote feeding site to provision the colony. Since older workers in the colony of *C. formosanus* are distributed farther away from the nest for foraging (Su et al., 2017), young workers remain near the nest, and they could participate in organic matter acquisition from the soil near the nest. However, future study is warranted to study such possibilities.

Compared to workers, we found different patterns from soldiers in *C. formosanus*. Soil type at the nest and foraging site did not have any impact on soldier proportion at the foraging site. Soldiers in termites, excluding soldierless termites in the family of Termitidae, are morphologically specialized for the defense task (Eggleton, 2010). In some termite species, soldiers also perform as scouts in the foraging group (Traniello, 1981; Traniello and Leuthold, 2000; Casarin et al., 2008; Almeida et al., 2016; Sacramento et al., 2020) and could help the tunneling process (Janowiecki and Vargo, 2022). Acting as a scout and helping with tunneling behavior could reduce predation risk in the early stage of foraging (Traniello, 1981). Soldiers in termites are fully dependent on workers for nutrition because of modified mandibles for a defensive purpose (Noirot and Darlington, 2000; Eggleton, 2010). Under normal conditions (i.e., no predation risk), a high number of soldiers at the foraging site will impose a burden on workers as they need to provide food to soldiers. A higher number or proportion of soldiers could reduce the potential predation risk, preventing the

cost of foraging during the early phase (Almeida et al., 2016) and may improve the decision of foraging (Sacramento et al., 2020). Despite the aforementioned studies reported the effect of soldiers in foraging, a recent study, however, showed that the presence of soldiers and different proportions of soldiers in *C. formosanus* did not affect the tunneling behaviors of workers (McCarthy et al., 2023). Therefore, it is possible that the proportion of soldiers in the foraging group could be set intrinsically at 10–20%, to maximize the protection of foraging workers from predation risk while limiting the burden on workers. As predicted, morphological polyethism could be less flexible to environmental factors compared to age polyethism (Oster and Wilson, 1978).

In conclusion, the current study showed the same perturbation could lead to different responses depending on castes in termites. Workers need to forage for food to provision their nestmates including the queen, king, younger broods, and soldiers, while soldiers are in charge of protecting their nestmates at the foraging site. Therefore, foraging worker proportion varied in different nutrition availability at the nest, whereas soldier proportions at the foraging site were maintained stable regardless of soil type in *C. formosanus*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

S-BL and N-YS conceptualized and designed the study, interpreted the results together, and wrote the first draft. S-BL carried out experiments, data collection, and data analysis. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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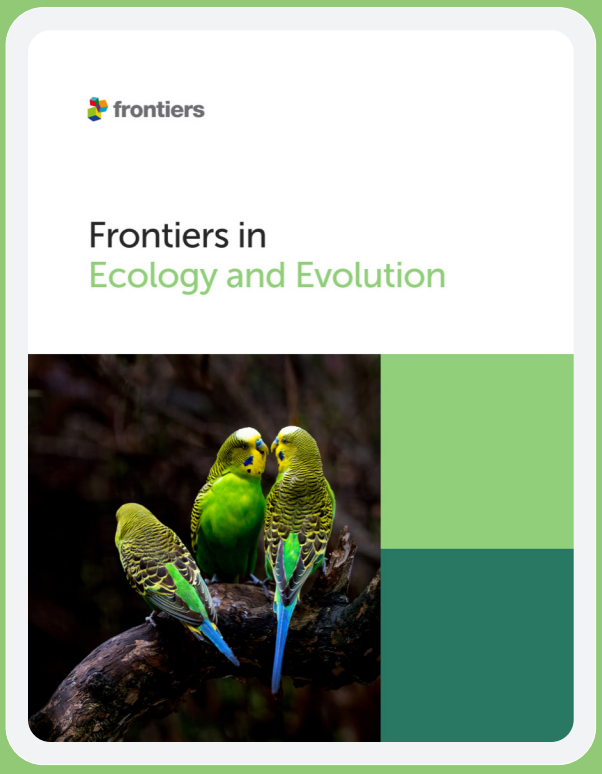

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