Cooperative Breeding in the Ambrosia Beetle *Xyleborus affinis* and Management of Its Fungal Symbionts

Peter H. W. Biedermann[1,2,3]*

[1] Research Group Insect-Fungus Symbioses, Department of Animal Ecology and Tropical Biology, University of Würzburg, Würzburg, Germany, [2] Chair of Forest Entomology and Protection, University of Freiburg, Freiburg, Germany, [3] Southern Research Station, USDA Forest Service, Pineville, LA, United States

Fungus-farming is known from attine ants, macrotermites, and ambrosia beetles (Scolytinae, Platypodinae). Farming ant and termite societies are superorganismal and grow fungal cultivars in monocultures. Social organization of ambrosia beetle groups and their farming systems are poorly studied, because of their enigmatic life within tunnel systems inside of wood. Ambrosia beetle-fungus symbioses evolved many times independently in both the beetles and their fungal cultivars. Observations suggest that there is evolutionary convergence between these lineages, but also a high variation in the degree of sociality and the modes of fungiculture. Using a laboratory observation technique, I here tried to give insights into the social system and fungus symbiosis of the sugar-cane borer, *Xyleborus affinis* Eichhoff (Scolytinae: Curculionidae), a currently poorly studied ambrosia beetle. The study revealed a cooperatively breeding system characterized by delayed dispersal of adult daughters, alloparental brood care by larvae and adults, and about half of the totipotent adult daughters laying eggs within the natal nest. Most interesting, there was a tendency of egg-laying females to engage more commonly in mutually beneficial behaviors than non-egg-layers. Fungus gardens covering gallery walls composed of five different filamentous fungi. A *Raffaelea* isolate was predominant and together with an unidentified fungus likely served as the main food for adults and larvae. Three isolates, a *Mucor*, a *Fusarium* and a *Phaeoacremonium* isolate were most abundant in the oldest gallery part close to the entrance; *Mucor*, *Fusarium* and the *Raffaelea* isolate in diseased individuals. Additionally, there was correlative evidence for some fungal isoaltes influencing beetle feeding and hygienic behaviors. Overall, *X. affinis* is now the second ambrosia beetle that can be classified as a cooperative breeder with division of labor among and between adults and larvae.

Keywords: cooperative breeding, bark beetle, insect agriculture, symbiosis, fungus community, social behavior, fungus-farming, mutualism

INTRODUCTION

Advanced fungus agriculture by insects has evolved once in ants, once in termites and more than a dozen times independently in wood-boring weevils (Mueller et al., 2005; Hulcr and Stelinski, 2017; Vanderpool et al., 2018; Biedermann and Vega, 2020). The latter are jointly termed ambrosia beetles, even though they are a polyphyletic group and their mutualisms with fungi evolved multiple times independently (in both beetles and fungi; Hulcr and Stelinski, 2017) and every beetle-fungus combination may have a different farming system (Harrington, 2005; Six, 2012; Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). In the best studied ambrosia beetle lineage, the Xyleborini (Curculionidae: Scolytinae), fungus-farming evolved along with social behavior and in some cases division of labor between and within developmental stages and adults (Mueller et al., 2005; De Fine Licht and Biedermann, 2012; Biedermann and Rohlfs, 2017; Nuotclá et al., 2019; Biedermann and Vega, 2020). At least one Xyleborini species classifies as cooperatively breeding (*Xyleborinus saxesenii*; Biedermann and Taborsky, 2011; Biedermann et al., 2012), which is defined in ambrosia beetles by adult daughters delaying dispersal from the natal nest and engaging in in hygienic brood care and fungus-farming tasks, while the mother is still present and breeding (Peer and Taborsky, 2007; Biedermann and Taborsky, 2011; Nuotclá et al., 2019). Some of the daughters may co-breed with the mother (Biedermann et al., 2012). They may disperse anytime to found their own nests (Biedermann et al., 2009). The remainder of described social systems in Xyleborini comprise maternal care and some kind of communal breeding (all daughters that stay co-breed with their mother in *Ambrosiophilus* spp.; Kirkendall et al., 2015; Kasson et al., 2016).

The morphology of ambrosia beetle galleries (i.e., tunnel systems within wood, where they nest) is highly variable (Kirkendall et al., 2015). Most common types in Xyleborini are brood-chambers as in *Xyleborinus* or *Xylosandrus* species and branching tunnel systems as in *Xyleborus* and *Euwallacea* species (Roeper, 1995; Biedermann, 2012). As adults, brood and fungus gardens are spatially more separated in branching tunnels than in communal chambers, gallery morphology has probably a major influence on social interactions and modes of farming. Cooperative breeding has been reported from *X. saxesenii* with a brood-chamber type of breeding system (Biedermann and Taborsky, 2011; Biedermann et al., 2012), but the social and farming behaviors of species that construct branching tunnel systems remain enigmatic. The sugar-cane borer *Xyleborus affinis* (Xyleborini, Scolytinae, Curcilionidae) is one of those species making branching tunnel systems.

Healthy fungus gardens of ants, termites, and ambrosia beetles harbor a rich community of fungi and bacteria, even though typically one or rarely two main cultivar fungi dominate (Francke-Grosmann, 1967; Haanstad and Norris, 1985; Kajimura and Hijii, 1992; Mueller et al., 2005; Rodrigues et al., 2008; Biedermann et al., 2013; Saucedo-Carabez et al., 2018; Skelton et al., 2018; Biedermann and Vega, 2020). In ambrosia beetles, these food fungi are transmitted by adult females from the natal to a newly established nest either within the gut (Francke-Grosmann, 1975) or more commonly within a specialized cuticular pouch, the mycetangium (Francke-Grosmann, 1956, 1967; Hulcr and Stelinski, 2017). The whole mycobiome and how monocultures of cultivar fungi are established by the beetles remains poorly studied (Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). In the cooperatively breeding *X. saxesenii* hygienic behaviors are induced in response to fungal pathogens (Biedermann and Taborsky, 2011; Nuotclá et al., 2019). Furthermore, removal of beetles from galleries does lead to the immediate outgrowth of cultivars by non-mutualistic, weedy fungi that are present in a resting form in the fungus gardens of all ambrosia beetles studied (Schneider-Orelli, 1913; Francke-Grosmann, 1956; Norris, 1993).

My study species, *X. affinis,* is best known for its damages in sugar-cane plantations, it is reported to also attack more than 248 other woody plant species (Schedl K. E., 1963). Galleries are founded by single females within a stressed or recently dead plant and may be inhabited by one to several generations of beetle offspring if the nesting substrate is durable enough (Schneider, 1987). While boring an entrance tunnel perpendicular into the wood, foundresses inseminate tunnel walls with spores of *Raffaelea* ambrosia fungus mutualists [possibly *Raffaelea arxii*, which might be the primary food fungus (Saucedo-Carabez et al., 2018)] they carry in oral mycetangia [i.e., fungal-spore pockets (Francke-Grosmann and Schedl, 1960; Schneider, 1987)]. If successful, layers of conidiophores with nutritional conidiospores by the *Raffaelea* fungi line tunnel walls and the foundresses start to lay eggs. By feeding solely on the fungi, larvae pass through three instars followed by pupation (Biedermann et al., 2009). When reaching adulthood many daughters do not disperse immediately, but remain in the natal nest, where they may help in gallery expansion and hygiene, brood and fungus care, and possibly co-breed. Delayed dispersers have been found to loose reserves in the natal nest either by egg-laying and/or investments in helping (Biedermann et al., 2011). Thus, initial tunnel systems with a length of about 20–30 cm after one generation have been found to expand over 6 m (within 4 years) by the work of several consecutive overlapping generations (Schneider, 1987). Staying and consecutive breeding within one gallery is possible because *X. affinis* (i) produces its own fungal food and (ii) is an inbreeding species with regular brother-sister mating and a haplodiploid sex-determination system. Haplodiploidy enables founder females to assign optimal brood sex-ratios (by laying unfertilized eggs that develop into males and fertilized eggs that develop into females), which are strongly female biased (Schedl K. E., 1963; Biedermann et al., 2009). Eighty-five percent of laboratory galleries contain only a single male, which is hatching first and obviously capable to fertilize several dozens of sisters (Roeper et al., 1980). Males are flightless and may disperse only by foot, which they do after all female offspring matured (Biedermann, 2010).

Here I used artificial observation tubes that contained entire colonies of reproducing *X. affinis* beetles and their fungus gardens (a) to determine the social system of an ambrosia beetle breeding in a branching tunnel system and (b) to describe the fungal community of gardens in relation to the presence and the behaviors of beetles and their larvae. Furthermore, I asked whether (c) larvae and adult offspring engage in alloparental

brood care and fungus maintenance, (d) decisions of adult females to help, to breed and to disperse relate to the number of potential beneficiaries, the number of potential competitors and depend on the location within the nest (old vs. freshly excavated gallery parts), and (e) ovary status affects the propensity to engage in cooperative behaviors.

MATERIALS AND METHODS

Beetle Collection, Laboratory Breeding and Phenology

Females used for breeding in this study were directly collected from oak logs in Pineville, LA, United States (123 ft asl; 31°20′, 92°24′) in June 2007. Carried in sterile glass vials, they were immediately brought to the laboratory and used for artificial rearing: Females were surface-sterilized (by washing them first for a few seconds with 95% ethanol and then with distilled water) and afterward singly placed on prepared sterile artificial medium in separate glass tubes (18 mm diameter × 150 mm length; Bellco Glass, Vineland, NJ, United States) that were covered by sterile plastic caps (Bellco Glass kap-uts, Vineland, NJ, United States). Artificial medium had been drying for 5 days before the placement of beetles and consisted of an autoclaved mixture of 0.35 g streptomycin, 1 g Wesson's salt mixture, 5 g yeast, 5 g casein, 5 g starch, 10 g sucrose, 20 g agar, 75 g oak tree sawdust (freshly grounded and oven dried), 2.5 ml wheat germ oil, 5 ml 95% ethanol and 500 ml deionized water (for details on the preparation see Biedermann et al., 2009). After introduction of the beetles, tubes were stored at room temperature (∼23°C) in darkness (wrapped in paper, but light could shine on the entrance). This way, females start digging tunnels as if in wood and often build them adjacent to the glass of the tube. Thus, this technique allows to observe behavior of adults and larvae in the tunnels when the paper is removed (Biedermann et al., 2009). At 23°C a fungal layer and first eggs appear in successfully founded galleries around 10 days after gallery foundation. The first adults hatch about a month later and females remain in the natal nest for at least a week before they disperse by crawling onto the surface of the media, where they try to fly off (and where they can be collected for consecutive rearing). Peak productivity is around 60 days after gallery foundation. At that time, offspring of all stages are present together and the first adult daughters start to disperse. In the laboratory, more offspring will develop typically for another 20–30 days until the medium dries out and all individuals leave the gallery (Roeper et al., 1980; Biedermann et al., 2011).

Behavioral Observations, Dissections of Galleries and of Female Ovaries

Here I used a pool of 23 successfully founded laboratory galleries. Tunnels were visible in 16 galleries, which were used for behavioral observations. Fungi were isolated from 15 galleries. Both behavior and fungal communities were determined for eight galleries among those (for details on these galleries see **Supplementary Table S1**). I started the treatment at about the peak of gallery productivity, when study galleries ranged between 51 and 60 days of age (after gallery foundation) and adult daughters started to disperse. In the 3 days before the treatment, I conducted daily behavioral observations of all visible individuals ($N = 16$ galleries). For each individual I noted the developmental stage and sex, its location within the gallery (main tunnel, side tunnel, brood tunnel; see **Figure 1**) and its behavior at the time of observation [i.e., "scan observation" technique (Biedermann and Taborsky, 2011)]. Larvae were classified by size in either 1st or 2nd/3rd instars. After pupation, females first have a brownish coloration and turn black when fully sclerotized. Light brown females were termed *tenerals* and dark brown females *adults*. Males are discernable from females by their smaller body size small horns on the pronotum. I differentiated between the larval behaviors *allogrooming*, *fungus cropping*, *cannibalism*, *locomotion*, *being pushed* by an adult female and *inactivity*, and the teneral/adult behaviors *shuffling frass*, *blocking*, *digging*, *allogrooming*, *self-grooming*, *fungus cropping*, *cannibalism*, *locomotion*, *pushing larva*, *inactivity* and the male *mating (attempt)* (for details see **Table 1**). For a video of *shuffling frass* by adults see **Supplementary Material**.
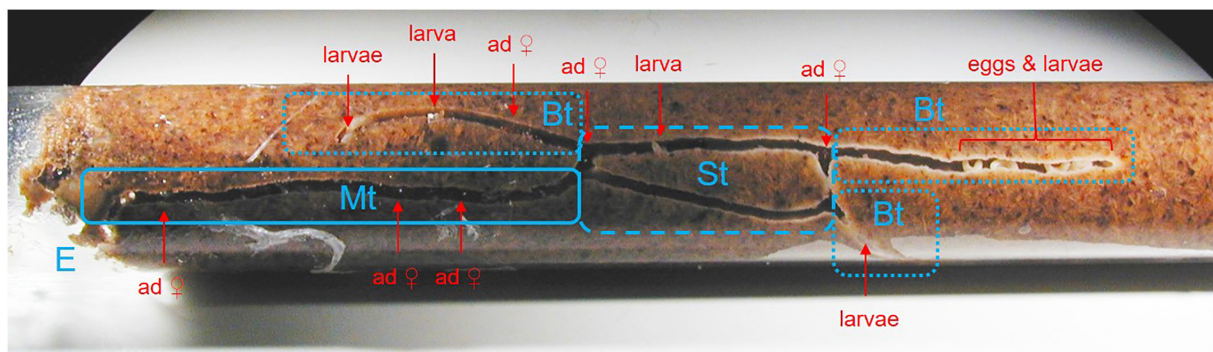

FIGURE 1 | Morphology of a *X. affinis* laboratory gallery in artificial medium at about 60 days after its foundation. Note the species-characteristic tunnel system with the four compartments: E, entrance; Mt, main tunnel; St, side tunnel; Bt, brood tunnel. A white fungal layer and white larvae are visible within the Sts and the Bts. Several adult beetles are sitting in the Mt and a few in the Sts and the Bts.

Behavior	Shown by	Definition	Mutual benefit
Digging	F	Excavating new tunnels	Gallery extension
Fungus cropping	L, F, M	Grazing and feeding on the fungal layer covering gallery walls with the maxillae and/or mandibles	Fungus care
Shuffling frass	F, M	Moving frass and sawdust with the legs and elytra	Hygiene
Cannibalism	L, F, M	Feeding on a larva, pupa or adult beetle that is usually dead	Potentially hygiene
Allogrooming	L, F, M	Grooming an egg, larva, pupa or adult beetle with the mouthparts (i.e., maxillae, labium)	Brood care, hygiene
Blocking	F	Staying inactive in the entrance tunnel and plugging it with the body (abdomen directed to the outside)	Protection
Pushing others	F	Shifting a larva or male within a tunnel	Protection
Self-grooming	F, M	Grooming oneself with the legs	–
Inactive	L, F, M	Being inactive without moving	–
Locomotion	L, F, M	Creeping (L), or walking on the tibia with back-folded tarsi (F, M)	–
Being pushed	L, M	Getting shifted by an adult female	–
Mating (attempt)	M	Mounting a female or copulating with her	–

An update of mutually beneficial behaviors has been published by Nuotclá et al. (2019).

Most galleries are not fully visible from the outside. Therefore, to assess the number of individuals within a gallery and the reproductive status of adult females, I broke the glass of eight galleries at an age between 55 and 61 days, when the first generation of offspring matured and just started to disperse (**Supplementary Table S1**). All developmental stages (eggs, 1st instar larvae, 2nd/3rd instar larvae, pupae and adults) from within the nests were counted and all 49 females from within nests as well as 14 dispersing females were preserved in 95% ethanol until dissection. For all the 49 females from within nests, I noted their location within the nest and for 21 of these females it was even possible to record their behavior immediately before the glass of the tube was destroyed. I dissected female ovaries on microscope slides within some drops of phosphate-buffered saline (PBS) buffer solution under a binocular (6.4×–40× magnification). Females were decapitated, their wings removed and ovaries were exposed by dorsal opening of the abdomen with high precision tweezers and a scissor. I discriminated between *non-developed* (the four ovarioles are all thin or not present), *developed* (four ovarioles present, but not thickened and no oocytes visible) and *egg-carrying ovaries* (one to two of the four ovarioles are thickened and contain one to two oocytes); numbers of oocytes (i.e., developing eggs) were noted (for figure of ovaries see Fischer, 1954).

Fungal Isolations and Identification of Filamentous Fungal Isolates

On the fourth day of the treatment (i.e., 55 to 64 days after gallery foundation), I brought some of the galleries ($N = 15$) to a sterile bench, carefully broke the glass of the tubes, and took 12 fungus samples each from the main tunnel, the side tunnel and the brood tunnel with a forceps that was flame-sterilized after each sample. Four samples were placed on malt agar plates (MA: 25 g malt extract, 20 g agar, 1 L deionized H_2O), four samples on cycloheximide-streptomycin malt agar plates (CSMA: 10 g malt extract, 15 g agar, 20 ml filter sterilized CSMA stock solution containing 2 mg of cycloheximide and 1 mg streptomycin, 1 L deionized H_2O) and the last four on benomyl malt agar plates (BMA: 10 g malt extract, 15 g agar, 1 ml filter sterilized BMA stock

solution containing 1 mg benomyl dissolved in dimethylsulfoxid, 1 L deionized H_2O). MA is unselective, CSMA is selective for Ophiostomatoid fungi (e.g., *Raffaelea* species; Harrington, 1981) and BMA for Basidiomycetes (Ross et al., 1992). Samples were spotted in the center of the plates. Afterward, I fully dissected the whole gallery system and counted all eggs, larvae, pupae, adult females and males (see above).

For obtaining fungal isolates from female cadavers ($N = 4$), I put diseased adult females (from within nests) singly in 1.5 ml Eppendorf tubes with 1 ml PBS buffer. Bodies were crushed with a pestle and vortexed within the PBS buffer for 5 s. Afterward 1:1000 and 1:10000 dilution series were generated and 100 ul of both dilutions were plated out on the three media above. Presence/absence of fungal isolates on these plates was recorded 10 days later.

Isolated fungi were first sorted into groups based on cultural growth and morphological colony characteristics (i.e., morphology and color of the mycelium) and these were compared with a database of fungal isolates from bark and ambrosia beetles available at the Southern Research Station of the USDA Forest Service. Afterward, I brought pieces of fungal tissue on microscope slides with drops of PBS buffer and examined their spore structures. Confirmation and final determination of four of the five fungal isolates was done using internet sources and the illustrated key to identify genera of imperfect fungi by Barnett and Hunter (1998). Representative samples were stored for DNA sequencing, but material got lost through a failure of a refrigerator and could neither be revived nor successfully sequenced. Therefore, only morphological, genus-level identifications are available.

Statistical Analyses

If not stated differently, all statistical analyses were done using generalized estimating equations (GEEs) in R [lmer; Version 2.12.1 (R-Development-Core-Team, 2008)]. GEEs are an extension of generalized linear models with an exchangeable correlation structure of the response variable within a cluster, which allows for controlling the variation between observations from a single gallery. This was necessary

because of variation in sample sizes between galleries (as for the fungal community analyses a few plates without microbial growth had to be excluded).

Using all 12 samples taken from each location (main, side, brood tunnel) of each gallery (independent variable), I estimated the frequency and presence (yes/no) of each fungal isolate (dependent variables) by controlling for medium (MA, CSMA, BMA; fixed factors) and gallery of origin (random factor). I also analyzed how number of eggs, larvae and adult females (dependent variable) were affected by the location within the gallery (fixed factor) and the presence of the different fungi (fixed factor) by controlling for gallery of origin (random factor). Differences in the frequency of male observations between locations within the gallery were tested using Fisher's exact test, because of the small number of males observed. In a third series of models, I analyzed whether frequencies of larval and adult behaviors (dependent variable) were influenced by the location within the gallery (fixed factor).

By correlating behavior with fungal communities, I first determined whether the frequency of larval and adult behaviors (dependent variable) were affected by the presence of the different fungi (fixed factor), by controlling for location within the gallery (fixed factor) and gallery of origin (random factor). Second, I modeled whether the frequency of larval and adult *fungus cropping* and *shuffling frass* (fixed factors) and gallery of origin (random factor) affected isolation frequency of each fungus (dependent variable). Finally, I used logistic regression models (GLMs with binary response variable) to model if the proportion of egg-laying and dispersing females and females with developed ovaries (dependent variables) were affected by the proportion of immatures relative to adults in the nest, the proportion of different female reproductive groups relative to all females inside the nest, and the proportion of dispersing relative to staying females (fixed factors).

RESULTS

Overlapping Generations, Factors Influencing Reproductive Division of Labor and Alloparental Care by Adult Daughters

Between day 55 and 61, when the first generation of offspring matured and started to disperse, galleries ($N = 8$) contained on average 33.6 (± 10 SE) eggs, 7.1 (± 2.2) larvae, 5.4 (± 3) pupae, 0.3 (± 0.3) immature females, 6.8 (± 0.9) adult females and 0.9 (± 0.1) adult males. Of the adult females a mean of 26.8% (± 9.7) had non-developed ovaries, 19.2% (± 7.9) had developed ovaries, and 54.1% (± 13.3) were laying eggs (**Supplementary Table S1**). All 14 dispersing adult females that were dissected had either non-developed ($N = 9$) or developed ovaries ($N = 5$).

Egg-laying was unequally distributed among females and there was no fixed proportion of females laying eggs per gallery: First, egg numbers were independent of the number of potential egg-layers (i.e., females with developed ovaries and egg-laying females) (GLM: $p > 0.05$) and second the proportion of egg-laying females were negatively affected by the proportion of females inside the nest with non-developed ovaries (GLM: $p = 0.016$; **Supplementary Table S2**) and developed ovaries ($p = 0.031$). The proportion of females with developed ovaries correlated negatively with the proportion of females with non-developed ovaries ($p = 0.013$) and with the proportion of egg-layers ($p = 0.042$), which might suggest that development of ovaries is triggered by the opportunity to breed. The propensity of females to disperse was reduced with increasing numbers of larvae dependent on brood care ($p = 0.019$), but independent of the relative proportion of immature offspring to adult females inside the nest as well as the proportion of female egg-layers and females with non-developed ovaries. In contrast, proportion of female dispersers correlated negatively with proportion of females with developed ovaries, possibly suggesting that ovary development typically leads to either dispersal or egg-laying in the natal nest (see above).

Both potential egg-layers and females with non-developed ovaries engaged in alloparental care (i.e., *allogrooming*, *fungus cropping* and *shuffling frass*) at the natal nest. However, despite the small sample size, there was a trend for adult females with non-developed ovaries ($N = 11$) to engage less in these mutually beneficial behaviors immediately before galleries were dissected, than females potentially laying eggs (Fisher's exact test: $p = 0.085$, $N = 21$; **Figure 2**). Additionally, potential egg-layers tended to be found more frequently close to the brood (i.e., in the side-tunnel) than in the main tunnel compared to females with non-developed ovaries (Chi^2-test: $\chi^2 = 2.94$, df $= 1$, $p = 0.087$, $N = 93$).

Offspring and Adult Numbers and Their Behaviors in Relation to the Three Gallery Compartments

Eggs and pupae were only found in the brood tunnels of the galleries. Larvae were most commonly found in the brood tunnels, followed by the side tunnels (GEE: $p = 0.03$; **Supplementary Table S3**, **Figure 3**), and the main tunnel ($p < 0.001$). When present in the main tunnel they only showed *locomotion*, which was also often observed in the side tunnel, but only rarely in the brood tunnel ($p < 0.001$; **Supplementary Table S4**). Larval *allogrooming* and *fungus cropping* were most common in the brood tunnel ($p < 0.05$). Larval *cannibalism* was only present at low rates in the side tunnel.

Adult females and males were least common in the brood tunnels ($p < 0.05$; **Supplementary Table S3**, **Figure 4**). Males tended to stay in the side tunnels ($p = 0.064$). The most common adult female behaviors were *shuffling frass* and *fungus cropping*, with the last most common in the brood tunnel ($p = 0.001$; **Supplementary Table S5**, **Figure 4**). *Inactivity* tended to be expressed most often in the main tunnel ($p < 0.066$). *Blocking* was, by definition, only found in the main tunnel, whereas *digging*, *cannibalism* and *pushing larva* was never expressed there. All other female behaviors were equally common in all three gallery compartments ($p > 0.05$). Adult males spent their time mainly with *fungus cropping* (27% of their time), *locomotion* (25%), *inactivity* (16%), followed by *attempting to*
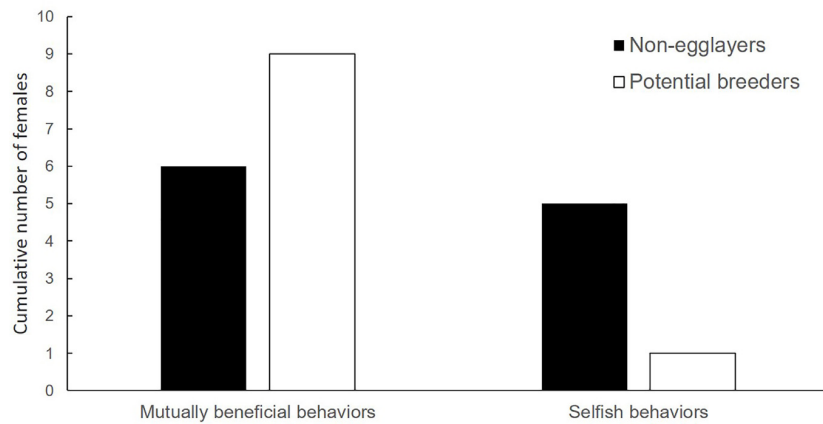
FIGURE 2 | Reproductive status in relation to adult female behavior. All females engage in mutually beneficial behaviors. However, relative to selfish behaviors, females with fully developed or egg-carrying ovaries (*potential breeders*) tended to engage more often in mutually beneficial behaviors (see **Table 1**) than females with non-developed ovaries (*non-egg-layers*) (Fisher's exact test: $p = 0.085$, $N = 21$).
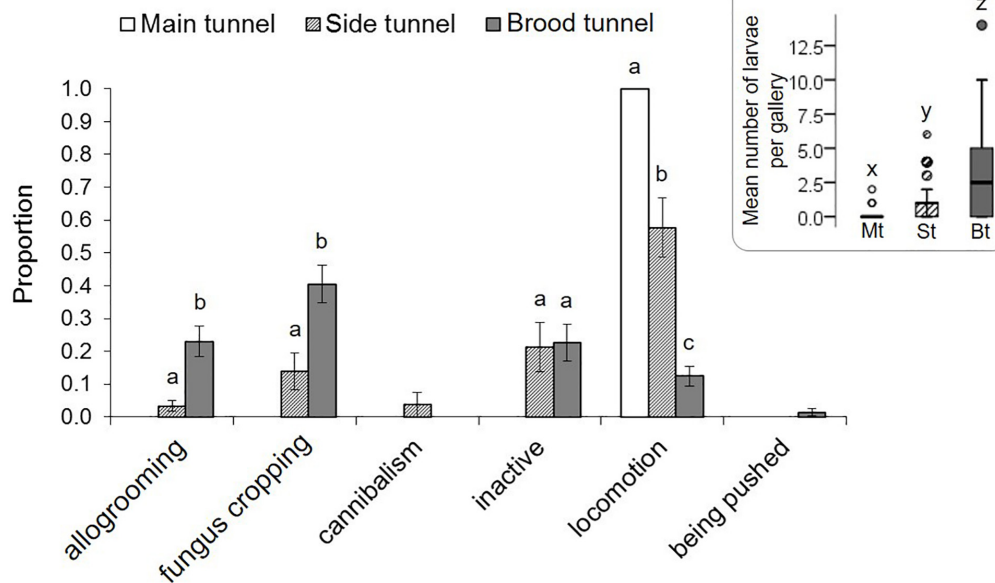

FIGURE 3 | Behaviors of *X. affinis* larvae in the three gallery compartments. Mean ± SE; different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S4**) in pairwise comparisons between compartments. The Mean (±SE) numbers of larvae observed in the three compartments are given in the box on the right.
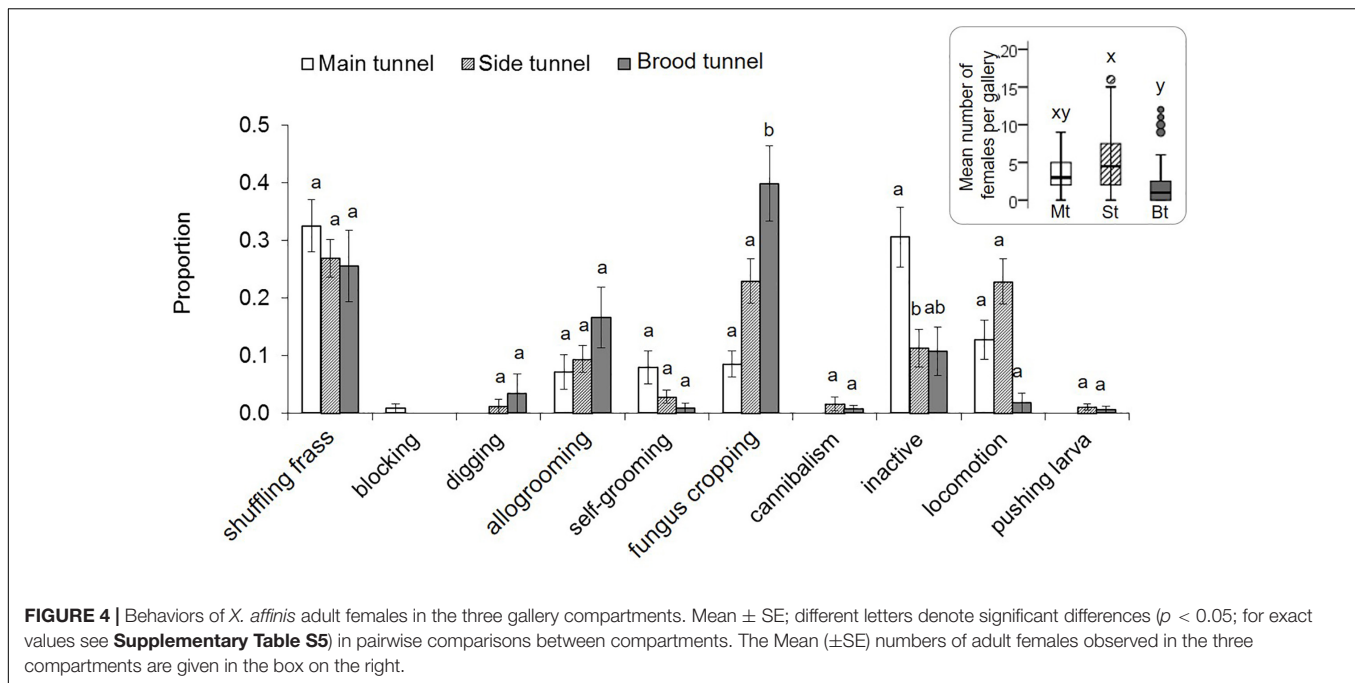
mate (11%), and *allogrooming* (11%). *Self-grooming, mating, being pushed* and *shuffling frass* were only rarely shown by males (<4%).

Fungal Isolations

Our isolations revealed five isolates of filamentous fungi associated with galleries of *X. affinis*: a *Fusarium*, a *Mucor*, a *Phaeoacremonium*, a *Raffaelea* and an unidentified filamentous fungus ("*Unknown*") isolate. The *Mucor* isolate was identified by its fluffy aerial mycelium and the black sporangiophores. The *Raffaelea* isolate was identified by its characteristic growth on plates (filamentous on the outside of the culture, yeast-like in the center) and the morphology of conidiophores and the budding conidiospores. The *Fusarium* isolate was identified by its characteristic sickle-shaped conidia. The identity of the *Phaeoacremonium* isolate was confirmed by macro- and micro-comparisons with a morphologically nearly identical *Phaeoacremonium rubrigenum* isolate in the fungal database of the USDA Forest Service. The *Raffaelea* isolate was the most common fungus isolated.

Overall, *Raffaelea*, *Phaeoacremonium* and *Unknown* were more commonly detected on CSMA than MA
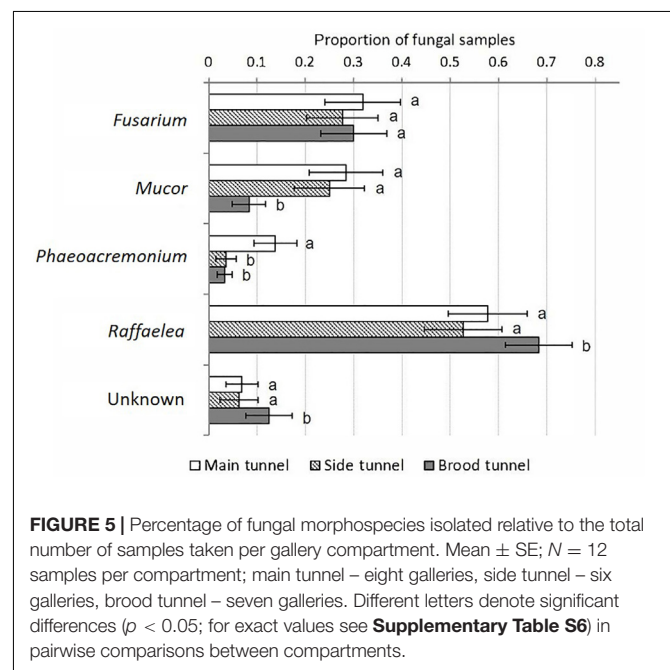
FIGURE 4 | Behaviors of *X. affinis* adult females in the three gallery compartments. Mean ± SE; different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S5**) in pairwise comparisons between compartments. The Mean (±SE) numbers of adult females observed in the three compartments are given in the box on the right.

(GEEs: $p = 0.017–0.001$; **Supplementary Table S6**), which shows that these three isolates are resistant to the antibiotic cycloheximide. Interestingly they grew also better on BMA than MA ($p = 0.007–0.001$). The opposite was found for *Mucor* and *Fusarium*, which were most frequently isolated from MA followed by BMA and CSMA ($p < 0.01$).

Fungal Diversity in Relation to Gallery Compartment and Age of Gallery

The *Raffaelea* was isolated from all compartments of all galleries, and was most commonly detected in the brood tunnels (GEEs: $p = 0.003–0.001$; **Supplementary Table S6**, **Figure 5** and **Supplementary Figure S1**), which strongly suggests its nutritional role for *X. affinis*. *Fusarium*, *Mucor*, and *Unknown* were present in about 60% of all galleries. *Unknown* and *Fusarium* were isolated equally often from all compartments ($p > 0.05$), whereas *Mucor* and *Phaeoacremonium* occurred more frequently near the entrance than the brood ($p = 0.003–0.006$). *Raffaelea*, *Fusarium* and *Mucor* were strongly associated with old galleries (i.e., isolation rate > 60%; **Supplementary Figure S1**). The other two fungi were detected in <20% within these samples. Also dead females harbored *Raffaelea*, *Fusarium* and *Mucor*; the latter in >60% of diseased individuals.

Influence of Fungal Species on Beetle Productivity and Their Farming Behaviors

The regular presence of *Raffaelea* in samples from all compartments of all galleries supports its essential function for the beetles, but inhibited testing the influence of its presence on beetle productivity and behaviors. It is possibly the only fungus with a nutritional role for the brood, because egg and

FIGURE 5 | Percentage of fungal morphospecies isolated relative to the total number of samples taken per gallery compartment. Mean ± SE; $N = 12$ samples per compartment; main tunnel – eight galleries, side tunnel – six galleries, brood tunnel – seven galleries. Different letters denote significant differences ($p < 0.05$; for exact values see **Supplementary Table S6**) in pairwise comparisons between compartments.

larval numbers were both not affected by any other isolate (GEEs: $p > 0.05$; **Supplementary Table S3**). In contrast, adult female numbers were negatively affected by the presence of *Phaeoacremonium* ($p = 0.005$). Adult females increased *fungus cropping* frequency in the presence of *Unknown* ($p = 0.038$; **Supplementary Table S7**, **Figure 6**), increased *shuffling frass* ($p = 0.048$) and general activity levels in the presence of *Fusarium* ($p = 0.02$) and decreased activity levels in the presence of *Phaeoacremonium* ($p = 0.002$). None of the fungal isolates
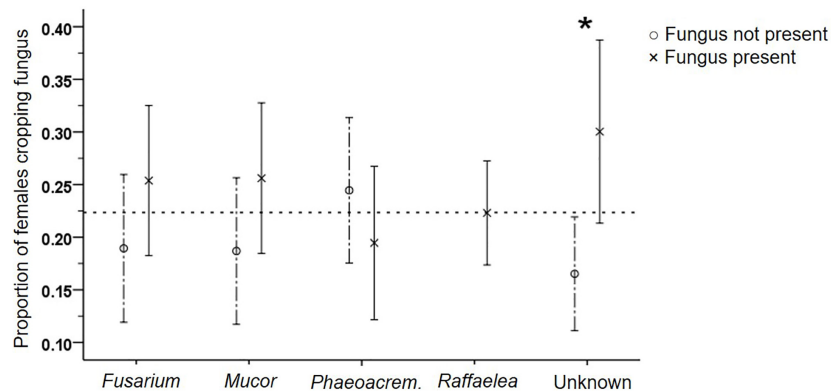
FIGURE 6 | Effect of the presence of the five different fungi on the frequency of adult female *fungus cropping*. Mean (±SE) frequencies, for fungus present or not per gallery, are given. *Raffaelea* was always present. *$p < 0.05$ (for exact values see **Supplementary Table S7**).

influenced the activity of larvae (i.e., their *fungus cropping* and *shuffling frass* behaviors; $p > 0.05$; data not shown).

Potential Influence of Larval and Adult Farming Behaviors on the Isolation Frequency of the Fungi

Raffaelea was less commonly isolated from those galleries with higher activity levels of larvae in the 3 days before the fungal isolations ($p < 0.05$; **Supplementary Table S8**). Isolation frequency of *Unknown* was positively correlated with larval *fungus cropping* ($p = 0.004$). Adult *fungus cropping*, *shuffling frass* or activity levels showed no significant correlation with the isolation frequency of the five fungi. There was, however, a very weak trend for a higher isolation frequency of *Mucor* and *Phaeoacremonium* from those galleries with lower activity levels of adults in the days before ($p < 0.12$).

DISCUSSION

This study is the first to correlate ambrosia beetle behavior with egg-laying, location within the gallery and frequency of different fungal symbionts. Ambrosia beetle behavior normally cannot be observed within solid wood, so here I reared beetles in artificial medium in glass tubes (Biedermann et al., 2009). That gave the chance for individual behavioral observations of females immediately before dissection of ovaries and targeted fungal samplings, in combination with behavioral observations. So for each of the three *X. affinis* gallery compartments – brood tunnel, side tunnel, and main tunnel – I was able to give an overview of the number of larval and adult inhabitants, their respective behaviors (including egg-laying) and the diversity of filamentous fungi. The social system was similar to that of other Xyleborini ambrosia beetles (**Table 2**) and there was correlative evidence for active management of fungus gardens by the beetles, i.e., behavioral changes in response to the fungal composition of their ambrosia gardens.

Observations and female dissections revealed that in *X. affinis* some adult daughters delay dispersal, help in hygienic tasks,

brood and fungus care (**Figure 2**; see **Supplementary Video**) and on average half of them reproduce in the natal gallery together with their mother (**Supplementary Table S1**). This social system is termed cooperative breeding or facultative eusociality (Boomsma and Gawne, 2017) and has already been described from the ambrosia beetle *Xyleborinus saxesenii* (Biedermann and Taborsky, 2011) (**Table 2**). Development, offspring numbers, number of egg-layers and dispersed females after 60 days almost equals the numbers reported from Biedermann et al. (2012) that used also the same rearing media. Even though we currently lack behavioral studies on other ambrosia beetle species, data on development and delayed dispersal of adult offspring indicate that cooperatively breeding can be possibly found also in some other *Xyleborinus* and *Xyleborus* species, even if it is probably not widespread (Schedl W., 1963; Kingsolver and Norris, 1977; Gagne and Kearby, 1979; Maner et al., 2013).

Xyleborus affinis galleries contained high numbers of individuals of all age groups at around day 60, which is shortly after adult daughters start to disperse from galleries (Biedermann et al., 2011). Multiple egg-layers were already present and on average, about half of the adult daughters that had remained in the natal nest had started to lay eggs. Similar numbers been reported for *X. saxesenii* (Biedermann et al., 2012). In contrast to *X. saxesenii*, the propensity of females to disperse did not correlate with brood depending on care (i.e., eggs, larvae, pupae). Although all adult females participate in social tasks in the natal nest, egg-laying females were more inclined to do so. This higher investment of egg-laying individuals than of non-reproducing helpers into brood care is typical for social groups that have not taken the transition to superorganismality (i.e., obligate eusociality; Boomsma and Gawne, 2017). Reproducing females in the equally cooperatively breeding *X. saxesenii*, for example, typically protect the gallery entrance against the introduction of predators, which is the most risky task in an ambrosia beetle society (Biedermann and Taborsky, 2011). By contrast, in superorganismal societies sterile workers are not only overtaking the most dangerous tasks, but usually all non-reproductive tasks that need to be done (Wilson, 1971; Bourke, 2011; Boomsma and Gawne, 2017).

TABLE 2 | Overview of social traits found in the ambrosia beetle tribe Xyleborini (Curculionidae: Scolytinae).

Social characteristic	Prevalence in Xyleborini	References
Sibling mating, haplodiploidy	All species	Kirkendall et al. (2015)
Subsocial behavior		
Maternal care (brood care, nest hygiene and protection, fungus farming)	All species	Jordal et al. (2011), Kirkendall et al. (2015)
Overlapping generations		
Delayed dispersal of adult daughters*	Widespread; shown for *Xyleborus affinis, X. ferrugineus, X. glabratus, X. monographus, Xylosandrus germanus* and *Xyleborinus saxesenii*	Schedl W. (1963), Kingsolver and Norris (1977), Peer and Taborsky (2007), Biedermann et al. (2011), Maner et al. (2013), this study
Reproductive division of labor		
Not present: Daughters stay but do neither breed nor help	Probably widespread; present in *Xylosandrus germanus*	Personal observation
Not present: All daughters co-breed with mother	Rare; shown only for *Ambrosiophilus* spp.	Kasson et al. (2013)
Present: Some, but not all staying daughters co-breed with mother*	Prevalence unknown; shown only for *Xyleborus affinis* and *Xyleborinus saxesenii*	Biedermann et al. (2012), this study **(Supplementary Table S1)**
Help by offspring in the natal nest		
Cooperative larvae (brood care, nest hygiene, fungus farming)	Probably widespread; shown for *Xyleborus affinis* and *Xyleborinus saxesenii*	Biedermann and Taborsky (2011), this study **(Figure 3)**
Alloparental care by adult daughters that do not breed (brood care, nest hygiene and protection, fungus farming)*	Prevalence unknown; shown only for *Xyleborus affinis* and *Xyleborinus saxesenii*	Biedermann and Taborsky (2011), this study **(Figures 2, 4** and **Supplementary Video)**

Overlapping generations, reproductive division of labor and alloparental brood care are characterizing cooperatively breeding animal societies, as present in the Xyleborini ambrosia beetles Xyleborus affinis and Xyleborinus saxesenii.

Observations of the three gallery compartments (**Figure 1**) revealed a spatial organization of developmental stages and behavioral tasks. Brood tunnels were surrounded by thick layers of fungal growth and inhabited mostly eggs, pupae and larvae, whereas males and females capable of laying eggs (i.e., developed ovaries or ones containing eggs) tended to be found in the side tunnels. Males were waiting in the side tunnels for freshly emerging females to mate. There was a trend for females with undeveloped ovaries to be found in the main tunnels. This compartmentalization of developmental stages reflects the spatial fidelity found in ants, where brood and adults are also unequally distributed within the nest (Mersch et al., 2013). Not surprisingly, this compartmentalization is also reflected by a spatial division of labor in the sense that most *fungus cropping* and *allogrooming* are found in the brood tunnels, whereas *shuffling frass, inactivity* and *locomotion* are found most often in the food-deficient side and main tunnels (**Figures 2, 3** and **Supplementary Video**). This compartmentalization of age groups and the resulting spatial division of labor has not been reported for any other ambrosia beetle and is probably caused by breeding in tunnels instead of brood chambers (where the fungal food resource and all age groups occur together; Biedermann and Taborsky, 2011; Biedermann et al., 2012). Given that all *Xyleborus* species make tunnel systems (Roeper, 1995; Kirkendall et al., 2015), it is likely that compartmentalization is present in other species of this genus. In the future, some more comparative analyses of brood-chamber- and branching-tunnel-building species need to be done, to check if gallery morphology allows inferences on the social structures of the societies.

This is the first study to look at fungal communities of *X. affinis* using artificial laboratory cultures. Five different morphospecies were identified: a *Raffaelea*, a *Fusarium*, a *Phaeoacremonium*, a *Mucor* and an *Unknown* fungus. This diversity of fungi is much lower than culture independent studies from *X. affinis* in the field reported (Kostovcik et al., 2014; Saucedo-Carabez et al., 2018), but also not surprising as many environmental microbes are excluded by the lab rearing. This study was not successful to determine a primary mutualistic fungus. Here I present correlative evidence that *Raffaelea* and *Unknown* are putative nutritional mutualists of *X. affinis*. *Raffaelea* formed thick ambrosia layers on the walls of the side and brood tunnels, which were fed upon by larvae and adults. *Raffaelea* species produce nutritional conidiospores and have been proven to be primary cultivars in other *Xyleborus* and *Xyleborinus* species (Francke-Grosmann, 1967; Roeper, 1996; Biedermann et al., 2013; Hulcr and Stelinski, 2017; Saucedo et al., 2017; Saucedo-Carabez et al., 2018). Saucedo-Carabez et al. (2018) predominately isolated *Raffaelea arxii* from field galleries of *X. affinis,* next to unfrequent *R. lauricola* and *R. subfusca*. *R. arxii* might therefore be the primary mutualist of *X. affinis* that I here could not determine to the species level. A recent metabarcoding study also confirmed the ubiquitous presence and nutritional role of a *Raffaelea* species for *X. affinis* (Ibarra-Juarez et al., 2020). *Unknown* may function as a secondary food source because it was particularly common in brood tunnels and its presence had a positive influence on adult female feeding.

It is unlikely that the other three fungal isolates, *Mucor*, *Fusarium*, and *Phaeoacremonium*, have a mutualistic role for the beetles. All three were predominantly found in the main tunnels, old galleries and associated with dead individuals. Presence of *Fusarium* also correlated with more active adult females and hygienic *shuffling* activity. *Phaeoacremonium* had negative correlative effects on the number of adult females

present. *Mucor* species are typical saprotrophs (Ritz, 1995), whereas *Fusarium* and *Phaeoacremonium* species are often plant pathogens (Larignon and Dubos, 2000; Ma et al., 2013) and might profit from transmission by ambrosia beetles. One clade of *Fusarium* species is known as primary mutualists of *Euwallacea* ambrosia beetles, however (Kasson et al., 2013; O'Donnell et al., 2015; Freeman et al., 2016; Aoki et al., 2019). Fungi in this clade have typically thickened conidiospores, which I did not see in the isolate found in this study. Clearly, molecular identifications and experimental manipulations are needed to determine the roles suggested here.

I found some significant correlations between ambrosia beetle behavior and presence of specific fungi. Whether these behavioral changes are actually caused by the fungi is unclear, however. Ambrosia beetles are termed "farmers," but currently there is very little data proving that the beetles indeed actively manage fungal communities in the sense that they have means to promote beneficial fungi over antagonistic fungi. The strongest evidence comes from a recent study on *X. saxesenii* by Nuotclá et al. (2019), who found that injection of *Aspergillus* pathogens in laboratory galleries may induce adult (allo-)grooming and - over time - reduced *Aspergillus* spore loads (Nuotclá et al., 2019). Previously it had been shown that *X. saxesenii* larvae are able to suppress the growth of not-identified fungal pathogens (Biedermann and Taborsky, 2011). Mechanistically, beetles may suppress fungal pathogens by application of antibiotic producing bacteria. Application of oral secretions containing bacteria have been observed (Cardoza et al., 2006) and bacteria selectively inhibiting fungal pathogens by producing cycloheximide (which does not harm *Raffaelea* species) have been isolated from *X. affinis* and *X. saxesenii* laboratory galleries (Grubbs et al., 2011, 2019).

In summary, *X. affinis* lives in a branching gallery in a cooperatively breeding social system with multiple egg-layers and division of labor between larvae and adults. Adult daughters have been shown previously to lose reserves during the delayed dispersal period (Biedermann et al., 2011). Future studies have to determine if some daughters lay eggs before dispersal, which may be possible as we found that one third of the dispersing females had developed ovaries. A similar social system has been described for *X. saxesenii*, which builds brood chambers, so interactions between adults and larvae are more common there (Biedermann and Taborsky, 2011; Biedermann et al., 2012). *X. affinis* is predominately associated with one or two fungal mutualists, of which the predominating one is a *Raffaelea* fungus (maybe *R. arxii*). This species has been reported by Saucedo-Carabez et al. (2018) and confirms the mutualism between the genus *Xyleborus* and *Raffaelea* species. Even though two recent articles suggest that yeasts (e.g., *Ambrosiozyma*) are additional mutualists of *X. affinis* and other *Xyleborus* species (Saucedo-Carabez et al., 2018; Ibarra-Juarez et al., 2020), their mutualisms appear less promiscuous then the first metabarcoding study on *Xyleborus* field galleries

suggested (Kostovcik et al., 2014). More experimental work on the fitness effects of single fungal species on the beetles is clearly needed to test how specific the ambrosia beetle-fungus symbioses are (see e.g., Skelton et al., 2019). Finally, it is important to mention that our findings from the Xyleborini lineage should not be extrapolated to other independently evolved ambrosia beetle-fungus symbioses (Kirkendall et al., 2015; Hulcr and Stelinski, 2017). There is some evidence that the many independent origins of the ambrosia symbiosis in beetles differ considerably in their behaviors, social systems and ways of farming (Biedermann and Vega, 2020). We are just at the beginning of getting a small glimpse in the diversity of these fascinating beetle farmers.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PB conceived the study, performed all analyses, and wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.518954/full#supplementary-material

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