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Phylogenomics for Chagas Disease Vectors of the *Rhodnius* Genus (Hemiptera, Triatominae): What We Learn From Mito-Nuclear Conflicts and Recommendations

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We provide in this study a very large DNA dataset on *Rhodnius* species including 36 samples representing 16 valid species of the three *Rhodnius* groups*, pictipes, prolixus* and *pallescens.* Samples were sequenced at low-depth with whole-genome shotgun sequencing (Illumina technology). Using phylogenomics including 15 mitochondrial genes (13.3 kb), partial nuclear rDNA (5.2 kb) and 51 nuclear protein-coding genes (36.3 kb), we resolve sticking points in the *Rhodnius* phylogeny. At the species level, we confirmed the species-specific status of *R. montenegrensis* and *R. marabaensis* and we agree with the synonymy of *R. taquarussuensis* with *R. neglectus.* We also invite to revisit the species-specific status of *R. milesi* that is more likely *R. nasutus*. We proposed to define a *robustus* species complex that comprises the four close relative species: *R. marabaensis, R. montenegrensis, R. prolixus* and *R. robustus.* As *Psammolestes tertius* was included in the *Rhodnius* clade, we strongly recommend reclassifying this species as *R. tertius*. At the *Rhodnius* group level, molecular data consistently supports the clustering of the *pictipes* and *pallescens* groups, more related to each other than they are to the *prolixus* group. Moreover, comparing mitochondrial and nuclear tree topologies, our results demonstrated that various introgression events occurred in all the three *Rhodnius* groups, in laboratory strains but also in wild specimens. We demonstrated that introgressions occurred frequently in the *prolixus* group, involving the related species of the *robustus* complex but also the pairwise *R. nasutus* and *R. neglectus*. A genome wide analysis highlighted an introgression event in the *pictipes* group between *R. stali* and *R. brethesi* and suggested a complex gene flow between the three species of the *pallescens* group, *R. colombiensis, R. pallescens* and *R. ecuadoriensis*. The molecular data supports also a sylvatic distribution

of *R. prolixus* in Brazil (Pará state) and the monophyly of *R. robustus*. As we detected extensive introgression events and selective pressure on mitochondrial genes, we strongly recommend performing separate mitochondrial and nuclear phylogenies and to take advantages of mito-nuclear conflicts in order to have a comprehensive evolutionary vision of this genus.

Keywords: phylogenomics, mito-nuclear conflict, introgression, selective pressure, *Rhodnius*, Chagas disease vectors

INTRODUCTION

The blood-sucking bugs (Hemiptera, Reduviidae, Triatominae) are vectors of the parasite *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastea, Trypanosomatida). Most species of live in natural habitats where they feed on a large variety of vertebrates. But some of them can be found in human dwellings responsible for the Chagas disease endemic to Latin America where about 6 million people are infected (PAHO, 2020). The Triatominae subfamily is distributed in five tribes, the most diverse among them being the Rhodniini and Triatomini, which represent more than 90% of the known species. The Rhodniini tribe is composed of two *Rhodnius* and *Psammolestes* genera, which show some morphological differentiations (Lent and Wygodzinsky, 1979) that has justified the erecting of two different genera. They also differ in their ecology, the *Rhodnius* species mainly associated with palm trees and the *Psammolestes* species with bird nests (Galvão and Justi, 2015). Whereas only three *Psammolestes* species were described, there are twenty-two *Rhodnius* species but one species, *R. taquarussuensis* (Rosa et al., 2017a), has been recently synonymized with *R. neglectus* (Nascimento et al., 2019) (see **Table 1** with authors and year of description). The *Rhodnius* genus is divided into three major groups, *pictipes, prolixus* and *pallescens* (Hernández et al., 2020). The three species of the *pallescens* group (*R. colombiensis, R. ecuadoriensis,* and *R. pallescens*) are *trans*-Andean, distributed in the west of the Andes. The seven species of the *pictipes* group (*R. amazonicus, R. brethesi, R. micki, R. paraensis, R. pictipes, R. stali,* and *R. zeledoni*) are *cis*-Andean, distributed in the east of the Andes and in the Amazon region. The same distribution is observed for ten species of the *prolixus* groups (*R. barretti, R. dalessandroi, R. domesticus, R. marabaensis, R. milesi, R. montenegrensis, R. nasutus, R. neglectus, R. prolixus,* and *R. robustus*), the eleventh *R. neivai* having also some *trans*-Andean populations (Zhao et al., 2021).

Some species are difficult to differentiate from each other on morphological criteria thus generating taxonomic conflicts. Pioneer studies using allozymes had questioned the species-specific status of closely related species such as Venezuelan populations of *R. robustus* and *R. prolixus* (Harry et al., 1992; Harry, 1993). Concerning some newly described species, molecular data are sparse for *R. barretti* (Abad-Franch et al., 2013), not released in a database for *R. marabaensis* (Souza et al., 2016) or lacking for *R. micki* (Zhao et al., 2021). Recently, molecular studies on a newly described species *R. montenegrensis* (Rosa et al., 2012) suggested that it is not a true species but a part of the *R. robustus* variability (Abad-Franch et al., 2013; Monteiro et al., 2018). However, the two species *R. montenegrensis* and *R. marabaensis* were recognized by Castro et al. (2020) based on the geographical origin of their samples. Moreover, some authors consider *R. milesi* to be a *R. neglectus* variant from south-eastern Amazonia (Abad-Franch et al., 2013). Otherwise, only a few specimens have been collected for *R. paraensis* first described as *R. domesticus* (Sherlock et al., 1977) and *R. amazonicus* close to *R. pictipes* but this latter species was revalidated by Bérenger and Pluot-Sigwalt (2002). Finally, the species-specific status for two species is questionable and difficult to deepen since *R. zeledoni* reported as morphologically close to *R. paraensis* was described from only one dead and dried male specimen (Jurberg et al., 2009), and *R. dalessandroi* is actually known only from its published description (Carcavallo and Barreto, 1976).

Some *Rhodnius* species are important vectors of *T. cruzi*, as *R. prolixus,* the main Chagas disease vector within the genus, which extends from Central America to the Andean countries and the Amazon basin. Control programs in Central America were thought to have achieved the elimination of this vector but it was reported again in Mexico in 2019 (Antonio-Campos et al., 2019). Three other species are domiciliated in some countries, namely *R. ecuadoriensis* in the northern zone of Peru and Ecuador, *R. stali* in Bolivia, and *R. pallescens* in Panama. In Brazil, *R. neglectus* and *R. nasutus* often colonize human environments (Galvão and Justi, 2015).

Phylogenetic studies performed on the Rhodniini tribe, mainly with mitochondrial genes, showed that *Psammolestes* was nested within the *Rhodnius* clade but discrepancies remained about its phylogenetic position within the *prolixus* group (reviewed in Hernández et al., 2020). Moreover, several molecular studies have also focused on the relationship between the three groups. Two tree topologies were obtained either *pictipes* and *pallescens*, or *pictipes* and *prolixus* groups as sister taxa, unrelated to the type of marker used, mitochondrial, nuclear, or both (reviewed in Hernández et al., 2020). Recently, the third configuration, *prolixus* and *pallescens* groups as sister taxa, was also reported by Kieran et al. (2021) using ultraconserved elements.

The principal sources of discrepancies for phylogenies may come from methodological pitfalls and/or reflect the biological reality. Methodological bias could therefore be induced by incomplete/different taxon samplings but also by the type and the number of markers used. However, discordance between mitochondrial and nuclear phylogenies are expected because the mitochondrial genome is haploid uniparentally inherited in most animals unlike the nuclear one and has faster rates of evolution. Incongruences between single gene phylogenies can be

TABLE 1 | Distribution and habitat of the species of the Rhodniini tribe and molecular species-specific status inferred from this study.

Species of the Rhodniini tribe	Distribution	Habitat	Molecular species-specific status
R. amazonicus (Almeida et al., 1973)	Brazil, French Guiana	Sylvatic	Valid
R. barretti (Abad-Franch et al., 2013)	Colombia, Ecuador	Sylvatic	Not studied
R. brethesi (Matta, 1919)	Brazil (Pará, Amazonas), Colombia, Venezuela	Sylvatic Sporadically in domiciles Amazon basin	Valid
R. colombiensis (Mejia et al., 1999)	Colombia	Sylvatic, Sporadically in domiciles	Valid
R. dalessandroi (Carcavallo and Barreto, 1976)	Colombia	Sylvatic (1 site)	Dubious, no data
R. domesticus (Neiva and Pinto, 1923)	Brazil (atlantic forest)	Sylvatic Sporadically in domicile	Valid
R. ecuadoriensis (Lent and Leon, 1958)	South Colombia, Ecuador, North Peru	Sylvatic, Domiciliar Principal vector in Ecuador	Valid
R. marabaensis (Souza et al., 2016)	Maraba, Para, Brazil Pará	Sylvatic	Valid
R. micki (Zhao et al., 2021)	Bolivia (Santa Cruz, Saavedra) 2 males	No data	Not studied
R. milesi (Carcavallo et al., 2001)	Brazil Pará	Sylvatic	*R. nasutus (?)*
R. montenegrensis (Da Rosa et al., 2012)	Brazil (Acre, Rondonia)	Sylvatic	Valid
R. nasutus (Stal, 1959)	Brazil (caatinga)	Sylvatic In domiciliation process	Valid
R. neglectus (Lent, 1954)	Brazil (Cerrado São Paulo)	Silvatic In domiciliation process	Valid
R. neivai (Lent, 1953)	Colombia, Venezuela	Sylvatic (some domiciliary focus)	Valid
R. pallescens (Lent, 1953)	Belize, Colombia, Costa Rica, Nicaragua, Panama, Venezuela	Silvatic, Domiciliar Principal vector in Panama	Valid
R. paraensis (Sherlock et al., 1977)	Brazil, French Guiana	Sylvatic	Not studied
R. pictipes (Stal, 1972)	Belize, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname, Trinidad, Venezuela	Sylvatic Sporadically in domiciles	Valid
R. prolixus (Stal, 1859)	Central America, Colombia, (Ecuador) (Suriname), Venezuela	Domiciliar Principal vector in Central America	Valid
R. robustus (Larousse, 1927)	Brazil, Colombia, Ecuador, French Guiana, Peru, Venezuela	Sylvatic, Ssporadically in domiciles (Colombia, Guyana)	Valid
R. stali (Lent et al., 1993)	Bolivia, Brazil (Matto Grosso, Acre)	Sylvatic, In domiciliation process in Bolivia	Valid
R. taquarussuensis (syn R. neglectus) (Da Rosa et al., 2017)	Mato Grosso do Sul, Brazil	In rural dwelling in the city of Taquarussu	*R. neglectus*
R. zeledoni (Jurberg et al., 2009)	Brazil	Aracajú-Sergipe, 1 specimen	Dubious, no data
Psammolestes arthuri (Pinto and Lent, 1935)	Colombia, Venezuela	Sylvatic	Not studied
Psammolestes coreodes (Bergroth, 1911)	Argentina, Bolivia, Brazil, Paraguay	Sylvatic	Not studied
Psammolestes tertius (Lent and Jurberg, 1965)	Brazil	Sylvatic	*R. tertius*

In blue: prolixus group species, in pale green: pictipes group species, in darker green: pallescens group species.

due to incomplete lineage sorting, deep coalescence, horizontal gene transfer, introgression, hybridization, hidden paralogy or lack of phylogenetic information (Toews and Brelsford, 2012; Campillo et al., 2019). For the Rhodniini tribe, only the complete mitogenome of *R. pictipes* (Zhao et al., 2019) and the complete nuclear genome of *R. prolixus* (Mesquita et al., 2015) are available. For all the phylogenies yet performed, the datasets were indeed limited in terms of the number of genes. So far, the most complete studies for the Rhodniini tribe were performed by Justi et al. (2014) using ten species and 1–4 genes depending on the species, Kieran et al. (2021) using from eight to sixteen species and ultraconserved elements and/or rDNA data, and Paula et al. (2021) using thirteen species and three genes.

In this study, we aimed to resolve major phylogenetic conflicts within the *Rhodnius* genus using phylogenomics from low-depth whole-genome shotgun sequencing and to explore mito-nuclear conflicts in order to have a better understanding of the evolution of this genus.

Data were obtained by Illumina technology for 36 samples of the Rhodniini tribe including 17 putative species identified using morphological characters. We performed both mitochondrial and nuclear phylogenies using a set 15 mitochondrial genes (13 protein-coding mitochondrial plus 2 rDNA genes) and two sets of nuclear markers (nu-rDNA genes and 51 protein-coding genes). The protein-coding datasets were tested for selection.

The outcome of this work led us to identified 16 valid *Rhodnius* species in our molecular dataset and to formulate recommendations for both taxonomic and phylogenetic issues.

MATERIALS AND METHODS

Material and Genomic Data

For this study, field specimens and laboratory strains were used with a special focus on the *prolixus* group for field specimens (**Table 2**). From our field collection, were selected specimens with doubts about their morphological identification (MILEP, NEGP), difficult to determine (INCP, NasG), or exhibited incoherent *cytb* sequences in a preliminary study (ROBB, ROBR, ROBQ). Some specimens were very old since they were also used by Harry (1993) and Harry (1994). We used laboratory strains from the Insetário de Triatominae da Faculdade de Ciências Farmacêuticas/Unesp/Araraquara, Brazil. Samples were either stored in absolute EtOH or air-dried.

DNA was extracted from legs and alary muscles from adult bugs, from 1 for field specimens to 6 for laboratory strains using the Qiagen DNEasy tissue kit. Genomic data was obtained from 36 samples of the Rhodniini tribe corresponding to 17 species identified using morphological characters. A whole-genome shotgun sequencing was performed using Illumina technology (100 bp paired-end, Imagif platform, Gif-sur-Yvette, France) generating from 3.7 to 21.1 Gb data per sample corresponding to a genome depth from 5 to 30x (**Table 3**).

Sequences have been deposited in the NCBI BioProject database *via* the accession number PRJNA429761.

Mitogenomes and Nuclear rDNA Operon Assembly and Annotation

In order to assemble the mitogenomes, the reads were subsampled (500 kb of reads for each sample) and assembled using the Trinity software with default parameters (Haas et al., 2013). This approach allows to assembly sequences that are highly abundant in the sequence pool like mitochondrial or ribosomal genes. In insect, mtDNA represents on average 0.42% of the total DNA in genome sequence project (Meng et al., 2019). As we used 500 kb of data/sample for the mitochondrial assembly which in average contains $(500 \times 0.42)/100 = 2{,}100$ kb of mtDNA and given that the mitochondrial genome is about 15 kb, we obtain coverage of 140x for this genome. This assembly strategy also prevent to pick up some NUMS that are mitochondrial pseudogenes in nuclear chromosomes, because if these elements were present in the data, as they are nuclear, their coverage would be very low (from 5 to 30x according to the samples) compared to that of the (true) mtDNA and with a low probability to be retrieved from the data used to assemble the mtDNA. Contigs corresponding to the mitogenomes and the nuclear rDNA operon were identified by BLAST using as templates the *Triatoma infestans* mitogenome and the *R. prolixus* reference genome rDNA. In most cases, the mitogenome and the nuclear rDNA operon of each sample appeared as a single contig, sometimes as two or three contigs.

The annotation of the *Rhodnius* mitogenomes was achieved using the MITOS pipeline (Bernt et al., 2013). Each mitochondrial and nuclear rDNA datasets was independently aligned with MAFFT[1] (Katoh et al., 2019), visualized with Bioedit (version 7.2.6.1; Hall, 1999), and ambiguously aligned regions or gapped positions were manually corrected or removed. Two concatenated datasets were generated for the mitochondrial data. The first included the 15 mitochondrial genes (15 mtG), namely the 13 mtPCG (*Nad2, COI, COII, Atp8, Atp6, COIII, Nad3, Nad5, Nad4, Nad4L, Nad6, Cytb* and *Nad1*) and the 2 rDNA genes (*16 rDNA* and *12 rDNA*). The second dataset including only the 13 mitochondrial protein-coding genes (mtPCG) was used for the selection signature analyses. This dataset was translated in order to test for the presence of pseudogenes (**Supplementary Table 1**).

For the nuclear rDNA genes (nu-rDNA), we retrieved from the data the complete *18S* and *28S* genes but the partial *ITS1, 5.8S,* and *ITS2* genes. The alignment for this dataset is given in the **Supplementary Table 1**.

Genome Assembly and Protein-Coding Gene Annotation

A second assembly was carried out for each of the 36 genomes with the SOAPdenovo2 software (Luo et al., 2012) with k-mers estimated using the Kmergenie program (Chikhi and Medvedev, 2014). Benchmarking Universal Single-Copy Orthologs (BUSCO) genes (Waterhouse et al., 2019) were searched in assemblies using the insecta_odb10 dataset comprising 1,367 genes universal to all insects. In a first step, we kept the 88 nuclear protein-coding genes (nu-PCGs) for which an entire or fragmented copy was found in each of the 36 Rhodniini genome assemblies and in the *T. brasiliensis* transcriptome (Marchant et al., 2015), as this species was used as an outgroup. Each set of genes was aligned with MAFFT (global homology option; Katoh et al., 2019) and then concatenated. The concatenated alignment was visualized using Bioedit (version 7.2.6.1; Hall, 1999). All regions without at least 29 aligned genomes were retrieved as well as gaps or misaligned sequences. This drastic final trimming resulted in 51 nu-PCGs with a sequence coverage of at least 80% except for *R. brethesi* (**Table 3**). In order to have *R. prolixus* samples validated without (or low) introgression, we also search the 51 nu-PCGs in both the *R. prolixus* used by Mesquita et al. (2015) for the reference genome (VectorBase: RproC3.4) and a Honduras strain reared in a French laboratory (**Table 1**). The

[1]https://mafft.cbrc.jp/alignment/server/

TABLE 2 | Specimens used in the study.

Specimens	Morphologicaldetermination	Moleculardetermination	Origin	Field/Strain	Storage inFrench Lab
PSAM	*P. tertius*	*R. tertius*	Bahia, Curaça, Brazil	F (sylvatic)	EtOH (2003)
Ama1A	*R. amazonicus*		Belizon, French Guyana	F (sylvatic)	air dried (2014)
BRE25WB	*R. brethesi*		Amazonia, Brazil	F (sylvatic)	EtOH (2003)
R9WX	*R. brethesi*		Igarapé Tucunaré, Brazil	S (CTA 222, 2009)	EtOH (2013)
R10B	*R. colombiensis*		Tolima, Colombia	S (CTA 050, 2001)	EtOH (2013)
DomC	*R. domesticus*		Santa Catarina, Brazil	F (sylvatic)	EtOH (2006)
ECUD	*R. ecuadorensis*		PD, Colombia	F (domiciliar)	EtOH (2003)
ECUE	*R. ecuadorensis*		Peru	S	EtOH (2006)
MILE	*R. milesi*	*R. nasutus*	Brazil	S (sylvatic)	EtOH (2006)
MILEP	*R. milesi*	*R. prolixus*	Pará, Brazil	F (sylvatic)	EtOH (2003)
R8F	*R. montenegrensis*		Monte Negro, Rondônia, Brazil	S (CTA 087, 2003)	EtOH (2013)
INCP	*R.* sp.	*R. neglectus*	Coronel José Dias, Piauí, Brazil	F (sylvatic)	EtOH (2003)
NASP	*R. nasutus*		Coronel José Dias, Piauí, Brazil	F (sylvatic)	EtOH (2003)
R7WU	*R. nasutus*		Brazil	S (CTA 054, 1999)	EtOH (2013)
NasG	*R. nasutus*		Piauí, Brazil	F (sylvatic)	EtOH (2003)
R5WU	*R. neglectus*		Frutal, Minas Gerais, Brazil	S (CTA 061, 1983)	EtOH (2013)
NEII	*R. neivai*		Maracay, Venezuela	S (sylvatic)	EtOH (2006)
R1J	*R. pallescens*		Colombia	S (CTA s/R)	EtOH (2013)
PIC34WC	*R. pictipes*		Belém, Brazil	F (sylvatic)	EtOH (2003)
PIC3L	*R. pictipes*		Pará, Brazil	F (sylvatic)	EtOH (2003)
PIC2WA	*R. pictipes*		Guyane	F (sylvatic)	air dried (2014)
R6WV	*R. pictipes*		Belém, PA, Brazil	S (CTA 072, 1998)	EtOH (2003)
R63K	*R. pictipes*		Belém, Pará, Brazil	S (CTA 072, 1998)	EtOH (2003)
Pro10O	*R. prolixus*		Guarico, Venezuela	S (domiciliar)	air dried (1990)
ProYRP	*R. prolixus*		Cojedes, Venezuela	S (domiciliar)	air dried (1990)
ProN	*R. prolixus*		Colombia	S (CTA 080, 1976)	EtOH (2013)
ProM	*R. prolixus*		Colombia	S (CTA 077, 1982)	EtOH (2013)
NEGP	*R. robustus*	*R. prolixus*	Coronel José Dias, Pará, Brazil	F (sylvatique)	EtOH (2003)
RobR	*R. robustus*	*R. prolixus*	Lima, Peru	S (CTA 085, 1982)	EtOH (2006)
Rob5s	*R. robustus*		Guyane	F (sylvatic)	EtOH (2014)
ROBB	*R. robustus*	*R. marabaensis*	Maraba, Brazil	F (sylvatic)	EtOH (2003)
RobQ	*R. robustus*	*R. prolixus*	Pará, Brazil	F (sylvatic)	EtOH (2003)
R4H	*R. robustus*	*R. prolixus*	Brazil	S (sylvatic)	EtOH (2013)
V-StaWZ	*R. stali*		Bolivia	S (sylvatic)	EtOH (2006)
U-StaWY	*R. stali*		Alto Beni, Bolivia	S (sylvatic)	EtOH (2006)
R3WT	*R. taquarussuensis*	*R. neglectus*	Taquarussu, Mato-Grosso do Sul, Brazil	S (CTA, 2011)	EtOH (2013)

Morphological determination prior the study and molecular determination posterior the study are indicated.
Specimens are either from the field, F or from laboratory strain, S, and the original habitat (sylvatic/domiciliary) is indicated in the brackets.
For strains, the reference and the year of the strain foundation are given, with CTA: Colônias de Triatominae, Araraquara, Brazil.
The storage conditions in the French laboratory is indicated, with EtOH: absolute ethanol.

corresponding BUSCO genes and the alignment are given in the **Supplementary Table 1**.

Diversity Parameters

In order to compare the variation rate between the datasets, the nucleotide diversity (Pi) was estimated for each dataset using DnaSP 6 (Rozas et al., 2017). Pairwise distances were calculated between samples using MegaX (Kumar et al., 2018) using the Maximum Composite Likelihood model and all the substitutions. The similarity between pairwise samples was calculated in percentage using the ratio of the total number of conserved sites and the total number of both conserved and variable.

Phylogenetic Analysis

All the individual mitochondrial genes and the three concatenated datasets namely the 15 mtG, the nu-rDNA, and the

TABLE 3 | Genomic data.

Samples	Data (Gb)	Genome depth (x)	mtG (bp)	mt sequence coverage (%)	nu-rDNA (pb)	nu-rDNA sequence coverage (%)	nu-PCGs (bp)	nu-PCG sequence coverage (%)
PSAM	9.3	13.29	13,300	1.00	5,189	1.00	33,793	0.93
Ama1A	5.9	8.43	13,300	1.00	5,189	1.00	34,434	0.94
BRE25WB	8.4	12.00	13,300	1.00	5,189	1.00	34,604	0.95
R9WX	7.7	11.00	13,300	1.00	5,189	1.00	28,108	0.77
R10B	12.2	17.43	12,301	0,92	5,189	1.00	34,137	0.94
DomC	16.7	23.86	13,300	1.00	5,189	1.00	35,078	0.96
ECUD	19	27.14	13,300	1.00	5,189	1.00	34,421	0.94
ECUE	6.6	9.43	13,300	1.00	5,189	1.00	30,504	0.84
MILE	11.2	16.00	13,245	1.00	5,189	1.00	35,692	0.98
MILEP	7.1	10.14	13,300	1.00	5,189	1.00	33,338	0.91
R8F	12.4	17.71	13,300	1.00	5,189	1.00	33,051	0.91
INCP	3.7	5.29	13,300	1.00	5,189	1.00	26,246	0.72
NasG	10.7	15.29	13,214	0.99	5,189	1.00	36,444	1.00
R7WU	11.4	16.29	12,203	0,92	5,189	1.00	32,565	0.89
NASP	4.1	5.86	13,300	1.00	5,189	1.00	29,385	0.81
R5WU	16.1	23.00	13,266	1.00	5,189	1.00	35,098	0.96
NEII	9.2	13.14	13,300	1.00	5,189	1.00	35,976	0.99
R1J	12.7	18.14	13,300	1.00	5,189	1.00	35,085	0.96
PIC34WC	9.5	13.57	13,300	1.00	5,189	1.00	33,932	0.93
PIC3L	19	27.14	13,300	1.00	5,189	1.00	31,363	0.86
PIC2WA	8.9	12.71	13,300	1.00	5,189	1.00	33,049	0.91
R6WV	12.6	18.00	13,300	1.00	5,189	1.00	33,95	0.93
R63K	12.6	18.00	13,300	1.00	5,189	1.00	34,032	0.93
Pro10O	8	11.43	13,300	1.00	5,189	1.00	36,135	0.99
ProYRP	7.3	10.43	13,300	1.00	5,189	1.00	33,794	0.93
ProN	7.6	10.86	13,300	1.00	5,189	1.00	35,202	0.97
ProM	12.7	18.14	13,300	1.00	5,189	1.00	35,437	0.97
NEGP	5.5	7.86	13,300	1.00	5,189	1.00	32,121	0.88
RobR	7.9	11.29	13,300	1.00	5,189	1.00	36,343	1.00
Rob5s	21.1	30.14	12,301	0.95	5,189	1.00	36,234	0.99
ROBB	6.4	9.14	13,300	1.00	5,189	1.00	29,699	0.81
RobQ	8.5	12.14	13,300	1.00	5,189	1.00	35,368	0.97
R4H	12.2	17.43	10,500	0.79	5,189	1.00	35,149	0.96
V-StaWZ	8.7	12.43	13,300	1.00	5,189	1.00	33,67	0.92
U-StaWY	10.8	15.43	13,300	1.00	5,189	1.00	30,769	0.84
R3WT	12.9	18.43	13,300	1.00	5,189	1.00	35,532	0.97
Tbra							30,347	0.83
Tdim			13,225	0.99				
Pruf			12,473	0.94				
Bcol					5,189	1.00		

For all the samples used are given: the quantity in gigabase (Gb) of the data generating by the whole-genome shotgun sequencing by Illumina technology (Data), the corresponding genome depth (x), given that 0.7 Mb (R. prolixus genome reference) was used for the calculation, the length sequences in base pair (bp) for the mtG, the nu-rDNA and the nu-PCGs datasets and the corresponding sequence coverage in percentage.
*See **Table 1** for the Rhodniini samples description.*
The Triatominae outgroups used were: T. brasiliensis (Tbra), T. dimidiata (Tdim), P. rufotuberculatus (Pruf), and B. colossus (Bcol).

nu-PCG alignments, were submitted to phylogenetic analyses. For each dataset, we used as outgroup the closest relatives identified in the sequence database. For the mitochondrial dataset, as some mitogenomes were sequenced for Triatominae, we used *T. dimidiata* (AF301594; Dotson and Beard, 2001) and *Panstrongylus rufotuberculatus* (NC_042682; Zhao et al., 2019). For the nu-rDNA dataset as only partial data is available for Triatominae, we used as outgroup the Reduviidae *Brontostoma colossus* (NC_024745; Kocher et al., 2014) and for the nu-PCGs, *T. brasiliensis* (Marchant et al., 2015). For the three concatened alignments, the sequence coverage is given for each sample in the **Table 3**. The optimal partitioning scheme and models of nucleotide substitution for each concatenated dataset were first analyzed using PartitionFinder v2.1.1 (Lanfear et al., 2012). For the concatenated mt-PCGs, alignments were also separated into single codon positions. Optimal partitioning

and nucleotide substitution models were subsequently analyzed using the Bayesian Information Criteria (BIC) for all datasets. For the concatenated datasets two types of trees were generated. MrBayes (Ronquist et al., 2012) was used, to generate Bayesian Inference (BI) topologies using the partitioning scheme and models of evolution suggested by PartitionFinder and by performing two independent 10-million generation runs. Maximum Likelihood phylogenies (ML) were inferred using IQ-TREE (Minh et al., 2020) using the partitioning scheme and models of evolution suggested by PartitionFinder and the approximate likelihood ratio test with the non-parametric Shimodaira–Hasegawa correction (SH-aLRT) (Guindon et al., 2010) was used to estimate the support for each node with 1,000 replicates. For individual mitochondrial genes, Maximum Likelihood phylogenies (ML) were inferred using IQ-TREE (Minh et al., 2020) after searching the best substitution model with ModelFinder (Kalyaanamoorthy et al., 2017) and SH-aLRT branch test with 1,000 replicates. Phylogenetic trees were displayed and modified using iTol (Letunic and Bork, 2019). Gene partitions and best substitution models for the concatenated dataset are given in the **Supplementary Table 1**.

Detection of Selection Signatures

Two approaches were used to unravel potential selective pressures during the evolution of *Rhodnius* mtDNA genomes and more specifically to identify whether the specific groups, namely *pallescens, pictipes* and *prolixus*, evolved at different rates and were under unique signatures of selection.

First, we applied the McDonald–Kreitman test (McDonald and Kreitman, 1991) using DnaSP 6 (Rozas et al., 2017) to investigate the rates of adaptive evolution in mitochondrial and nuclear genes. We used the test to compare synonymous and non-synonymous variations within and between species groups. Under neutrality (estimated with the neutrality index NI and the Fisher exact test), the ratio of replacement to synonymous fixed substitutions between species groups should be the same as the ratio of replacement to synonymous polymorphisms within species groups.

Positive selection was also investigated using branch-site models to test for differential selection on the three species groups with CodeML (PAML package v4.9; Yang, 2007) using the graphical user interface EasyCodeML (Gao et al., 2019). The mt-PCG and the nu-PCG datasets were used for this test but also the four mitochondrial genes usually used for *Rhodnius* phylogenies, namely *Cytb, COI, COII, Nad1*.

Introgression Analysis

To detect signatures of introgression in the *pallescens* and *pictipes* groups, we used the ABBA-BABA test (Green et al., 2010). The ABBA-BABA test considers that for phylogenetically-informative di-allelic sites in a four-taxon bifurcating tree, the most frequent configuration should be (A,(A,(B,B))), congruent with the species tree. Incomplete lineage sorting or homoplasy could lead to equal amounts of loci with (A,(B,(B,A))) and (B,(A,(B,A))) incongruent configurations. However, introgression could be detected if either one of the two configurations significantly exceeds the other. For the *pallescens* group, we used the three samples, *R. pallescens* R1J, *R. colombiensis* R10B and *R. ecuadoriensis,* ECUD with *R. neivai* NEII as the outgroup and for the *pictipes* outgroup, *R. stali* STAWZ, *R. brethesi* R9WX, *R. pictipes* R63K and *R. amazonicus* Ama1A as the outgroup. Raw Illumina reads of each species were mapped to the *R. prolixus* reference sequences[2] using minimap2 (v. 2.17-r941; Li, 2018) with default parameters. The mapped reads were filtered for a mapping quality of 20 with samtools (v. 1.9; Li et al., 2009). BAM files were cleaned by removing unmapped reads and sorted by coordinate using Picard v. 1.4.2[3]. The sorted BAM files were then combined in a mpileup file with samtools and finally converted to a synchronized file (one species per column) with custom scripts from PoPoolation2 (v. 1.201; Kofler et al., 2011). Using a customized perl script on the synchronized file, we counted the three configurations for all diallelic loci with a minimum coverage of 10 reads in the four species.

RESULTS

Mitochondrial Phylogenetic Inferences

The mitochondrial dataset is composed of 36 samples from the Rhodniini tribe and the 15 mitochondrial genes. The concatenated alignment totalized 13.3 kb with a nucleotide diversity (Pi) of 0.12389. No pseudogene was retrieved from our dataset as attesting by the functional proteins obtained after the traduction (**Supplementary Table 1**). For the concatenated dataset mtG, highly congruent trees were obtained from both the Maximum-Likelihood (**Figure 1**) and the Bayesian Inference (**Figure 2**) methods and showed *pallescens* and *pictipes* groups as sister clades with high statistical support (ML: 98.8, BI:100). For the individual genes, various topologies were obtained (**Supplementary Figures 1–15**). For nine genes, the same topology as for the concatenated dataset was obtained, namely for *Cytb* (**Supplementary Figure 8**), Nad1 (**Supplementary Figure 9**), *Nad5* (**Supplementary Figure 14**), *Nad6* (**Supplementary Figure 15**) and also for *Atp6* (**Supplementary Figure 3**), *Atp8* (**Supplementary Figure 4**), *COI* (**Supplementary Figure 6**), *COIII* (**Supplementary Figure 7**), and *Nad2* (**Supplementary Figure 10**), but for these five genes a paraphyly was observed for the *prolixus* group, *R. neivaii*, *R. domesticus*, and/or *P. tertius*, according to genes being outside the prolixus group. For four genes, the *pictipes* and the *prolixus* groups were sister clades, namely for *12S* (**Supplementary Figure 1**), *16S* (**Supplementary Figure 2**), *Nad3* (**Supplementary Figure 11**), *Nad4* (**Supplementary Figure 12**), and the *Nad4L* but for this gene the *pictipes* group is less supported (**Supplementary Figure 13**). The third topology, that is the *pallescens* and *prolixus* groups as sister clades, was displayed for the COII gene (**Supplementary Figure 6**) but the position of *R. neivaii* and *R. domesticus* were weak supported.

The analysis of rare insertion/deletion (InDel) patterns in the alignment indicated the presence of an InDel of 40 pb in the tRNA Cys that supported the genetic proximity between the *pallescens*

[2]www.vectorbase.org

[3]http://broadinstitute.github.io/picard/

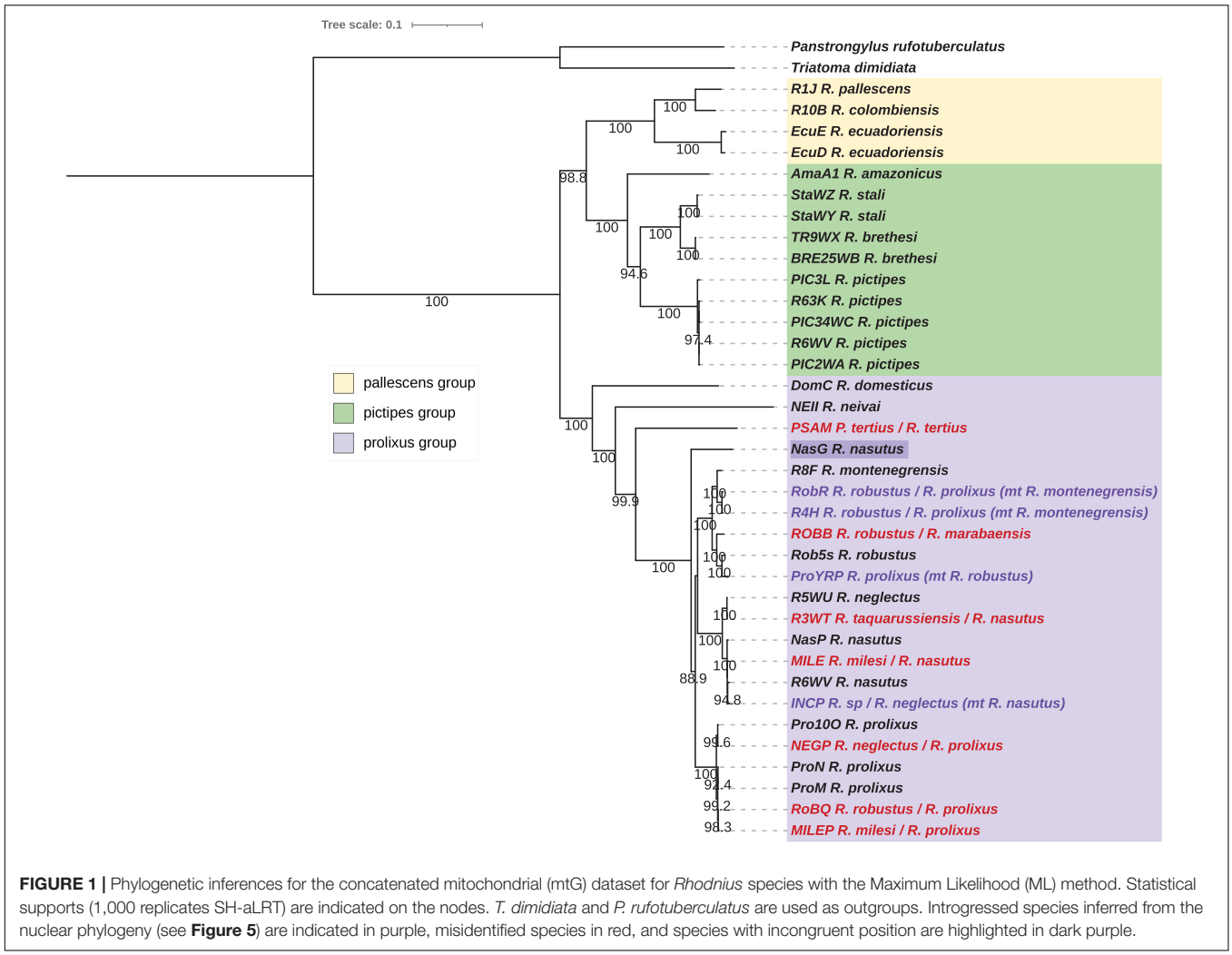

FIGURE 1 | Phylogenetic inferences for the concatenated mitochondrial (mtG) dataset for *Rhodnius* species with the Maximum Likelihood (ML) method. Statistical supports (1,000 replicates SH-aLRT) are indicated on the nodes. *T. dimidiata* and *P. rufotuberculatus* are used as outgroups. Introgressed species inferred from the nuclear phylogeny (see **Figure 5**) are indicated in purple, misidentified species in red, and species with incongruent position are highlighted in dark purple.

and the *pictipes* groups and the split with the *prolixus* group (**Supplementary Figure 16**).

According to the concatenated mtG trees (**Figures 1**, **2**), for the *pictipes* group, *R. stali* and *R. brethesi* were closer to each other than to *R. pictipes*. In the *pallescens* group, *R. pallescens* and *R. colombiensis* were closer to each other than to *R. ecuadoriensis*. The *prolixus* group comprised samples of *R. domesticus, R. neivai, R. montenegrensis, R. taquarussuensis* (syn *R. neglectus*), *R. neglectus, R. nasutus, R. robustus, R. prolixus* and also *P. tertius*. Indeed, the *Psammolestes* species was robustly included in the *prolixus* group independently of the considered mitochondrial dataset. However, in the two trees, ML (**Figure 1**) and BI (**Figure 2**), the genetic proximity between the three clades (*R. montenegresis-R. robustus/R. nasutus-R. neglectus/R. prolixus*) is not resolved. For *R. amazonicus* some discrepancies were observed according to the mitochondrial dataset. For the concatenated dataset, this taxon was related to the *pictipes* group at a basal position but for the individual datasets it was either related to the *pallescens* group or the *pictipes* group.

Three Brazilian specimens seemed to have an aberrant position in the trees. Specimens NEGP and MILEP, morphologically identified as *R. neglectus* and *R. milesi* respectively, were related to *R. prolixus*. The NASG specimen, identified as *R. nasutus,* is at a basal position in the ML tree (but with a weak bootstrap) and nested within the *prolixus* group but not related to the *R. nasutus-R. neglectus* clade in the BI tree. The non-identified INCP sample was related to *R. nasutus*.

For samples identified as *R. robustus* and *R. prolixus,* two clades were inferred, each of them comprising samples from the two species. One is composed of all the *R. robustus* samples except the field specimen RobQ from Brazil, the laboratory strain *R. montenegrensis* and one *R. prolixus* sample, YRP, a Venezuelan laboratory strain from Cojedes. The other grouped all other *R. prolixus* samples with the RobQ.

Concerning species with questionable species-specific status in addition to the topologies observed in the phylogeny, the pairwise distances (**Supplementary Table 2**) and the similarity index can be considered using the mitochondrial PCG concatenated dataset. *R. neglectus* (R5WU) and *R. taquarussuensis* (syn *R. neglectus*) (R3WT) appeared to be very closely related taxa (pairwise distance = 0.0012; similarity = 99.88%). Unexpectedly, in the phylogeny, *R. milesi* (MILE) was included in the same

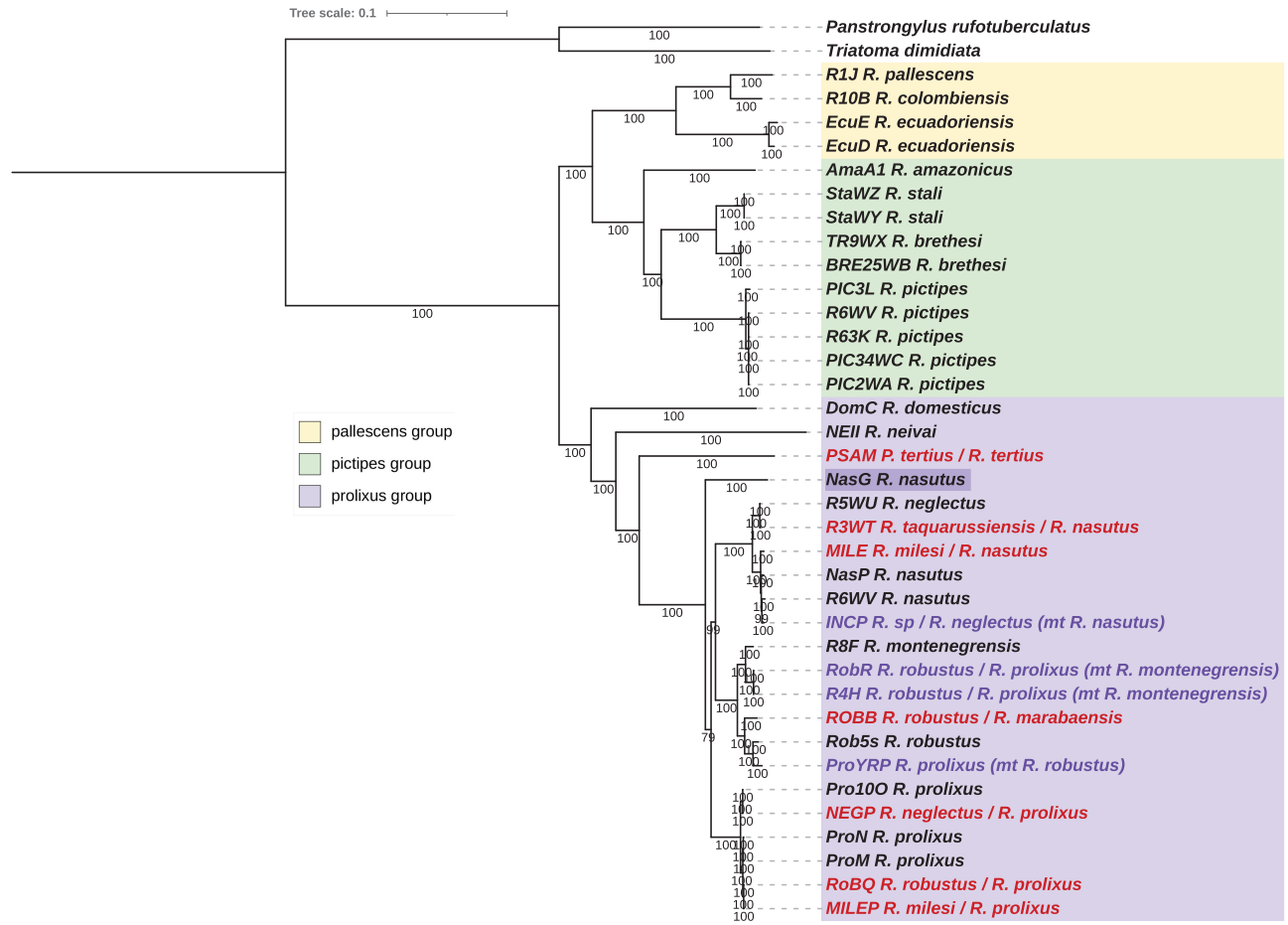

FIGURE 2 | Phylogenetic inferences for the concatenated mitochondrial (mtG) dataset for *Rhodnius* species using Bayesian Inference (BI) method. Statistical supports (specify Bayesian posterior probabilities in percent) are indicated on the branches. *T. dimidiata* and *P. rufotuberculatus* are used as outgroups. Introgressed species inferred from the nuclear phylogeny (see **Figure 6**) are indicated in purple, misidentified species in red, and species with incongruent position are highlighted in dark purple.

clade as the laboratory reference R5WU *R. nasutus* sample (MILE R7WU: 0.0069; 98.29%). More distant pairwise values were obtained between *R. montegrenensis* and *R. robustus,* the smallest being with the *R. robustus* samples from Peru (R8F RobR: 0.0149; 98.56%) and the highest with the Amazonian ones, the Brazilian ROBB (R8F ROBB: 0.0311; 97.13%) and the Guyanese Rob5s (R8F Rob5s: 0.0326; 97%). For comparison, the pairwise values between *R. nasutus* (RW7) and *R. neglectus* (R5WU) that are close but clearly distinct species, were smaller (0.0202; 98.09%) than those between *R. robustus* and *R. montenegrensis*.

Nuclear rDNA Phylogenetic Inference

The nuclear rDNA dataset included 36 taxa from the Rhodniini tribe for an alignment length of 5.2 kb and a Pi of 0.0089. ML and BI rDNA phylogenetic analysis confirmed the three major Rhodniini groups (*pallescens, pictipes*, and *prolixus*) but not supported by high bootstrap values (**Figures 3**, **4**). However, an InDel of 19 pb in the 28S rDNA genes supported the genetic proximity between the *pallescens* and the *pictipes* groups (**Supplementary Figure 16**).

For the resolution within the *Rhodnius* groups, only the BI tree gave some reliable results (**Figure 4**). The *pallescens* group exhibited a good resolution level, with the two species *R. pallescens* and *R. ecuadoriensis* closer related than to *R. colombiensis*. For the *prolixus* group, only the R7WU *R. nasutus* is differentiated from the other *R. neglectus* samples including *R. taquarenssensis* (syn. *R. neglectus*) (R3WT). Two *R. robustus* specimens (RobR, Rob5s) were clustered with *R. montenegrensis* R8F. Note that *Psammolestes* was clustered with the *R. prolixus* species. The *pictipes* group was the least resolute.

Nuclear Protein-Coding Gene Phylogenetic Inferences

The nu-PCG dataset included 36 taxa from the Rhodniini tribe. The trees were reconstructed using 51 PCGs for an alignment length of 36.3 kb with a Pi of 0.02648. The tree topologies between

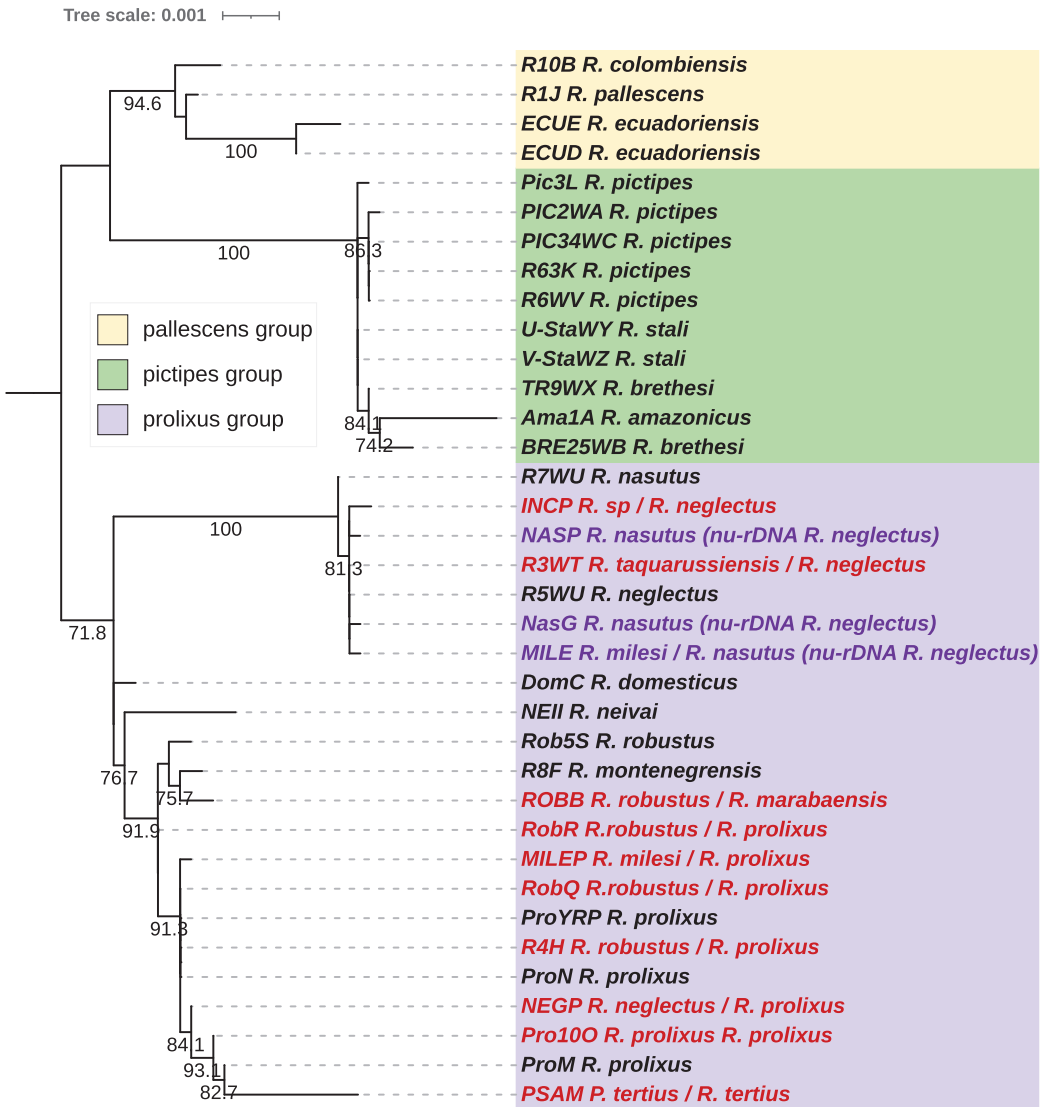

FIGURE 3 | Phylogenetic inferences for the concatenated nuclear rDNA (nu-rDNA) dataset for *Rhodnius* species with the Maximum Likelihood (ML) method. Statistical supports (1,000 replicates SH-aLRT) are indicated on the nodes. *B. colossus* was used as outgroup. Introgressed species inferred from the nuclear phylogeny (see **Figure 5**) are indicated in purple, and misidentified species in red.

the reconstruction methods, ML (**Figure 5**) and BI (**Figure 6**) are fully congruent and robust.

As for the mitochondrial dataset, the three groups, *pictipes*, *pallescens*, and *robustus,* were robustly identified (MI and BI trees) with the same topology with *pallescens* and *pictipes* as sister groups. Moreover, the same topology was found for the *pallescens* group, *R. pallescens* and *R. colombiensis* being closer to each other than to *R. ecuadoriensis.* Discrepancies were found for the *pictipes* and the *prolixus* groups. Unlike the relationship revealed with mitochondrial data, *R. stali* and *R. pictipes* appeared with nuclear data closer to each other than to *R. brethesi*. Similar to what was observed with the concatenated mitochondrial dataset, *R. amazonicus* was related to the *pictipes* group at a basal position. For *R. robustus* and *R. prolixus* samples, two well supported related clades were observed. The Guyanese *R. robustus* (Rob5s), the Brazilian *R. robustus* (ROBB) and the *R. montenegrensis* (R8F) samples were grouped in the same clade. All the other *R. robustus* and *R. prolixus* samples were in another clade.

Concerning the pairwise distances (**Supplementary Table 2**) and the similarity index, *R. neglectus* (R5WU) and *R. taquarussuensis* (syn *R. neglectus*) (R3WT) were very closely related (pairwise distance = 0.00076; similarity = 99.82%), and *R. milesi* (MILE) was closest to R7WU *R. nasutus* (MILE R7WU: 0.00192; 99.81%). More distant pairwise values than for the two previous pairs of species were obtained between *R. montegrenensis* and *R. robustus,* e.g., RobB (R8F RobB: 0.00309; 99.62%) and Rob5s (R8F Rob5s: 0.00408; 99.59%) and were of the same order of magnitude as between the Guyanese *R. robustus* (Rob5s) and *R. prolixus* samples e.g., Pro100 (Rob5s Pro100: 0.00485; 99.52%). Similar distant pairwise values were

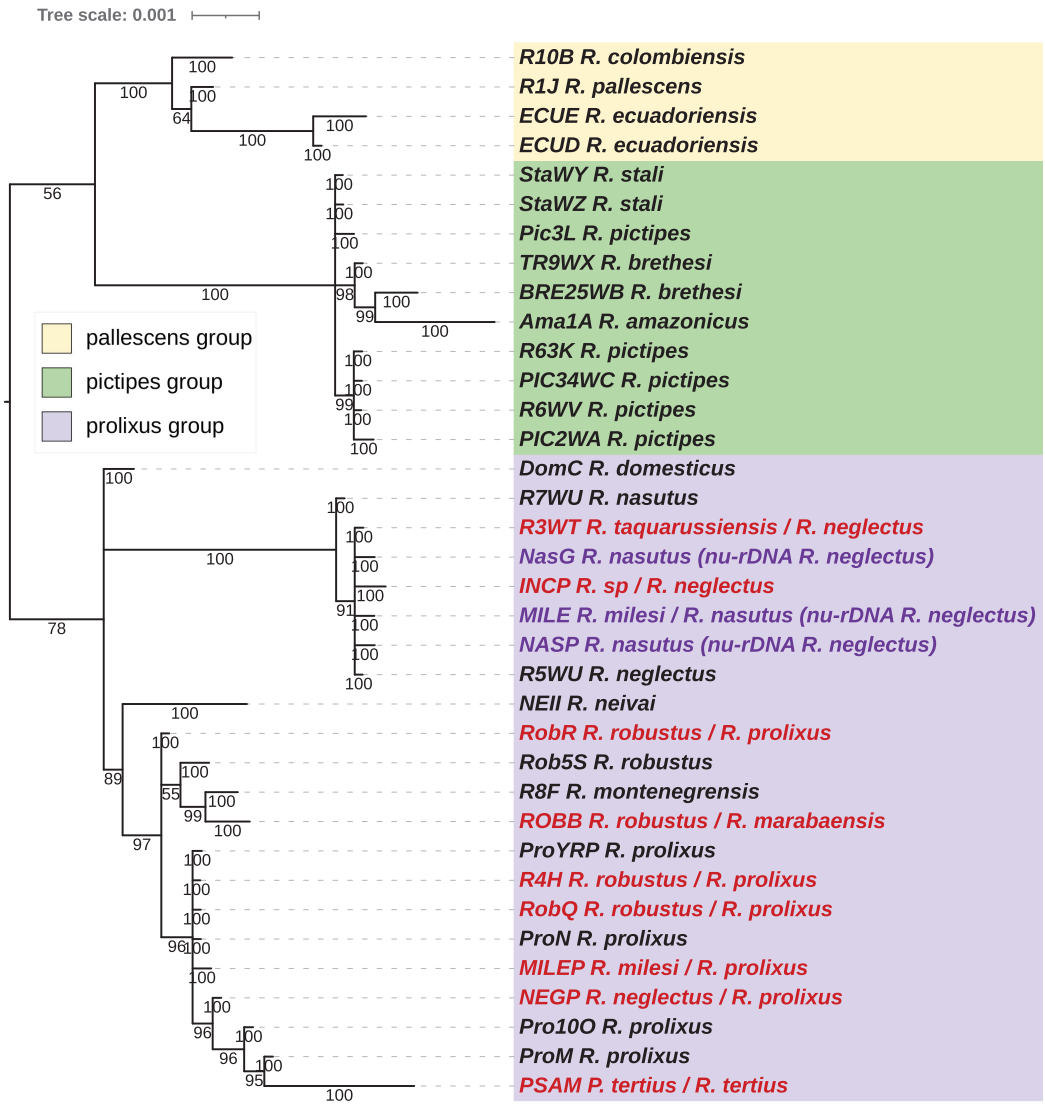

FIGURE 4 | Phylogenetic inferences for the concatenated nuclear rDNA (nu-rDNA) dataset for *Rhodnius* species using Bayesian Inference (BI) method. The statistical supports (specify Bayesian posterior probabilities in percent) are indicated on the branches. *B. colossus* was used as outgroup. Introgressed species inferred from the nuclear phylogeny (see **Figure 5**) are indicated in purple, and misidentified species in red.

obtained between *R. nasutus* and *R. neglectus* (R7WU R5WU: 0.0036; 99.64%) or between the R5WU *R. neglectus* and INCP (R5WU INCP: 0.004; 98.16%) or NASP (R5WU NASP: 0.00396; 99.65%). It should be of interest to note that NasG, placed outside the *R. nasutus-R. neglectus* clade for the concatenated mitochondrial dataset, was close related to R7WU *R. neglectus* (NASG R7WU: 0.00182; 99.82%).

Test of Selection

The McDonald–Kreitman test detected significant positive selection in the concatenated mitochondrial dataset for the three group comparisons, *prolixus-pallescens* groups (NI = 0.696; *p*-value = 0.023), *prolixus-pictipes* groups (NI = 0.609; *p*-value = 0.003), and *pallescens-pictipes* groups (NI = 0.779; *p*-value = 0.04), revealing an excess of divergent non-synonymous mutations between the group tested. For the concatenated nuclear PCG dataset no selection was detected for any pairwise, *prolixus-pallescens* groups (NI = 0.754; *p*-value = 0.41), *prolixus-pictipes* groups (NI = 0.583, *p*-value = 0.199), and *pallescens-pictipes* groups (NI = 0.931; *p*-value = 0.792).

Selective pressure was also tested using PAML method (**Table 4**). For the concatenated mitochondrial dataset, selective pressure was detected in each *Rhodnius* group test against the others (LRT *p*-value = 2×10^{-9} for *pallescens* vs. *pictipes* and *prolixus* groups; 0.0044 for *pictipes* vs. *pallescens* and *prolixus* groups; 9.6×10^{-7} for *prolixus* vs. *pictipes* and *pallescens* groups). For the single mitochondrial genes tested, namely *cytb, COI, COII, Nad1*, all were under selective pressure for the *prolixus* group tested against the *pictipes* and *pallescens* groups (LRT *p*-value = 13×10^{-6}; 13×10^{6}; 0.0009; 0.02 respectively) and

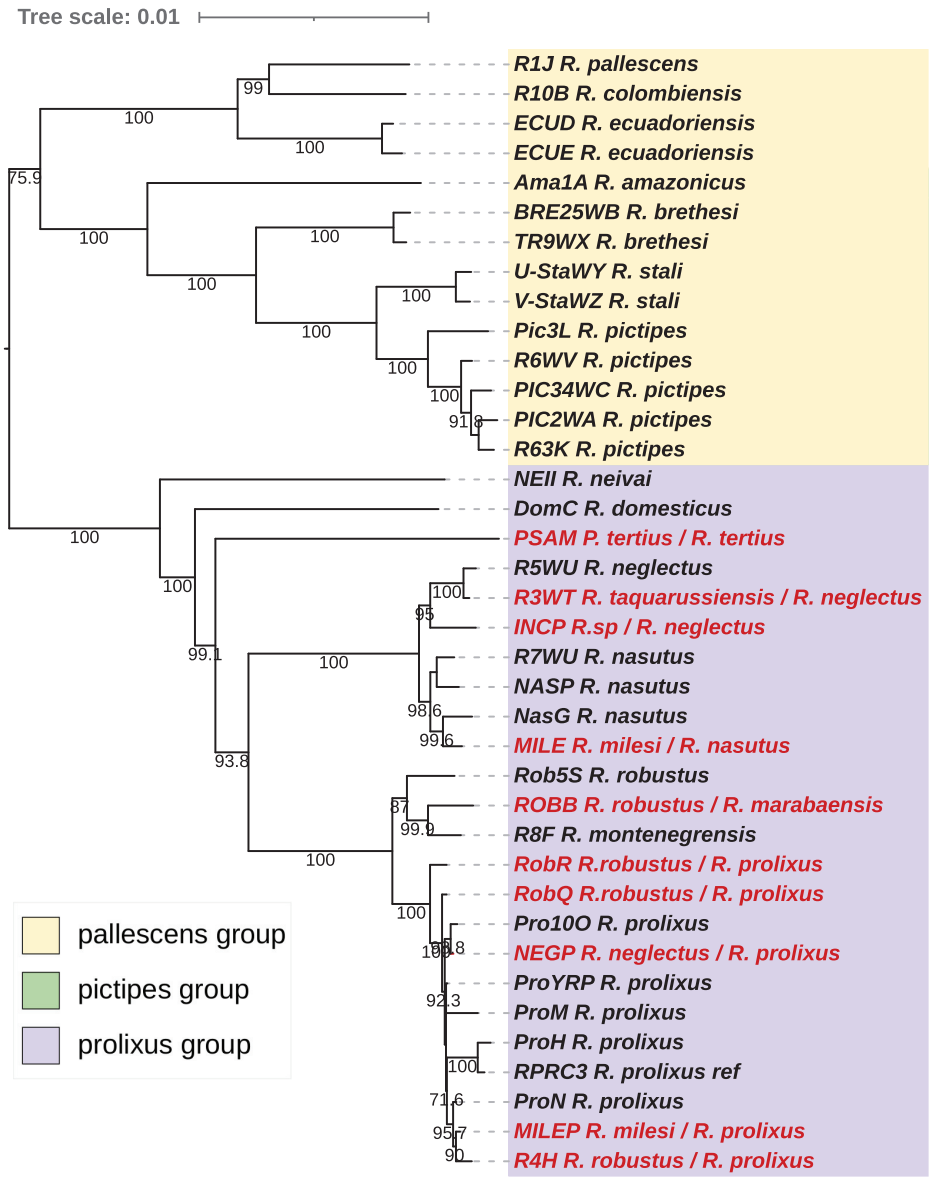

FIGURE 5 | Phylogenetic inferences for the concatenated nuclear protein-coding gene (nu-PCGs) dataset for *Rhodnius* species with the Maximum Likelihood (ML) method. The statistical supports (1,000 replicates SH-aLRT) are indicated on the nodes. *T. brasiliensis* was used as outgroup. Misidentified species are indicated in red.

two, *COI* (LRT *p*-value = 0.046) and *COII*, for the *pallescens* group tested against the *pallescens* and *prolixus* groups (LRT *p*-value = 0.04). For the concatenated nuclear dataset no selective pressure was detected for any of the groups (LRT *p*-value = 0.47; 0.10; 0.14 for *pallescens, pictipes* and *prolixus* groups, respectively, tested against the others groups).

Genome-Wide Examination for *pallescens* and *pictipes* Groups

We conducted the ABBA-BABA test of introgression in the *pictipes* group for which a discordance between the mitochondrial and nuclear PCG trees was noticed. *R. stali* and *R. brethesi* were indeed clustered together in the mitochondrial tree while *R. stali* was closer to *R. pictipes* in the nuclear tree.

The three species *R. brethesi*, *R. stali* and *R. pictipes* were tested for introgression and *R. amazonicus* as the outgroup. The ABBA-BABA results were in accordance with the nuclear phylogeny of the species and provided a strong support for a mitochondrial *R. stali* introgression in *R. brethesi*. Out of 6,437 bi-allelic SNPs, we counted 5,571 AABB, namely *R. pictipes* closer to *R. stali*, 409 ABBA, namely *R. brethesi* closer to *R. pictipes* and 457 BABA, namely *R. brethesi* closer to *R. stali*. No signal of further nuclear introgression was found between *R. brethesi* and *R. stali* since

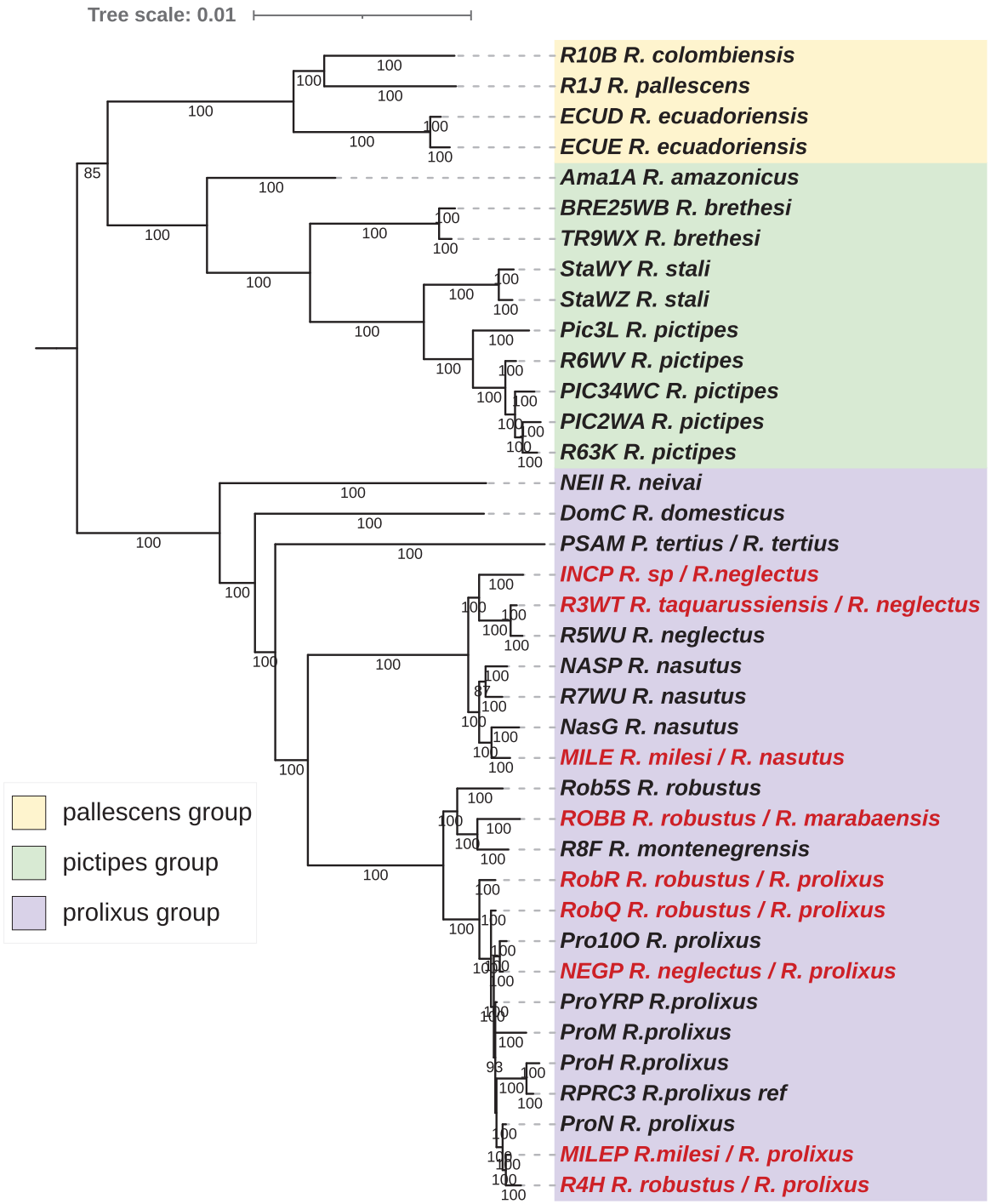

FIGURE 6 | Phylogenetic inferences for the concatenated nuclear protein-coding gene (nu-PCGs) dataset for *Rhodnius* species using Bayesian Inference (BI) method. The statistical supports (specify Bayesian posterior probabilities in percent) are indicated on the branches. *T. brasiliensis* was used as outgroup. Misidentified species are indicated in red.

both ABBA and BABA counts were almost equal and far below the AABB (i.e., the nuclear tree topology) SNP counts.

We conducted the same analysis in the *pallescens* group, for which the mitochondrial and nuclear PCG trees are congruent showing *R. colombiensis* and *R. pallescens* clustered together, but in the rDNA phylogeny, *R. pallescens* and *R. colombiensis* are closer. We found a strong signal for pervasive and widespread phylogenetic incongruence between the three species,

TABLE 4 | Test of selection results using the PAML package.

Datasets	*pallescens* group	*pictipes* group	*prolixus* group
mt-PCGs	**0.000000002** ω0 = 0.06533 ωpall = 0.04302	**0.004452367** ω0 = 0.06222 ωpic = 0.04958	**0.000000958** ω0 = 0.05644 ωpro = 0.07712
Cytb	0.141282571 ω = 0.03490	0.248087413 ω = 0.03490	**0.000013280** ω0 = 0.000013280 ωpro = 0.06698
COI	**0.046034763** ω0 = 0.01252, ωpall = 0.00639	0.521251673 ω = 0.01134	**0.000013280** ω0 = 0.02806 ωpro = 0.06698
COII	**0.040155883** ω0 = 0.02126, ωpall = 0.01023	0.139838995 ω = 0.01887	**0.000910664** ω0 = 0.01453 ωpro = 0.04180
Nad1	0.202681777 ω = 0.01952	0.440764212 ω = 0.01952	**0.020486354** ω0 = 0.01366 ωpro = 0.03990
nu-PCGs	0.696800235 ω = 0.04575	0.373073414 ω = 0.04575	**0.001175575** ω0 = 0.05701 ωpro = 0.02452

For each gene or the concatenated datasets, the null model (ω for the whole tree) is compared by an LRT test to the model with 2 ratios: ω0, the ratio for branches of the two Rhodnius groups not tested and ωpall or ωpic or ωpro, the ratio for the Rhodnius group tested.
The model that best explains the data is reported in the table with the p-value. Significant p-values are indicated in bold.

R. pallescens, R. colombiensis, and *R. ecuadoriensis*. Out of a total of 18,910 di-allelic SNPs, the ABBA-BABA test gave 7,666 sites with the configuration AABB, namely *R. pallescens* is closer to *R. colombiensis*, 4,084 with the ABBA configuration supporting a closer relationship between *R. ecuadoriensis* and *R. colombiensis*, and 7,160 with the configuration BABA supporting a closer relationship between *R. ecuadoriensis* and *R. pallescens*. Remarkably, the BABA counts even approached those of the AABB species tree counts, indicating that the exact position of *R. pallescens* could not be determined with certainty. In order to test if the ABBA or BABA configurations are restricted to specific genomic regions, the evaluated SNPs were mapped to the reference genome of *R. prolixus*. We did not observe any specific localization, the three configurations were widespread and overlapping on all scaffolds of the reference genome of *R. prolixus*.

DISCUSSION

Phylogenetic Relationship Between the Three *Rhodnius* Groups

In this study, our goal was to provide a large genomic dataset in order to elucidate key issues on phylogenetic relationships for yet the most taxonomically controversial Tritaominae tribe, the Rhodniini, using both mitochondrial and nuclear datasets analyzed separately. Previous *Rhodnius* phylogenetic studies used a limited number of genes, which could explain the divergent topologies found for the *Rhodnius* groups. Three topologies were recovered independently of the type of marker used. Considering studies with at least eight *Rhodnius* species and reliable phylogenetic reconstruction methods (Maximum Parsimony, ML, BI), the *pictipes-pallescens* clade was recovered using only mitochondrial markers, as *cytb* (Maia da Silva et al., 2007), as well as using a combination of mitochondrial and nuclear genes, as *16S* + *cytb* + *28S* (Monteiro et al., 2000). The *pictipes-prolixus* clade was obtained using also only mitochondrial markers: 16S (Paula et al., 2005, 2007) or *16S* + *cytb* (Hypša et al., 2002), as well as mitochondrial and nuclear markers together, namely *18S* + *28S* + *16s* + *Cytb* + *COI* + *COII* (Justi et al., 2014) or *18S* + *28S* + *wg* + *16S* (Justi et al., 2016). It is to highlight that Paula et al. (2021) by combining the three genes, *16S, 18S, and wg*, obtained contrasted results according to the type of analysis used, namely *pictipes-prolixus* clade with MP and *pictipes-pallescens* with ML. Using ultraconserved elements and/or rDNA data, Kieran et al. (2021) obtained the *pallescens-prolixus* clade. To limit the methodological bias due to a limited dataset, we used in this study a large genomic dataset of both mitochondrial (13.3 kb) and nuclear data (5.3 kb of rDNA; 51 PCGs, 36.3 kb). The large dataset was able to resolve the relationship between the three *Rhodnius* groups. Except for some single mitochondrial genes, both the concatenated mitochondrial and nuclear datasets showed *pictipes* and *pallescens* as sister groups, irrespective of the outgroup used.

Variation Rate Among Partitions

The incongruence of phylogenetic signals between different genomic regions can result from the rate of variation among partitions. Our study indicates that the nuclear rDNA markers (including *18S, 5.8S, 28S*, and *ITS*) led to a poorly resolved tree. Indeed, the nucleotide divergence of the rDNA marker dataset is low (max 1.99%) compared to the mitochondrial *Rhodnius* dataset (max 15.06%), and the nucleotide diversity (Pi) is 14 times slower than the mitochondrial dataset one. It results that slow-evolving rDNA genes are less suitable to infer phylogenetic relationships. Mitochondrial genes, which evolve more rapidly than the rDNA and the set of insect universal nuclear genes (BUSCO genes), seem to be candidates for more accurate phylogenetic reconstruction. In our dataset, based on Pi estimates, the mitochondrial genes evolve 4.7 times faster than the nuclear PCGs, which is in accordance with what was found in insects, the mitochondrial genes being estimated to evolve 2–9 times faster than the nuclear protein-coding genes (Lin and Danforth, 2004). Moreover, mitochondrial genes are easier to use for both technical and evolutionary reasons. Indeed, mitochondrial primers are widely available and as these mitochondrial genes are clonally inherited and non-recombining, they are not subjected to recombination and heterozygosity like the nuclear markers. Nevertheless, mitochondrial genes have some clear disadvantages (Lin and Danforth, 2004). Since all mitochondrial genes are linked to the same DNA unit, they cannot provide an independent estimate of phylogeny in the same way that unlinked single-copy, as the nuclear genes do (Harrison, 1989). Furthermore, the higher rate of substitution can be disadvantageous to resolve divergences between taxa of more than 5–10 million years, while they are useful to resolve

relationships between closely related taxa that have diverged relatively recently.

Selective Pressure on Mitochondrial Genes

In our study, we demonstrated that mitochondrial genes widely used for phylogenies, namely *cytb* and *COI,* were under selection for all the three groups, *pallescens*, *pictipes* and *prolixus.* By contrast, no selective pressure was evidenced for the nuclear PCGs. In the literature, there is increasing evidence that the genetic diversity of mtDNA may therefore be shaped not only by random genetic drift but also by natural selection given the functional importance of mitochondrial polymorphisms. Positive selection on the mitogenome has been shown for various species including vertebrates and insects (reviewed by Sun et al., 2018). This process may be related to energy production linked to migration but also aging, size, diet, neuronal function, and thermoregulation (see: Garvin et al., 2015; Sloan et al., 2017). For the *Rhodnius* species, selective pressure is likely linked to fitness-related traits that have to be investigated.

Species Diversity in the *Rhodnius* Genus

A delicate point of this work is to give molecular pertinent arguments about the species-specific status of some controversial or recently described species.

Concerning the status of the *Psammolestes* genus, some morphological differentiations with the *Rhodnius* genus has justified the erecting of two distinct genera as shorter head, stronger legs, wider femora and wider rostrum but also the lifestyle of the *Psammolestes* species in nests of birds while *Rhodnius* species live mainly in palm tree (Lent and Wygodzinsky, 1979). Our work clearly established using both large mitochondrial and nuclear datasets that *P. tertius* is included into the *Rhodnius* genus and the *prolixus* group. This position into the *Rhodnius* genus was also pointed out by previous studies (e.g., Lyman et al., 1999; Monteiro et al., 2000; Hypša et al., 2002; Justi et al., 2014; Paula et al., 2021). Thus, according to the cladistic principles and the rule of species name precedence, we strongly recommended reclassifying the *P. tertius* species as *Rhodnius tertius*. Complementary studies combining morphological and molecular approaches will be useful to know to what extent the morphological characters put forward in *Psammolestes* are part of some adaptive processes acting on *Rhodnius* species from differentiated environments. It is interesting to note that among the species of the *Rhodnius* genus, *R. paraensis* which presents a particular ecology because found in the nests of arboreal rodents, is a species of small size as *Psammolestes* with also short head, stout legs, short and stout antennae that led Lent and Wygodzinsky (1979) to suggest that these characters "make this one of the most plesiomorphic species of *Rhodnius*, if the progressive elongation of the head and appendages found in most other species is considered as derived." Phylogenetic studies including *R. paraensis* and *Psammolestes* species are required to determine the polarity (ancestral vs. derived) of the character states. Since the three *Psammolestes* species are morphologically and ecologically close (Lent and Wygodzinsky, 1979), share cytogenetic characteristics indicating a chromosomal homogeneity (Oliveira et al., 2018), and are phylogenetically closely related (rDNA, Kieran et al., 2021), we recommended to re-examine also the taxonomic status of the two other *Psammolestes* species, *P. arthuri* and *P. coreodes*.

The species *R. amazonicus* was initially described by Almeida et al. (1973). It was then invalidated by Lent and Wygodzinsky (1979) because of the description made on only one female collected in the municipality of Manaus (Amazonas, Brazil) with several *R. pictipes* specimens and was then seen as a poorly preserved specimen of *R. pictipes*. The species was then revalidated by Bérenger and Pluot-Sigwalt (2002) from both male and female specimens collected in French Guyana and more recently recorded by Rosa et al. (2017b) in Pará, Brazil. We provided the first molecular data for this species and confirmed by both our robust mitochondrial and nuclear phylogenies its species-specific status as *bona species*. Moreover, this taxon belonged to the *pictipes* group at a basal position.

In contrast, the large molecular data set of our study definitively closes the question about the species-specific status of *R. taquarussuensis* (syn *R. neglectus*), described by Rosa et al. (2017a) from a female, which invaded a rural dwelling in the city of Taquarussu, Mato Grosso do Sul, Brazil. Because of its very close genetic similarity for both mitochondrial and nuclear genes, we demonstrated that this sample belongs undoubtedly to the *R. neglectus* species. This result corroborated the revision performed by Nascimento et al. (2019) using two mitochondrial and four nuclear genes leading the authors to state that the morphological and constitutive heterochromatin pattern differences between *R. taquarussuensis* and *R. neglectus* are likely intraspecific polymorphism of *R. neglectus* and consequently to synonymize *R. taquarussuensis* with *R. neglectus*.

In our dataset, we included *R. milesi,* a less studied species. This species was described from Pará, Brazil and morphologically similar to *R. dalessandroi* (Valente et al., 2001). Yet, for this latter species, only the published description and illustration are available leading Lent and Wygodzinsky (1979) to not include it in their taxonomical work. Doubts about the species-specific status of *R. milesi* had been issued by Monteiro et al. (2018) in their review. The authors concluded to the proximity of *R. milesi* with *R. neglectus* based on molecular *cytb* and *ITS* sequences but the accession number of the *R. milesi* sequences are not provided. In our dataset, for both the nu-PCG and mitochondrial phylogenies, MILE is congruently related to the R7WU *R. nasutus* sample with low nuclear genetic pairwise values (<0.002) but is clustered with *R. neglectus* in the rDNA phylogeny. A possible explanation is that the MILE specimen used in this study is a *R. nasutus* with a probable introgression with some nuclear DNA from *R. neglectus*. This molecular finding is in accordance with the morphological observation made by Carcavallo et al. (in Valente et al., 2001) observing that *R. milesi* male genitalia have a second phallosome process, only found in *R. nasutus*. With respect to our study, we conclude that *R. milesi* should be considered as synonym to *R. nasutus* and we recommend reconsidering the species-specific status of this species with a larger sampling.

A crucial point concerns two species closely related to *R. robustus*, namely the two newly described *R. montenegrensis* (Rosa et al., 2012) and *R. marabaensis* (Souza et al., 2016). *Rhodnius montenegrensis* was described from a strain established from eight specimens collected in a palm tree in the municipality of Monte Negro, Rondonia, Brazil and morphologically identical to specimens previously caught in the same locality (Rosa et al., 2012). The authors assessed their description on morphological and *cytb* sequence divergences with *R. robustus*. But, previously, using a large set of *R. prolixus* and *R. robustus* samples, Monteiro et al. (2003) based on *cytb* phylogeny have described four clades for *R. robustus* and proposed to see *R. robustus* as a complex of species and concluded for a paraphyly for *R. robustus*. Thus, the validity of the newly described species was pointed out by Abad-Franch et al. (2013) by declaring that "*R. montenegrensis* likely represents one of the *R. robustus* lineages of Monteiro et al. (2003)." Using *cytb* and ITS2 genes from transcriptomic data, Brito et al. (2019) evidenced that *R. montenegrensis* and *R. robustus* clade II are likely the same species but this does "not invalidate the former as a separate species." Concerning *R. marabaensis,* this species was described from Marabá, Pará, Brazil (Souza et al., 2016). Unfortunately, no DNA sequence is available in public database for the described species, the authors only stated that the *cytb* sequence shown 99% of identity with that of *R. robustus*. In this study, based on the nuclear genes, we evidenced similar genetic distance values between the three pairwise involving Rob5s *R. robustus* from field French Guyana, *R. montenegrensis* (R8F), and ROBB. Similar distant pairwise values were also obtained between the two species *R. nasutus* and *R. neglectus*. Indeed, if we recognized *R. nasustus* and *R. neglectus* as valid species, the three entities must be too, especially since they displayed congruent position in all trees, mitochondrial and nuclear ones. Thus, we state that the sample Rob5s is representative of *R. robustus,* R8F of *R. montenegrensis* and the specimen ROBB has also a species-specific status. It is worth noting that ROBB was caught in a palm tree near the Benfica Field Station (5°16′S, 49°50′E) at 50 km from Marabá, Pará, Brazil. Since *R. marabaensis* was described from Marabá (Souza et al., 2016), even if molecular data for this are not publicly available, a parsimonious hypothesis could be formulated that ROBB is putatively *R. marabaensis*. The same assumption was made by Castro et al. (2020) assimilated the *R. robustus* clade III to *R. marabaensis* based on the geographical origin of the samples. For the four close related species *R. marabaensis, R. montenegrensis, R. robustus,* and *R. prolixus*, we proposed to use the terminology of "*robustus* species complex."

In order to corroborate the inference about the *R. robustus* clades defined using *cytb* sequences (Monteiro et al., 2003), despite we demonstrated that this gene was under selective pressure and that *Rhodnius* phylogeny is not well resolved using this marker, we nonetheless performed an ML phylogeny including the *R. robustus* clade sequences and also cytb sequences from *R. barretti* (**Figure 7**). In the *cytb* phylogeny: (i) *R. montenegrensis* R8F was robustly included in the ROB II clade, (ii) ROBB in the ROB III clade assimilated to *R. marabaensis* and (iii) *R. robustus* Rob5s in the ROB IV clade.

Note that the new species *R. barretti* described from both Colombia and Ecuador and morphologically close to *R. robustus* (Abad-Franch et al., 2013), showed on the *cytb* phylogeny in Monteiro et al. (2018) a basal position to *R. neglectus, R. nasutus,* and the *robustus* species complex. In the *cytb* phylogeny (**Figure 7**), the NASG has a position similar to *R. barretti*. If only mitochondrial genes were analyzed, due to the high divergence observed, the proposal of a specific status for this specimen could arise. But the nuclear data are unequivocal, NasG is relative to *R. nasutus* but having a mitochondrial introgression likely by DNA from a species of the *robustus* complex (see below). If the position of *R. barretti* in the *cytb* is irrefutable, more molecular data and especially nuclear ones are needed to discard putative introgression and definitively assess its phylogenetic position.

Discordance Between Mitochondrial and Nuclear Phylogenomic Inferences

If the mitochondrial genes used for phylogenetics suffer from disadvantages, discordance between mitochondrial and nuclear phylogenies could highlight evolutionary processes and be a potential source of additional information about the evolutionary history of a recent clade. Such mito-nuclear discordance is not rare and was reported in numerous taxa. Incomplete lineage sorting (ILS) could explain this discordance by the conservation of ancestral polymorphisms in multiple lineages after species splits (Degnan and Rosenberg, 2009). However, polymorphisms in mtDNA are expected to be lost relatively rapidly through genetic drift because of the reduced effective population size in mitochondrial genomes. This leads ILS to unlikely be the predominant cause of mito-nuclear discordance, notably when a large number of nuclear loci are congruent in phylogenies but in conflict with mitochondrial ones (Good et al., 2015). Another more likely cause of discordance could be introgression, highlighted between otherwise well-defined species by differentially affecting nuclear vs. mitochondrial DNA. In many cases, mitochondrial introgression occurs with few or no detectable movement of nuclear genes (Sloan et al., 2017). Nonetheless, evidence for mitochondrial introgression with accompanying nuclear genes usually selected against in the recipient species was described (Rubinoff and Holland, 2005; Matute et al., 2020). A least plausible explanation is hybrid speciation, though more common in nature than previously thought (Mavárez and Linares, 2008; Schumer et al., 2014; Payseur and Rieseberg, 2016). In this study, for the mitochondrial genes, misleading phylogenetic reconstructions could be due to sequence convergence as we found the occurrence of selective pressure on these genes, but also to introgression, with mitochondrial introgression being the most likely process rather than the nuclear one (Good et al., 2015).

The *pictipes* Group

In our study, we aim to test signatures of introgression using the ABBA-BABA test (Green et al., 2010) for the *pictipes* group for which a discordance between the mitochondrial and nuclear PCG trees was noticed. However, the mapping on *R. prolixus,*

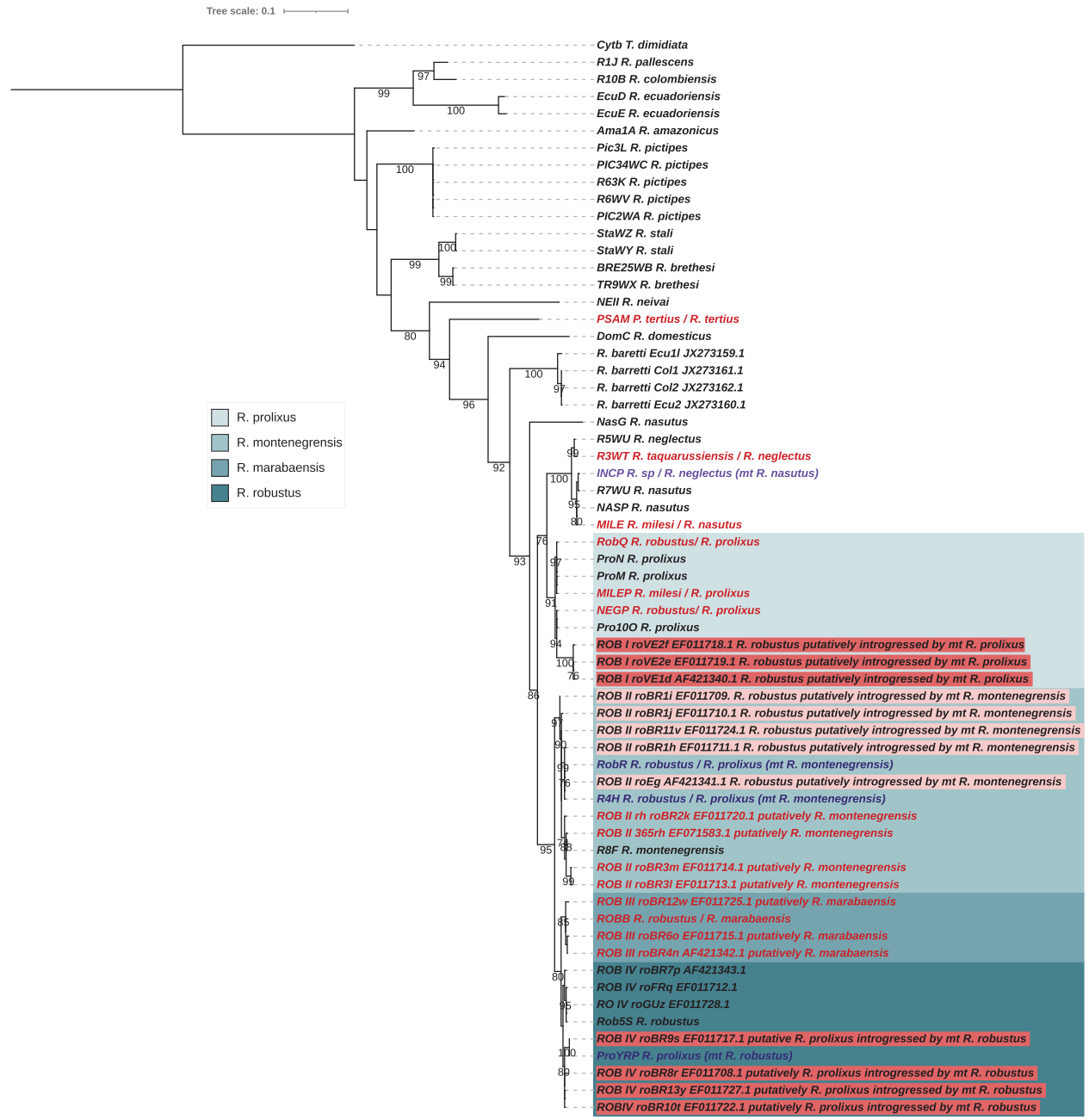

FIGURE 7 | Phylogenetic inferences and interpretation for the *Cytb* gene for *Rhodnius* species with the Maximum Likelihood (ML) method including Monteiro et al. (2003) samples. The statistical supports (1,000 replicates SH-aLRT) are indicated on the nodes. *T. dimidiata* was used as outgroup. For the dataset of this study, introgressed species inferred from the nuclear phylogeny (see **Figure 5**) are indicated in purple, misidentified species in red. For Monteiro et al. (2003) samples, based on the inferences made for our samples, we proposed putative introgressed species, highlighted in the tree and putative misidentifications for some others, indicated in red. For ROB I clade clustered with *R. prolixus* samples and composed of Venezuelan *R. robustus* samples, they putatively are *R. robustus* introgressed by mitochondrial DNA from *R. prolixus*. For ROB II clade, some samples clustered with the *R. montenegrensis* could be synonymized with *R. montenegrensis* while the other could be *R. prolixus* introgressed by mitochondrial DNA from *R. montenegrensis*. For the ROB III clade, specimens clustered with *R. marabaensis* and could be synonymize as *R. marabaensis*. For the ROB IV clade, some samples are clustered with *R. robustus* while others could be *R. prolixus* introgressed by mitochondrial DNA from *R. robustus*.

the only reference genome available for the *Rhodnius* species, could be a limitation for this study. Withal, the genome-wide analysis supports in the *pictipes* group the hypothesis of a mitochondrial introgression between *R. brethesi* and *R. stali* as the source of incongruence. Based on the nuclear phylogenies, we can suggest an introgression of *R. stali* with mitochondrial DNA from *R. brethesi*. This assumption is supported by the present distribution of the two species, *R. stali* being further

south (Bolivia, Brazil: Mato Grosso and Acre) than *R. brethesi* (Colombia, Venezuela, Brazil: Pará and Amazonas) and also with the morphological similarity of the two species. However, an unsampled (or undiscovered) species of the *pictipes* group as the source of the introgressed mitogenome cannot be excluded. The recent discovery of a new species *R. micki* (Zhao et al., 2021) from Bolivia could be a putative candidate as the source of mitochondrial introgression since this species is morphologically similar to *R. pictipes* and *R. stali* but *R. brethesi* was not included in the morphometric study and molecular data from *R. micki* is still lacking. A selective sweep of mitochondrial DNA linked to an expending endosymbiont could also be considered. In this way, a study of *Wolbachia* genomic data (Filée et al., unpublished) showed that the same *Wolbachia* strain is shared by species of the *pictipes* group (*R. brethesi, R. amazonicus, R. stali,* and *R. pictipes)* but that it differs from that of the *prolixus* group (*R. robustus, R. prolixus, R. neglectus,* and *R. nasutus*). Indeed, this endosymbiont does not seem to be involved in this mechanism.

The *pallescens* Group

For the *pallescens* group, we tested signatures of introgression because if the mitochondrial and nu-PCG trees are congruent showing *R. colombiensis* and *R. pallescens* clustered together, in the rDNA phylogeny, *R. pallescens* and *R. colombiensis* are closer. Such divergent topologies were already obtained in the literature, indicating either a close relationship between *R. pallescens* and *R. colombiensis* (Hypša et al., 2002) or between *R. ecuadoriensis and R. colombiensis* (Justi et al., 2016). However, the first topology is coherent with the vector distribution since the Chocó rainforests along the Colombian Pacific coast is assumed to separate *R. ecuadoriensis* from *R. pallescens–R. colombiensis* (Abad-Franch et al., 2009). Although the tests were carried out using *R. prolixus* for the mapping, three hypotheses could explain the numerous SNPs supporting either each grouping (ABBA- or BABA distribution): Incomplete Lineage Sorting (ILS), hybrid speciation and introgression. ILS could be the result of a fast radiation of the three species that led to the sharing of ancestral polymorphisms. However, this hypothesis cannot explain alone the under-representation of the ABBA configuration, i.e., less allele sharing between *R. ecuadoriensis* and *R. colombiensis* than between *R. pallescens* and *R. colombiensis*. A least conventional yet plausible explanation is hybrid speciation. The ambiguous positioning of *R. pallescens* as closer to either *ecuadoriensis* or *colombiensis* could be the result of crosses between *R. ecuadoriensis* males and *R. colombiensis* females leading to the third species *R. pallescens*, which will reflect the mitochondrial tree topology. But hybrid speciation remains to be demonstrated in *Rhodniu*s. Introgression, which results from the occurrence of gene flow after speciation, could explain the ABBA under-representation if *R. ecuadoriensis* and *R. colombiensis* were genetically isolated but each still hybridizing with *R. pallescens*. Indeed, Díaz et al. (2014) demonstrated in the laboratory the crossbreeding between *R. pallescens* and *R. colombiensis* although the laboratory hybrids were infertile. We did not observe ABBA or BABA configurations restricted to specific genomic regions, the three configurations were widespread and overlapping on all scaffolds of the reference genome of *R. prolixus* used for the read mapping. Nonetheless, we cannot rule out the possibility that multiple rearrangements have taken place since the divergence of the three species and *R. prolixus*. At least, nuclear *R. ecuadoriensis* introgression in *R. pallescens* could explain the ABBA under-representation since the nuclear rDNA showed a closest genetic proximity between these two species. Therefore, genomic studies with large population sampling in the *R. pallescens* species group wherein different demographic models could be tested are strongly needed in the future.

The *prolixus* Group

In this study, the robust nu-PCG phylogeny splits the *prolixus* group into two major clades, the *neglectus-nasutus* clade and the *robustus* species complex clade (*R. marabaensis, R. montenegrensis, R. robustus,* and *R. prolixus*). In these two clades, we observed several discrepancies between mitochondrial and nuclear trees.

The wild Piauí-Brazil NasG specimen showed an incongruent position between the mitochondrial and nuclear PCG trees. This specimen showed a basal position for the two clades in the mitochondrial tree but it is clustered with the *R. neglectus* samples in the nu-rDNA tree and with R7WU *R. nasutus* in the nu-PCGs tree. Considering both its morphology is similar to *R. nasutus* and the substantial data for the nu-PCG dataset (36.3 kb), the mitochondrial introgression is the most likely process rather than the nuclear one. The nature of the mitochondrial introgression remains speculative. Unexpectedly, an introgression with a member of the *robustus* complex species cannot be ruled out. Moreover, some nuclear introgression for this *R. nasutus* sample could be hypothesized probably by rDNA from *R. neglectus.*

Mitochondrial introgression was also observed for two other field specimens from Piauí. The INCP sample exhibited a dark morph and an atypical phenotype which cannot be assigned to *R. nasutus* or *R. neglectus*. The molecular study evidenced that this sample is related to *R. neglectus* in the nuclear tree but to *R. nasutus* in the mitochondrial one, thus INCP could be considered as a *R. neglectus* introgressed by mitochondrial DNA from *R. nasutus*. The reverse introgression is exhibited by the NASP sample, that is a *R. nasutus* introgressed by mitochondrial DNA from *R. neglectus*. Introgressions between *R. nasutus* and *R. neglectus* was not yet reported, including from field specimens but are not surprising due to the close genetic proximity between the two species as revealed by genetic distance values but also to the geographic distribution of the species. Results obtained by Abad-Franch et al. (2009) *via* geometric morphometrics showed that *R. neglectus* and *R. nasutus* are sympatric in the Brazilian Cerrado-Caatinga transitional area. There is also some evidence of populations of *R. neglectus* occurring also in the true Caatinga biome where *R. nasutus* is distributed (Dias et al., 2011), which could favor hybridization. Moreover, chromatic variation in *R. nasutus* was already observed and adaptive phenotypic plasticity was suggested related to microhabitat features (Abad-Franch et al., 2009). It will be of interest using molecular markers to test the various *R. nasutus* chromatic forms for introgression with *R. neglectus*, that could explain the darker morph observed.

For the *robustus* species complex, three field specimens NEGP, RobQ and MILEP, all originated from Pará Brazil,

and morphologically identified as *R. neglectus, R. robustus* and *R. milesi* respectively, a misidentification is likely since all the trees (mtG, rDNA, nu-PCGs) are congruent with *R. prolixus* species. This exemplifies the difficulty to identify the species of the *prolixus* group and to admit the occurrence of sylvatic *R. prolixus*. If the occurrence of *R. prolixus* in Venezuela is now well documented (i.e., Fitzpatrick et al., 2008), in Brazil the controversy persists but it is actually accepted that *R. prolixus* occurs basically in the states of Amazonas and Pará (Pinho et al., 1998). Indeed, the discovery of *R. neglectus* in this region (Lent, 1954) but also the widely distribution of *R. robustus* and the description of *R. milesi* from Pará cast doubt on past identifications of *R. prolixus* and guided the future ones. By instance, it was suggested that *R. neglectus*, and not *R. prolixus*, was the species invading houses in central Brazil (Gurgel-Gonçalves et al., 2008). In our study, we then evidence by molecular data the presence of sylvatic *R. prolixus* in the Brazilian Pará state.

For two strain samples, R4H and RobR, a mito-nuclear conflict was evidenced. In the nuclear tree, these two samples were clustered with *R. prolixus* samples but in the mitochondrial tree, they were closely related to *R. montenegrensis* and likely have experienced mitochondrial introgression with this species. In addition, for the RobR sample, the rDNA tree suggested an introgression by *R. robustus*. The domiciliary Trujillo-Venezuelan *R. prolixus* specimen (ProYRP) was also introgressed by *R. robustus* but for mitochondrial DNA. Similar results were obtained for Venezuelan populations by Fitzpatrick et al. (2008) using both the mitochondrial *cytb* gene and the *D2* nuclear gene, a population of domiciliated *R. prolixus* identified as *R. robustus* was introgressed with mitochondrial DNA from *R. robustus* (Hap3, **Figure 7**). Introgression events of *R. prolixus* with *R. robustus* in Venezuela could explain the results obtained by Harry (1994) with morphometric data in which no clear-cut differentiation was evidenced between Venezuelan *R. prolixus* and *R. robustus* samples from Trujillo.

Considering the *cytb* phylogeny including Monteiro et al. (2003) samples, the apparent paraphyly of *R. robustus* pointed out by the authors is resolved by the consideration of introgression events (**Figure 7**):

(i) ROB I clade is clustered with *R. prolixus* samples and is composed of Venezuelan *R. robustus* samples. Due to the widespread of introgressions between *R. robustus* and *R. prolixus* especially in this region, we suggested that samples from ROB I clade are *R. robustus* introgressed by mitochondrial DNA from *R. prolixus*, but nuclear markers are needed to affirm or refute this hypothesis;

(ii) ROB II clade is split into two. One comprises samples clustered with the *R. montenegrensis* strain of reference (R8F) while the other comprises samples clustered with two of our samples (RobR, R4H) that we demonstrated the introgression of *R. prolixus* by mitochondrial DNA from *R. montenegrensis*. Then, we putatively suggest that field specimens from Carauari, Amazonas (RoBR1, haplotypes h, i, j) are introgressed, and only a part of ROB II could be synonymized with *R. montenegrensis*.

(iii) specimens of the ROB III clade clustered with ROBB from Marabá and assimilated to *R. marabaensis*. So, we conclude that this clade could be synonymize as *R. marabaensis* has used by Castro et al. (2020).

(iv) ROB IV clade is also split into two. One comprises samples including *R. robustus* from French Guyana and clustered with also our sample from this region. The same clustering was found by Barnabé et al. (2018) for an extensive survey of *R. robustus* from French Guyana. The other comprises samples clustered with our Venezuelan sample ProYRP for which we demonstrated the introgression of *R. prolixus* by mitochondrial DNA from *R. robustus* and the Hap3 also a *R. prolixus* introgressed by mitochondrial DNA from *R. robustus* and sharing the same haplotype than the roBR9 (Fitzpatrick et al., 2008). Then, we suggested that this part of samples from ROB IV clade are *R. prolixus* introgressed by mitochondrial DNA from *R. robustus*.

In this study, we demonstrated that introgression could be a biological event as field samples were introgressed. Misplacement in phylogeny for some *Rhodnius* lab strains could also result from a past biological event and not only be the fact of mixed or misidentified samples as pointed out by Brito et al. (2019). Indeed, at this time, it is impossible to state if lab strains with such pattern were accidentally introgressed or were founded with specimens naturally introgressed.

CONCLUSION

In this study, we provide a very large dataset on *Rhodnius* species including 36 samples. With low-depth whole-genome shotgun sequencing, we resolve sticking points in the *Rhodnius* phylogeny using phylogenomic approaches based on 15 mtG (13.3 kb), nu-rDNA genes (5.2 kb), and 51 nu-PCGs (36.5 kb).

Out the 17 putative species determined on phenotype, using molecular data, we identified 16 valid *Rhodnius* species (**Table 1**). We confirmed the species-specific status of *R. montenegrensis* and *R. marabaensis* and we agree with the synonymy of *R. taquarussuensis* with *R. neglectus*. We also invite to revisit the species-specific status of *R. milesi* that is more likely *R. nasutus*. We added *R. marabaensis* in our dataset, first identified as *R. robustus*. We propose the *robustus* complex species for including the four close related species *R. marabaensis, R. montenegrensis, R. prolixus,* and *R. robustus*. Moreover, we confirm the position of *P. tertius* into the *Rhodnius* genus and strongly recommended reclassifying *P. tertius* as *R. tertius*. For the newly described species *R. micki* and *R. barretti*, molecular data and especially nuclear ones are needed to assess their phylogenetic position. The same is true for *R. paraensis* rarely sampled since this species has been collected only from arboreal rodent nests in the Amazon region in Brazil (Sherlock et al., 1977) but also with light trap in French Guyana (Bérenger et al., 2009). For *R. zeledoni* and *R. dalessandroi* only known from their description, no molecular data can be obtained.

Both mitochondrial and nuclear data coherently support that the *pictipes* and *pallescens* groups are more related to each other

than they are to the *prolixus* group. We can hypothesize that the *pictipes* and *pallescens* groups more likely diverged from an ancestral form when the Northern Andean uplift before the formation of the Pebas system around 23–10 MYA and that the latter event has induced the diversification of the *prolixus* group when this system started to be drained eastward into the Atlantic Ocean. This subsequently led to hydrological changes promoting allopatric speciation or favoring dispersals (Albert et al., 2018). However, robust time-calibrated phylogenies are now needed to infer the historical and spatial origin of the *Rhodnius* lineages.

By comparing the topologies obtained for mitochondrial and nuclear phylogenies but also by performing genome-wide analysis, our results demonstrated unexpected introgression events in all the different *Rhodnius* groups, in laboratory strains but also in wild specimens. In the *pictipes* group, we formulated the hypothesis of cytoplasmic introgression between *R. brethesi* and *R. stali* and in the *pallescens* group the occurrence of complex introgression. In the *prolixus* group introgression occurred between various species: (i) from *R. robustus* or *R. montenegrensis* mitochondrial DNA to *R. prolixus*,(ii) from *R. robustus* mitochondrial DNA to *R. prolixus*, (iii) from *R. nasutus* mitochondrial DNA to *R. neglectus,* (iv) the reverse introgression from *R. neglectus* mitochondrial DNA from to *R. nasutus* and (v) putatively from one species of the *robustus* complex to *R. nasutus.* The taking into account the introgression events makes it possible to explain the paraphyly observed for *R. robustus* in some mitochondrial phylogenies but leads to abolish it by considering nuclear genes and restores the monophyly for this species. The extensive introgression especially in the *prolixus* group does not question the species boundaries for the pairwise *R. nasutus* and *R. neglectus* nor for the species of the *robustus* complex, and even that complicates the identification of the species, it is challenging from an evolutionary point of view. Further studies are needed in order to date the introgressions found in field samples which can result from old events at the time of the speciation or from recent hybridization between sympatric species.

This study exemplifies that the evolutionary inferences from only mitochondrial markers notably by using only the *cytb* gene or incomplete dataset could be misleading. Because of the persuasive introgression which confuse not only the molecular issue but surely also the phenotypic one, we strongly recommend the use of both mitochondrial and nuclear markers prior any study on *Rhodnius* species and to perform separately mitochondrial and nuclear phylogenies in order to take advantages from mito-nuclear conflicts for having a comprehensive evolutionary vision.

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 below: NCBI (accession: PRJNA429761).

AUTHOR CONTRIBUTIONS

JF, MM, and HB: data analysis and writing. FM and EF-R: study design. CA: writing. J-MB: field collections and *Rhodnius* taxonomy. MH: study conception, DNA extractions, data analysis, and writing. All authors edited the manuscript 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.2021.750317/full#supplementary-material

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