

## Article

# One Genome, Multiple Phenotypes: Would *Rhodnius milesi* Carcavallo, Rocha, Galvão & Jurberg, 2001 (Hemiptera, Triatominae) Be a Valid Species or a Phenotypic Polymorphism of *R. neglectus* Lent, 1954? <sup>†</sup>

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**Citation:** Campos, F.F.; de Oliveira, J.; Santos Santana, J.K.; Ravazi, A.; dos Reis, Y.V.; Marson Marquioli, L.; Galvão, C.; de Azeredo-Oliveira, M.T.V.; Aristeu da Rosa, J.; Alevi, K.C.C. One Genome, Multiple Phenotypes: Would *Rhodnius milesi* Carcavallo, Rocha, Galvão & Jurberg, 2001 (Hemiptera, Triatominae) Be a Valid Species or a Phenotypic Polymorphism of *R. neglectus* Lent, 1954? *Diversity* **2024**, *16*, 472. <https://doi.org/10.3390/d16080472>

Academic Editor: Michael Wink

Received: 5 June 2024

Revised: 30 July 2024

Accepted: 31 July 2024

Published: 5 August 2024



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**Abstract:** Species of the *Rhodnius* genus have a complex taxonomy because the events of phenotypic plasticity and cryptic speciation make it difficult to correctly classify these vectors. During the taxonomic history of the genus, five synonymization events occurred. Additionally, some authors suggest that *R. milesi* possibly represent only phenotypic polymorphisms of *R. neglectus*. Thus, we analyzed the specific status of *R. milesi* in relation to *R. neglectus* using phylogenetic studies with the mitochondrial gene *cytochrome B* and the study of reproductive barriers. The phylogenetic reconstruction grouped *R. milesi* together with *R. neglectus* from different localities, demonstrating that these taxa represent the same species based on the phylogenetic species concept. Experimental crosses demonstrate the absence of pre- and postzygotic barriers under laboratory conditions. Additionally, when the hatch rates of crosses are compared to intraspecific crosses, it can be noted that they are high and very similar. Finally, the mortality rate of the hybrids does not indicate hybrid inviability, the absence of chromosome pairing errors does not indicate hybrid sterility, and the proportion between male and female hybrids demonstrates that Haldane's rule was not acting. Therefore, we perform the formal synonymization of *R. milesi* with *R. neglectus*.

**Keywords:** taxonomy; triatomines; phylogenetic systematics; experimental crosses; synonymization

## 1. Introduction

Triatomines (Hemiptera, Reduviidae, Triatominae) are hematophagous insects of great importance for public health, as they are considered the main form of transmission of the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae), the etiological agent of Chagas disease [1]. Currently, there are 159 species, grouped into 18 genera and five tribes (Alberproseniini Martínez & Carcavallo, 1977, Bolboderini Usinger,

1944, Cavernicolini Usinger, 1944, Triatomini Jeannel, 1919 and Rhodniini Pinto, 1926), all being species considered potential vectors of the Chagas disease [2–7].

The main species from an epidemiological point of view are in the Triatomini and Rhodniini tribes [8]. The Rhodniini tribe is composed by 23 species, 20 belonging to the *Rhodnius* Stål, 1859 genus and three to the *Psammolestes* Bergroth, 1911 genus [2,7]. The *Rhodnius* genus is considered a paraphyletic group [8–11], as species in the *prolixus* group are evolutionarily closer to *Psammolestes* spp. than to the other *Rhodnius* groups [8–11].

Species of the *Rhodnius* genus have a complex taxonomy, because although species differentiation was initially based only on morphological distinctions and similarities [12,13], events of phenotypic plasticity and cryptic speciation make it difficult to correctly classify these vectors [14]. Intraspecific variations have already been reported in the species *R. nasutus* Stål, 1859 [15], *R. robustus* Larrousse, 1927 [16], *R. ecuadoriensis* Lent & León, 1958 [17], *R. brethesi* Matta, 1919 [18] and *R. neglectus* Lent, 1954 [19]. Furthermore, the main cryptic speciation event in the genus *Rhodnius* was signaled for *R. robustus* [11], although intraspecific genotypic variations were already observed for the species *R. ecuadoriensis* [20] and *R. pallescens* Barber, 1932 [21].

During the taxonomic history of the genus, five synonymization events occurred, namely, *R. brumpti* Pinto, 1925 with *R. nasutus*, *R. dunni* Pinto, 1932 with *R. pallescens*, *Conorhinus limosus* Walker, 1873 with *R. pictipes* Stål, 1872 and *R. prolixus* Stål, 1859 and, more recently, *R. taquarussuensis* Rosa et al., 2017 with *R. neglectus* and *R. zeledoni* Jurberg, Rocha & Galvão, 2009 with *R. domesticus* Neiva & Pinto, 1923 [7,19,22]. In addition to formal synonymization events, some authors suggest that, possibly, valid species represent only phenotypic polymorphisms: Abad-Franch et al. [17] and Monteiro et al. [11], for example, suggested that *R. milesi* Carcavallo, Rocha, Galvão & Jurberg, 2001 (in: Valente et al., 2001) is probably *R. neglectus*. Furthermore, recently Filée et al. [23] carried out a phylogenomic study in *Rhodnius* and suggested that *R. milesi* should be synonymized with *R. nasutus*. However, the authors themselves highlighted that a possible explanation for *R. milesi* approaching *R. nasutus* instead of *R. neglectus* is related to a probable event of introgression of nuclear genetic material between *R. neglectus* and *R. nasutus*.

*Rhodnius milesi* is a species reported in the states of Pará and Rondônia [24,25] that was described based on comparative morphological studies with *R. dalessandroi* Carcavallo & Barreto, 1976 [24]. However, phylogenetic systematic studies have grouped *R. milesi* with *R. neglectus* and, therefore, suggested that they are the same species [11]; Although some morphological differences in the external morphology [26], as well as in the structures of female genitalia [27] and the exochorial cells of eggs [28], have been observed, Galvão [29] and Jurberg [30] did not include this species in the dichotomous keys for adult *Rhodnius* due to the absence of external diagnostic characters when compared to *R. neglectus*. Furthermore, Alvarez et al. [31] recently carried out geometric morphometric studies between *Rhodnius* spp. and suggested that *R. milesi* is a variant of *R. neglectus* (emphasizing the need for the formal synonymization of these species).

Given the events of cryptic speciation and phenotypic plasticity, as well as the taxonomic problems associated with *Rhodnius* [11,14], integrative taxonomy has been used to characterize new species of the Rhodniini tribe [14,32,33]. Among the different tools that can be used in integrative taxonomy, phylogenetic systematics studies and analyses of pre- and postzygotic interspecific reproductive barriers are of great importance for evaluating the specific status of taxa (based on the phylogenetic [34] and biological concept of species, respectively [35,36]).

Thus, considering that morphological [12,29,30] and morphometric [31] studies have already been carried out and pointed out many similarities (suggesting, even, the formal synonymization of taxa [31]), we analyzed the specific status of *R. milesi* in relation to *R. neglectus* using phylogenetic studies with the mitochondrial gene *cytochrome B* (*cyt B*) and the study of reproductive barriers through experimental crosses.

## 2. Materials and Methods

### 2.1. Molecular Analyses

#### 2.1.1. DNA Extraction

For DNA extraction, two specimens of *R. milesi*, obtained from colonies at the Triatominae Insectary of the School of Pharmaceutical Sciences of Araraquara, São Paulo, were used. The extraction protocol used was based on Adams et al. [37], in which two legs of each specimen were added to a microtube containing Digsol Buffer (50 mM Tris, 20 mM EDTA, 117 mM NaCl and 1% SDS) and macerated, being subsequently incubated overnight with proteinase K. The solution was then precipitated using an ammonium acetate solution, which was homogenized in a vortex for 15 min and centrifuged. The supernatant was then transferred to a new microtube, precipitated in absolute ethanol and centrifuged. Finally, a wash in 70% ethanol was performed and centrifuged again. After drying, the DNA was resuspended in 40 µL of TE buffer (Tris-EDTA) and stored at  $-20^{\circ}\text{C}$ . DNA concentration and quality were assessed using the NanoDrop<sup>TM</sup> spectrophotometer (Thermo Scientific<sup>TM</sup>, Waltham, MA, USA).

#### 2.1.2. Cytochrome B Amplification

For the amplification of *cyt B*, forward (5'-GGACAAATATCATGAGGAGCAACAG-3') and reverse (5'-ATTACTCCTCCTAGCTTATTAGGAATTG-3') primers were used, following the methodology of Lyman et al. [38]. The PCR products obtained were verified on an agarose gel (2%) stained with GelRed<sup>TM</sup> 20x (Biotium Inc.<sup>TM</sup>, San Francisco Bay, CA, USA). Subsequently, the material was purified using ExoSAP-IT<sup>TM</sup> (Applied Biosystems<sup>TM</sup>, Foster City, CA, USA) and submitted for sequencing on the ABI 3730 DNA Analyzer (Applied Biosystems<sup>TM</sup>). Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems<sup>TM</sup>). The sequences obtained were evaluated in BioEdit 7.2.5 [39], and the consensus sequence for each specimen was obtained from the forward and reverse sequences, which were evaluated in BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 4 June 2024) to confirm the amplified region.

#### 2.1.3. Phylogenetic and Genetic Distance Analyses

The *cyt B* sequences obtained were grouped with sequences available in Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed on 4 June 2024) for *Rhodnius* spp. and *Triatoma* spp. (outgroup) (Table 1) and aligned in the Mega 11 program [40], using the Muscle method [41]. The resulting alignment containing 699 nucleotides was submitted to the jModeltest 2.10 [42] program to evaluate the best nucleotide substitution model based on AIC calculation, the best model being HKY [43] with invariant sites (+I) and gamma distribution (+G). Subsequently, this alignment was submitted to the Mega 11 program [40] to evaluate genetic distance (Kimura-2-parameters) and perform phylogenetic analysis by Neighbor-Joining [44]. In addition, a phylogenetic reconstruction using Bayesian inference was also performed in the Beast 1.8.4 program [45].

For Neighbor-Joining phylogenetic analysis, a total of 10,000 bootstrap replicates were performed [46]. The Kimura 2-parameter [47] method was used to calculate the evolutionary distance between sequences. The run was performed with the partial deletion option (all positions with less than 95% site coverage were eliminated). There were a total of 404 positions in the final dataset.

For phylogenetic reconstruction by Bayesian inference, an analysis was carried out with 100 million generations, using the HKY +I +G, strict clock and yule process prior [48,49]. Analysis stabilization (ESS > 200) was assessed in Tracer 1.8 [50]. Burn-in was adjusted for 25% of the samples, and the resulting tree was visualized and edited in Figtree program 1.4 [51].

**Table 1.** Genbank accession number for each marker used in the phylogenetic analyses. \* Sequences obtained in this study.

Specie	Acession Number	Country	State—City
<i>R. milesi</i>	PQ094218 *	Brazil	Pará—Bragança
	PQ094219 *	Brazil	Pará—Bragança
<i>R. neglectus</i>	AF045716	Brazil	-
	MZ399364	Brazil	Bahia—Ibotirama
	MZ399370	Brazil	Bahia—São Desiderio
	KT317037	Brazil	Bahia—Xique-Xique
	KT317036	Brazil	Bahia—Xique-Xique
	MH704748	Brazil	Goiás—Formoso
	MH704749	Brazil	Goiás—Formoso
	MH704751	Brazil	Goiás—Formoso
	MH704750	Brazil	Goiás—Formoso
	MZ399362	Brazil	Goiás—Mambai
	MZ399366	Brazil	Maranhão—Vargem Grande
	MZ399365	Brazil	Maranhão—Loreto
	MZ399367	Brazil	Minas Gerais—Buritizero
	MZ399368	Brazil	Minas Gerais—Januaria
	KT317058	Brazil	Paraíba—Olivedos
	KT317053	Brazil	Piauí—Canto do Buriti
	KT317056	Brazil	Piauí—Canto do Buriti
	KT317052	Brazil	Piauí—Canto do Buriti
	KT317054	Brazil	Piauí—Canto do Buriti
	KT317055	Brazil	Piauí—Canto do Buriti
	KT317063	Brazil	Piauí—Colônia do Gurgueia
	KT317065	Brazil	Piauí—Colônia do Gurgueia
	KT317068	Brazil	Piauí—Colônia do Gurgueia
	KT317064	Brazil	Piauí—Colônia do Gurgueia
	KT317067	Brazil	Piauí—Colônia do Gurgueia
	KT317066	Brazil	Piauí—Colônia do Gurgueia
	KT317045	Brazil	Piauí—Jaicos
	KT317042	Brazil	Piauí—Jaicos
	KT317043	Brazil	Piauí—Jaicos
	KT317044	Brazil	Piauí—Jaicos
	MZ399363	Brazil	Piauí—Monte Alegre do Piaui
	KT317057	Brazil	Piauí—Oeiras
	OQ785647	Brazil	Piauí—Sao Raimundo Nonato
	JX273156	Brazil	Tocantins—Palmeirantes
MZ399369	Brazil	Tocantins—Taguatinga	
<i>R. montenegrensis</i>	MZ396184	Bolivia	-
	MZ396185	Bolivia	-
	MZ396186	Bolivia	-
	MZ396187	Bolivia	-
	MZ396188	Bolivia	-
	MZ396189	Bolivia	-
	MZ396190	Bolivia	-
	MZ396191	Bolivia	-
<i>R. robustus</i>	JX273158	Brazil	-
<i>R. prolixus</i>	AF421339	Honduras	-
	EF043579	Venezuela	-
	EF043585	Venezuela	-
	EF043586	Venezuela	-
	EF043587	Venezuela	-
	EF043588	Venezuela	-
	KP126733	Colombia	-
	KP126734	Colombia	-

Table 1. Cont.

Specie	Acession Number	Country	State—City
<i>R. nasutus</i>	MG735124	-	Pernambuco—Serra Talhada
	MG735123	-	Pernambuco—Serra Talhada
	MG735122	-	Pernambuco—Serra Talhada
	MG735121	-	Pernambuco—Serra Talhada
	MG735109	-	Paraíba—Sousa
	MG735108	-	Paraíba—Sousa
	MG735107	-	Paraíba—Sousa
	MG735106	-	Paraíba—Sousa
	MG735080	-	Piauí—Piracuruca
	MG735079	-	Piauí—Piracuruca
	MG735078	-	Piauí—Piracuruca
	MG735077	-	Piauí—Piracuruca
	MG735071	-	Piauí—Parnaíba
	MG735070	-	Piauí—Parnaíba
	MG735069	-	Piauí—Parnaíba
	MG735068	-	Piauí—Parnaíba
	MG735054	-	Ceará—Jaguaruana
	MG735053	-	Ceará—Jaguaruana
	MG735022	-	Rio Grande do Norte—Carnaúba dos Dantas
	MG735021	-	Rio Grande do Norte—Carnaúba dos Dantas
MG734997	-	Piauí—Campo Maior	
MG734996	-	Piauí—Campo Maior	
<b>Outgroup</b>			
<i>T. infestans</i>	KC249262	Uruguai	-
<i>T. infestans</i>	KC249258	-	-
<i>T. rubrofasciata</i>	HQ333233	-	-

## 2.2. Experimental Crosses

Reciprocal experimental crosses were conducted between *R. milesi* and *R. neglectus* to evaluate the potential pre- and/or postzygotic reproductive isolation barriers [34,35,52]. The experimental crosses were conducted in the Triatominae Insectary of the School of Pharmaceutical Sciences (FCFAR/UNESP, Brazil), according to the experiments of Mendonça et al. [53] and Ravazi et al. [54]: the insects were sexed as 5th instar-nymphs, and males and females were kept separately until they reached the adult stage to guarantee the virginity of the insects used in the crosses. For the experimental crosses, five couples from each set were placed separately in five plastic jars (diameter 5 cm × height 10 cm) and were kept at room temperature (average of 24 °C) and a relative humidity of 63% [55]. Furthermore, intraspecific crosses were also performed for group control. The eggs were collected weekly throughout the female's oviposition periods, and the egg fertility rate was calculated. Additionally, after the hybrids hatched, the development of 1st instar-nymphs until adults was also monitored weekly to assess the mortality rate. As the nymphs did not die before reaching the adult stage, ten new couples of first-generation hybrids (F1) (five for each direction) were separated for intercrossing, with the same parameters described above being used in the evaluation. Furthermore, intercrosses between second-generation hybrids (F2) were also carried out in both directions. Finally, the hybrids that reached adulthood were sexed to assess whether Haldane's rule [56] was acting. This rule predicts that if hybrids hatch, the heterogametic sex is the first affected by the evolutionary events that make this organism unfeasible or lead to sterility [56,57]. We justify that for all quantitative data collected, the relative frequency was calculated.

### 2.3. Cytogenetic Analysis

Five adult male hybrids from each generation were dissected and their testes removed and stored in a methanol: acetic acid solution (3:1). Slides were prepared by the cell-crushing technique (as described by Alevi et al. [58]), and cytogenetic analyses were performed to characterize spermatogenesis, with emphasis on the degree of pairing between the homologous chromosomes, using the lacto-acetic orcein technique [58,59]. The slides were examined under a light microscope (Jenamed; Carl Zeiss, Jena, Germany) that was coupled with a digital camera with a 1000-fold magnification; AxioVision LE version 4.8 imaging software (Carl Zeiss) was used for analysis.

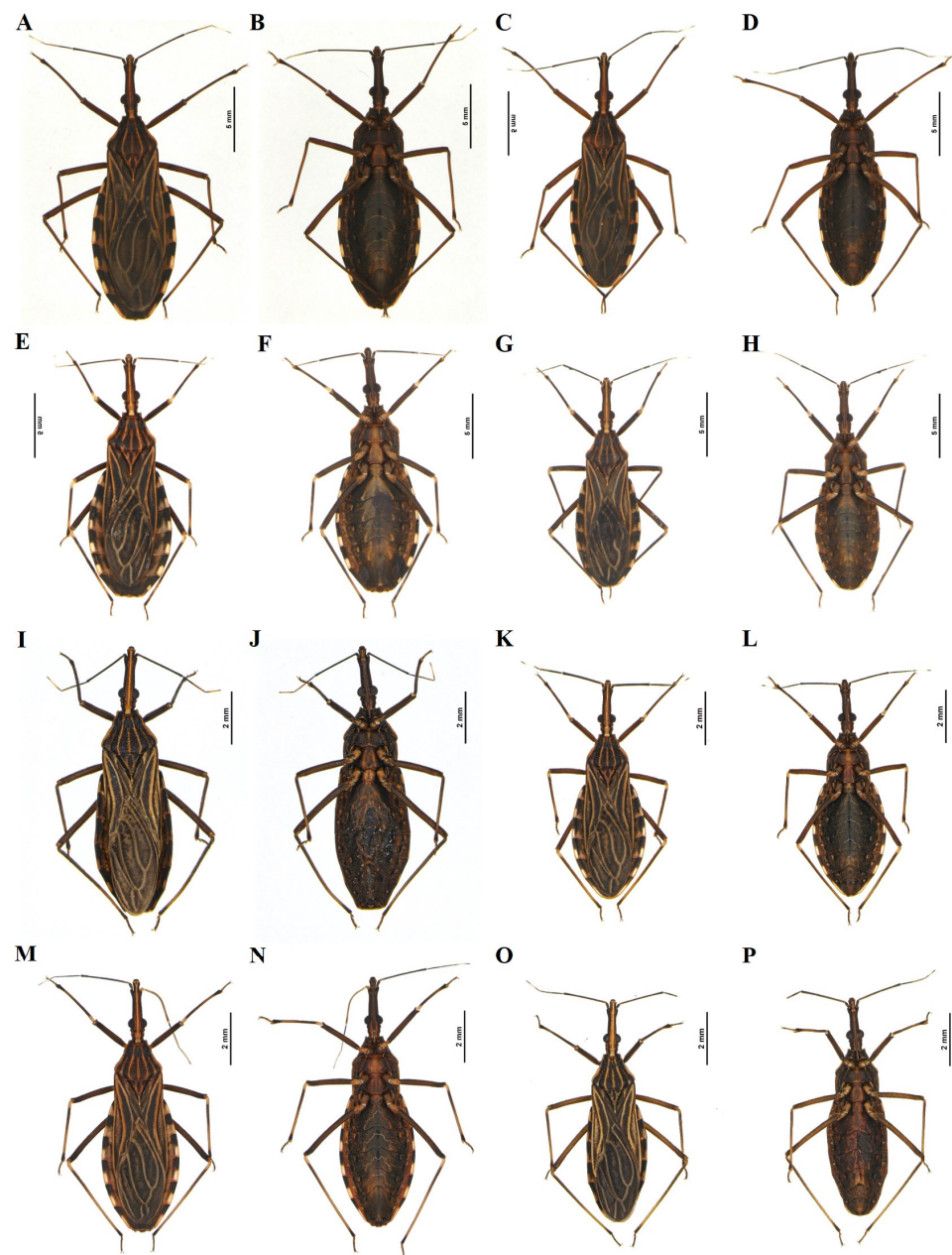
### 3. Results and Discussion

The phylogenetic reconstruction grouped *R. milesi* (Figure 1A–D) together with *R. neglectus* (Figure 1E–G) from different localities (Figures 2, 3 and S1), demonstrating that these taxa represent the same species based on the phylogenetic species concept [34]. Monteiro et al. [11] also analyzed the relationship of these species with the *cyt B* gene and the nuclear marker Internal Transcribed Spacer 2 (ITS2) and observed high genetic similarity between *R. milesi* from southeastern Amazonia and *R. neglectus*. Furthermore, Filée et al. [23], using mitochondrial and nuclear markers, also analyzed the phylogenetic position of this species with *Rhodnius* spp. and suggested that *R. milesi* be synonymized with *R. nasutus*. Although the nuclear marker analyzed by Monteiro et al. [11] demonstrated evolutionary proximity between *R. nasutus*, *R. neglectus* and *R. milesi*, the phylogeny with the *Cyt B* gene separated *R. nasutus* from *R. neglectus* + *R. milesi* into distant clades [11] (Figures 2, 3 and S1), which led to Filée et al. [23] suggesting possible introgression between *R. neglectus* and *R. nasutus*.

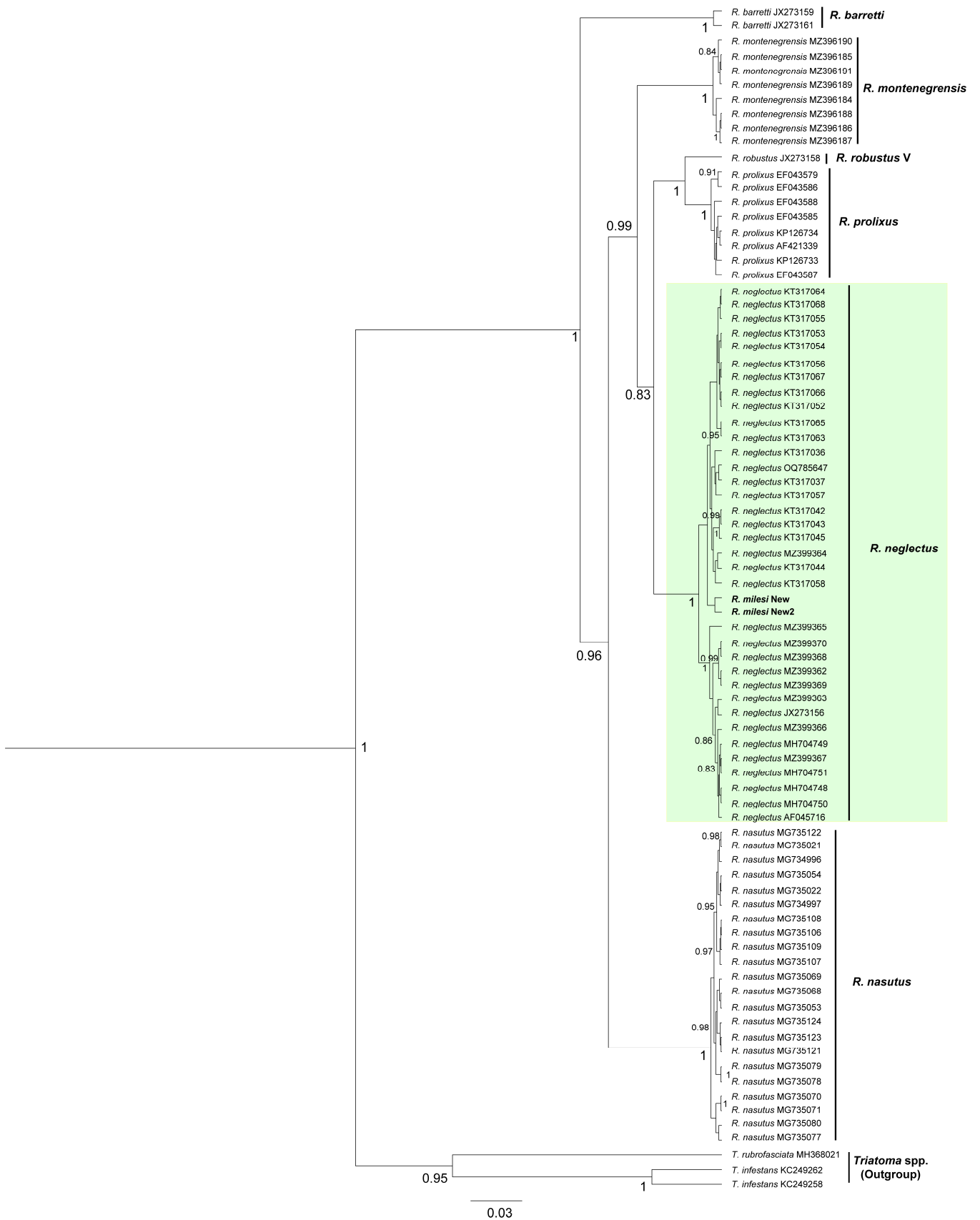
The genetic similarities and divergences reported above by Monteiro et al. [11] are congruent with the chromosomal analyses carried out by Pita et al. [20], once *R. neglectus* and *R. milesi* present 45S rDNA marking clusters on the X and Y sex chromosomes and *R. nasutus* presents marking only on the X sex chromosome. The fluorescence in situ hybridization (FISH) results highlight that the position of the 45S rDNA probes are species-specific markers [20,60,61]. These data, together with the phylogenetic analyses, demonstrate that *R. milesi* and *R. neglectus* represent the same taxon.

Experimental crosses between *R. milesi* and *R. neglectus* resulted in F1 hybrids in both directions (Figure 1I–P), demonstrating the absence of prezygotic reproductive barriers (Table 2). Furthermore, postzygotic barriers were also not observed; as the hybrids reached adulthood (Figure 1I–P) (Table 2) (absence of hybrid inviability), they were intercrossed, and F2 hybrid offspring were obtained (Table 2) (absence of hybrid sterility). Subsequently, F2 hybrids were intercrossed, and third-generation hybrids (F3) were obtained (Table 2) (possible absence of hybrid collapse). The characterization of reproductive barriers in laboratory conditions makes it possible to confirm the specific status of the parental species based on the biological species concept [35,36]. On the other hand, when reproductive barriers are not observed, this parameter alone cannot be used to synonymize species, as laboratory crossings can break several natural barriers that may exist between different species in nature.

Nascimento et al. [19] performed an integrative taxonomy study to assess the specific status of *R. taquarussuensis*. The authors relied on data from phylogenetic systematics and experimental crosses to synonymize this species with *R. neglectus*, because mitochondrial markers demonstrated that both were grouped into a single clade, did not present interspecific reproductive barriers and had very high hatching rates of 92% for the cross between *R. taquarussuensis* ♀ × *R. neglectus* ♂ and 88% between *R. neglectus* ♀ × *R. taquarussuensis* ♂. Likewise, when the hatch rates of crosses between *R. milesi* and *R. neglectus* are compared to intraspecific crosses, it can be noted that, in both directions, they are high and very similar (ranging between 81% and 89%) (Table 2).

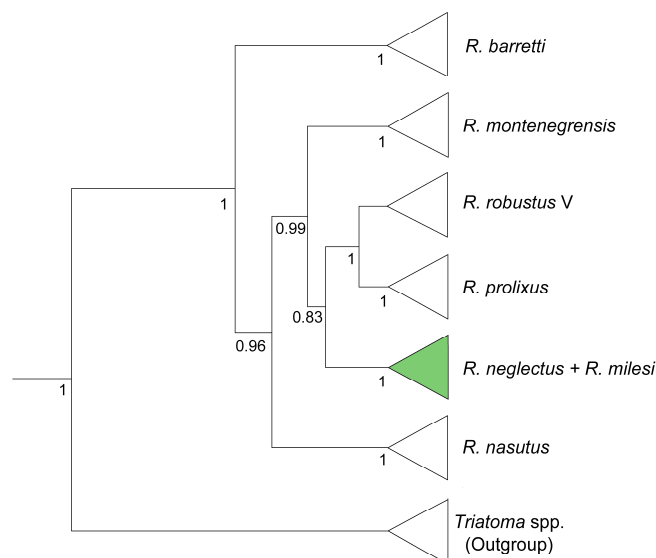


**Figure 1.** (A): Female *R. milesi* in dorsal view; (B): female *R. milesi* in ventral view; (C): male *R. milesi* in dorsal view; (D): male *R. milesi* in ventral view; (E): female *R. neglectus* in dorsal view; (F): female *R. neglectus* in ventral view; (G): male *R. neglectus* in dorsal view; (H): male *R. neglectus* in ventral view; (I): female hybrid resulting from the crossing between *R. neglectus* female x *R. milesi* male in dorsal view; (J): female hybrid resulting from the crossing between *R. neglectus* female x *R. milesi* male in ventral view; (K): male hybrid resulting from the crossing between *R. neglectus* female x *R. milesi* male in dorsal view; (L): male hybrid resulting from the crossing between *R. neglectus* female x *R. milesi* male in ventral view; (M): female hybrid resulting from the crossing between *R. milesi* female x *R. neglectus* male in dorsal view; (N): female hybrid resulting from the crossing between *R. milesi* female x *R. neglectus* male in ventral view; (O): male hybrid resulting from the crossing between *R. milesi* female x *R. neglectus* male in dorsal view; (P): male hybrid resulting from the crossing between *R. milesi* female x *R. neglectus* male in ventral view. Bar: 2 mm.



**Figure 2.** Bayesian phylogeny based on *cyt B* gene. The number in the nodes indicates the posterior probabilities for each clade. The *R. neglectus* clade is highlighted in green. Note that the *R. milesi* specimens are together with *R. neglectus* in the *R. neglectus* clade.





**Figure 3.** Simplified tree showing the phylogenetic relationship between taxa, based on Bayesian inference phylogeny of Figure 1. The numbers at the nodes indicate the posterior probability.

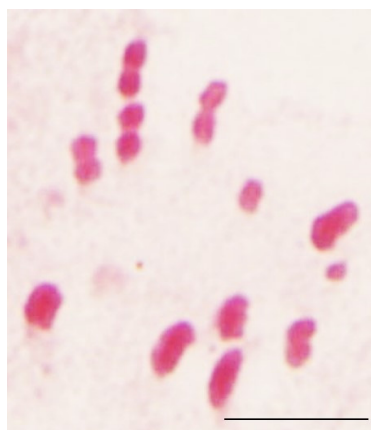
**Table 2.** Intra- and interspecific crosses between *R. milesi* and *R. neglectus*. \* F1 hybrids resulting from the cross between *R. milesi* ♀ × *R. neglectus* ♂, \*\* F1 hybrids resulting from crossing *R. neglectus* ♀ × *R. milesi* ♂, \*\*\* F2 hybrids resulting from the crossing between F1 of *R. milesi* ♀ × *R. neglectus* ♂, \*\*\*\* F2 hybrids resulting from the crossing between F1 of *R. neglectus* ♀ × *R. milesi* ♂.

Crosses	Eggs	Hatching Rate
<b>Experimental crosses (to obtain F1)</b>		
<i>R. milesi</i> ♀ × <i>R. neglectus</i> ♂	357	82%
<i>R. neglectus</i> ♀ × <i>R. milesi</i> ♂	366	89%
<b>Intercrosses (F1 × F1) (to obtain F2)</b>		
Hybrid F1 × Hybrid F1 *	284	79%
Hybrid F1 × Hybrid F1 **	233	81%
<b>Intercrosses (F2 × F2) (to obtain F3)</b>		
Hybrid F2 × Hybrid F2 ***	546	50%
Hybrid F2 × Hybrid F2 ****	703	83%
<b>Control group</b>		
<i>R. milesi</i> ♀ × <i>R. milesi</i> ♂	386	81%
<i>R. neglectus</i> ♀ × <i>R. neglectus</i> ♂	901	89%

In addition to hatching rates, we evaluated the mortality rates of F1 hybrids, namely, around 39% for crosses between *R. milesi* ♀ × *R. neglectus* ♂ and 33% for crosses between *R. neglectus* ♀ × *R. milesi* ♂. The analysis of mortality rate in F1 hybrids can be an important taxonomic tool, as it allows evaluating, under laboratory conditions, the reproductive barrier of hybrid inviability. Alevi et al. [62] reported the presence of this barrier in hybrids resulting from crosses between *T. sordida* (Stål, 1859) and *T. rosai* Alevi et al. (2020) and used these results to confirm the specific status of *T. rosai* (until then, considered as *T. sordida*). Furthermore, Mendonça et al. [62] performed crosses between *T. lenti* Sherlock & Serafim, 1967 and *T. bahiensis* Sherlock & Serafim, 1967 and also observed the presence of this postzygotic barrier, which resulted in the revalidation of the specific status of *T. bahiensis* (until then, considered synonymous with *T. lenti*). Although F1 hybrids were obtained in both directions from the crosses performed above, the authors [63,64] observed high mortality rates (ranging between 70 and 80% as well as 84% to 98%, respectively). Therefore,

the mortality rates observed for crosses between *R. milesi* and *R. neglectus* do not allow us to confirm the presence of this reproductive barrier between these species.

Cytogenetic analyses of the gonads allowed us to observe that the hybrids presented normal meiosis, with 100% pairing between the homologous chromosomes [karyotype equal to parental:  $2n = 22$  (20 autosomes + sex chromosomes X and Y)] (Figure 4). Furthermore, when dissecting the gonads, we observed that the testicles of these insects were not atrophied (absence of gonadal dysgenesis). Gonadal dysgenesis is a postzygotic reproductive barrier recently characterized in Triatominae [64]. Gonads atrophied by this evolutionary phenomenon do not carry out gametogenesis [64]. Both this event and pairing errors between homologous chromosomes—already reported for hybrids resulting from crosses between *P. tertius* Lent & Jurberg, 1965 and *P. coreodes* Bergroth, 1911 [54], *T. lenti* and *T. bahiensis* [65], *Mepraia gajardoi* Frias, Henry & Gonzalez, 1998 and *M. spinolai* (Porter, 1934) [66], *T. infestans* (Klug, 1834) and *T. rubrovaria* (Blanchard, 1843) [67], *Panstrongylus chinai* (Del Ponte, 1929) and *P. howardi* (Neiva, 1911) [68]—lead to sterility of the hybrid [64]. In this way, we can confirm that the hybrids of *R. milesi* and *R. neglectus* are fertile and present gametogenesis without chromosomal changes.



**Figure 4.** Meiotic metaphase of hybrids resulting from the cross between *R. milesi* and *R. neglectus*. Note 100% pairing between chromosomes. Bar: 10  $\mu$ m.

Finally, the analysis of the proportion between male and female hybrids demonstrated that Haldane's rule was not acting, as 98 adult males and 80 females resulted from the cross between *R. milesi* ♀ × *R. neglectus* ♂, and 115 adult males and 102 females resulted from the cross between *R. neglectus* ♀ × *R. milesi* ♂. Although Perlowagora-Szumlewicki and Correia [69] observed that a distortion of the sex ratio in favor of the female was occurring in crosses between *T. pseudomaculata* Corrêa & Espínola, 1964 and *T. sordida*, *T. pseudomaculata* and *T. infestans*, *T. pseudomaculata* and *T. brasiliensis* Neiva, 1911, and between *R. neglectus* and *R. prolixus*, and, with this, they suggested that the rule was acting in triatominae crosses, all other results in the literature suggest that the rule does not apply in Triatominae [66,68] (as observed in our experiments for *Rhodnius* spp., interspecific crosses between *Mepraia* spp. [66] and between *Panstrongylus* spp. [68] also produce adult hybrids of both sexes, suggesting that Haldane's rule may not be applicable to these insect vectors).

#### 4. Conclusions

Therefore, based on the results presented that connect *R. milesi* (Figure 5A–C) and *R. neglectus* (Figure 5D–F) as a single taxon, we performed the formal synonymization of these species:

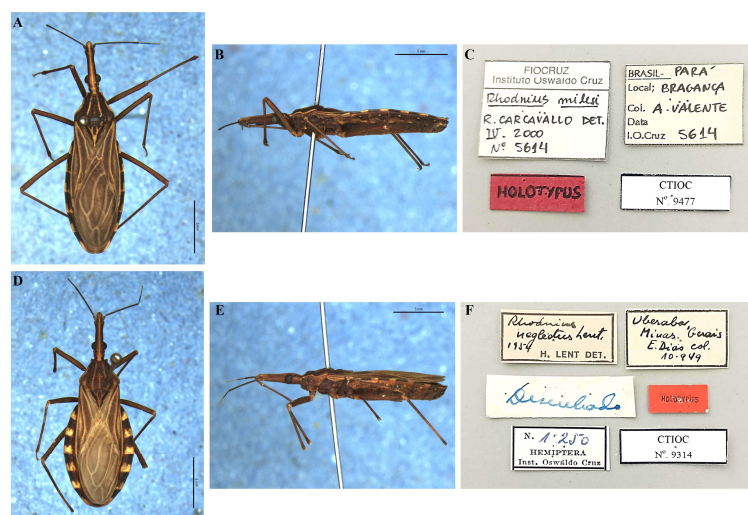
Taxonomy

Kingdom Animalia Linnaeus, 1758

Phylum Arthropoda von Siebold, 1848

Class Insecta Linnaeus, 1758

Order Hemiptera Linnaeus, 1758  
 Suborder Heteroptera Latreille, 1810  
 Family Reduviidae Latreille, 1807  
 Subfamily Triatominae Jeannel, 1919  
 Tribe Rhodniini Pinto, 1926  
 Genus *Rhodnius* Stål, 1859  
*Rhodnius neglectus* Lent, 1954 (Figure 5D–F)  
*Rhodnius taquarussuensis* da Rosa et al., 2017 (syn. *R. neglectus* [19])  
*Rhodnius milesi* Carcavallo, Rocha, Galvão & Jurberg, 2001 (in: Valente et al. 2001), syn. nov.  
 (Figure 5A–C)  
 urn:lsid:zoobank.org:pub:DC76CE99-5F14-4D6C-886A-4AD0DD20073E  
<http://zoobank.org/DC76CE99-5F14-4D6C-886A-4AD0DD20073E>



**Figure 5.** *Rhodnius milesi* [male holotype, deposited in Triatomines Collection of the Oswaldo Cruz Institute (CTIOC)] (A–C) and *Rhodnius neglectus* (male holotype, deposited in CTIOC) (D–F). A and D: Dorsal view; (B,E): Lateral view; (C,F): Labels. Bar: 5 mm.

Based on this synonymization, the Triatominae subfamily now has 158 valid species (Table 3).

**Table 3.** Tribes, genera and number of valid species that belong to the subfamily Triatominae.

Tribes	Genera	Species (n)
Alberproseniini	<i>Alberprosenia</i>	2
Bolboderini	<i>Belminus</i>	9
	<i>Bolbodera</i>	1
	<i>Microtriatoma</i>	2
	<i>Parabelminus</i>	2
Cavernicolini	<i>Cavernicola</i>	2
Rhodniini	<i>Psammolestes</i>	3
	<i>Rhodnius</i>	19
Triatomini	<i>Dipetalogaster</i>	1
	<i>Eratyrus</i>	2
	<i>Hermanlenticia</i>	1
	<i>Linshcosteus</i>	6
	<i>Mepraia</i>	3
	<i>Nesotriatoma</i>	3
	<i>Panstrongylus</i>	18
	<i>Paratriatoma</i>	2
	<i>Triatoma</i>	81
	<i>Paleotriatoma</i>	1
Total		158

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16080472/s1>, Figure S1: Neighbor-Joining phylogenetic analysis based on *cyt B* gene. The number in the nodes indicates the posterior probabilities for each clade. The *R. neglectus* clade is highlighted in green. Note that the *R. milesi* specimens are together with *R. neglectus* in the *R. neglectus* clade.

**Author Contributions:** Conceptualization, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M., C.G., M.T.V.d.A.-O., J.A.d.R. and K.C.C.A.; methodology, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M. and K.C.C.A.; formal analysis, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M., C.G., M.T.V.d.A.-O., J.A.d.R. and K.C.C.A.; investigation, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M., C.G., M.T.V.d.A.-O., J.A.d.R. and K.C.C.A.; resources, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M., C.G., M.T.V.d.A.-O., J.A.d.R. and K.C.C.A.; writing—original draft preparation, F.F.C., J.d.O., C.G. and K.C.C.A.; writing—review and editing, F.F.C., J.d.O., J.K.S.S., A.R., Y.V.d.R., L.M.M., C.G., M.T.V.d.A.-O., J.A.d.R. and K.C.C.A.; supervision, K.C.C.A. and M.T.V.d.A.-O.; project administration, K.C.C.A. and M.T.V.d.A.-O.; funding acquisition, K.C.C.A., C.G. and M.T.V.d.A.-O. All authors have read and agreed to the published version of the manuscript.

**Funding:** Appreciation to the São Paulo Research Foundation (FAPESP) for funding the researcher, Jociel Kleyton Santos Santana (process 23/00423-0), Jader de Oliveira (process 2019/02145-2), Coordination for the Improvement of Higher Education Personnel, Brazil (CAPES)—Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Carlos Chagas Filho Research Foundation of the State of Rio de Janeiro (FAPERJ).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All relevant data are within the manuscript.

**Acknowledgments:** We are very grateful to João Paulo Sales Oliveira Correia for the photos and information provided about the *R. milesi* and *R. neglectus* holotypes deposited in CTIOC, Brazil.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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