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Review

Taxonomy, Evolution, and Biogeography of the Rhodniini Tribe (Hemiptera: Reduviidae)

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Abstract: The Triatominae subfamily includes 151 extant and three fossil species. Several species can transmit the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, significantly impacting public health in Latin American countries. The Triatominae can be classified into five tribes, of which the Rhodniini is very important because of its large vector capacity and wide geographical distribution. The Rhodniini tribe comprises 23 (without *R. taquarussuensis*) species and although several studies have addressed their taxonomy using morphological, morphometric, cytogenetic, and molecular techniques, their evolutionary relationships remain unclear, resulting in inconsistencies at the classification level. Conflicting hypotheses have been proposed regarding the origin, diversification, and identification of these species in Latin America, muddying our understanding of their dispersion and current geographic distribution. Clarifying these factors can help for the design of vector control strategies. The aim of this review is to depict the different approaches used for taxonomy of the Rhodniini and to shed light on their evolution and biogeography.

Keywords: Triatominae; Chagas Disease; *Rhodnius*; *Psammolestes*; evolution; taxonomy; Rhodniini; biogeography

1. Introduction

Chagas disease is caused by the protozoan parasite and hemoflagellate *Trypanosoma cruzi*. This pathology mainly affects Latin American countries, where there are an estimated 6–8 million people infected and causes approximately 50,000 deaths per year [1]. The parasite can be transmitted by insect vectors, blood transfusion, vertical transmission, organ transplantation, laboratory accidents, and oral route [1,2]. The major transmission mechanism is via contact between humans and the feces of infected insects of the subfamily Triatominae (Hemiptera: Reduviidae) [1–4]. Human infection occurs accidentally during urbanization and environmental imbalances (deforestation and habitat loss) which cause triatomine insects infected with the parasite to invade human dwellings [3–7].

In the Triatominae subfamily, 151 extant and three fossil species have been reported and classified into five tribes according to their morphological, biological, and ecological characteristics [8,9]. Most subfamily diversity is restricted to Latin America, where 135 species have been described [10]. The

Rhodniini tribe is one of the most diverse and has 23 described species (20 of the genus *Rhodnius* and three of *Psammolestes*). Within the genus *Rhodnius*, some species are important vectors of *T. cruzi* [3,8,11,12], and some have been found to have the capacity to produce metacyclic trypomastigotes of *T. rangeli* in their salivary glands [13–20].

Rhodnius prolixus is one of the main vectors of Chagas disease. Its wide geographic distribution (extending from Central America through the Andean countries and the Amazon basin), its capacity for domiciliation, its high dispersion, and its strong vector capacity are a threat to the future of vector control programs [21–23]. In addition to *R. prolixus*, three species within the Rhodniini tribe have been found domiciled: *Rhodnius ecuadoriensis* in the northern zone of Peru and Ecuador, *Rhodnius stali* in Bolivia, and *Rhodnius pallescens* in Panama. *Rhodnius prolixus*, *R. ecuadoriensis*, *R. pallescens*, and *R. stali* are infected with *T. cruzi* at prevalence rates of 12.0–82.0% [24–26], 10.0–42.0% [15,27,28], 42.0–87.4% [24,29–33], and 7.7% [34], respectively. Additionally, *R. neglectus* and *R. nasutus* are epidemiologically relevant species in Brazil, where they often invade and colonize human environments [7,35–37].

Although the taxonomy of the genus *Rhodnius* has been widely studied, there are controversies regarding the number of species, the classification of these species into groups, and the phylogenetic relationships and monophyletic status of these groups. These have arisen from the conflicting results of morphometric analyses, cytogenetic analyses, and analyses of isoenzyme markers and molecular markers [8,12,38]. There is also controversy regarding the paraphyly of two genera belonging to the Rhodniini tribe (*Rhodnius* and *Psammolestes*): although morphological differences are present, other techniques used for taxonomic analysis group *Psammolestes* with *Rhodnius* [8,38].

The objective of this review is to describe the current state of knowledge of the phylogenetic and biogeographical relationships among the species of the Rhodniini tribe and to highlight the need for new studies to develop a more comprehensive understanding of the systematics of these species.

2. The Rhodninii Tribe: the Current Taxonomy

The Rhodniini tribe is composed of two genera: the genus *Rhodnius* consisting of 20 species and the genus *Psammolestes* consisting of three species (Table 1). Initially, it was proposed that the three species belonging to the genus *Psammolestes* should be grouped into a tribe called Psammolestini given their marked morphological differences compared with the genus *Rhodnius* [39]. Later the two genera were grouped into the Rhodniini tribe, based mostly on the presence of tuberosities posterior to the eyes and bearing in mind that they represented mostly arboreal species with the exception of some of the *Rhodnius* [8,40,41]. The Rhodniini tribe has been extensively studied due to its epidemiological importance and wide geographical distribution. Additionally, members of the genus *Rhodnius* have been classified into three groups—*pictipes*, *pallescens*, and *prolixus*—based on geographical distribution, biogeography, and morphology. Initially these groups were called lineages; however, since their monophyletic origin has been questioned, the term group is currently preferred [8].

Table 1. Genera and species of the Rhodniini tribe.

Genus	Group	Species
Rhodnius	*pictipes*	*R. amazonicus*, *R. brethesi*, *R. paraensis*, *R. pictipes*, *R. stali*, *R. zeledoni*
	pallescens	*R. colombiensis*, *R. ecuadoriensis*, *R. pallescens*
	prolixus	*R. barretti*, *R. dalessandroi*, *R. domesticus*, *R. milesi*, *R. marabaensis*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. prolixus*, *R. robustus*, *R. taquarussuensis* *
Psammolestes		*P. arthuri*, *P. coreodes*, *P. tertius*

* *R. taquarussuensis* is currently considered a phenotypic form of *R. neglectus*.

3. Geographical Distribution of Members of the Rhodniini Tribe

The Rhodniini tribe has a wide geographical distribution ranging from Central America to the Southern Cone (Figure 1A). The distribution of species richness within the tribe is unimodal, with a greater number of species identified towards the northern hemisphere at low latitudes and some species identified in the southern hemisphere, reaching latitudes of 30° south [10].

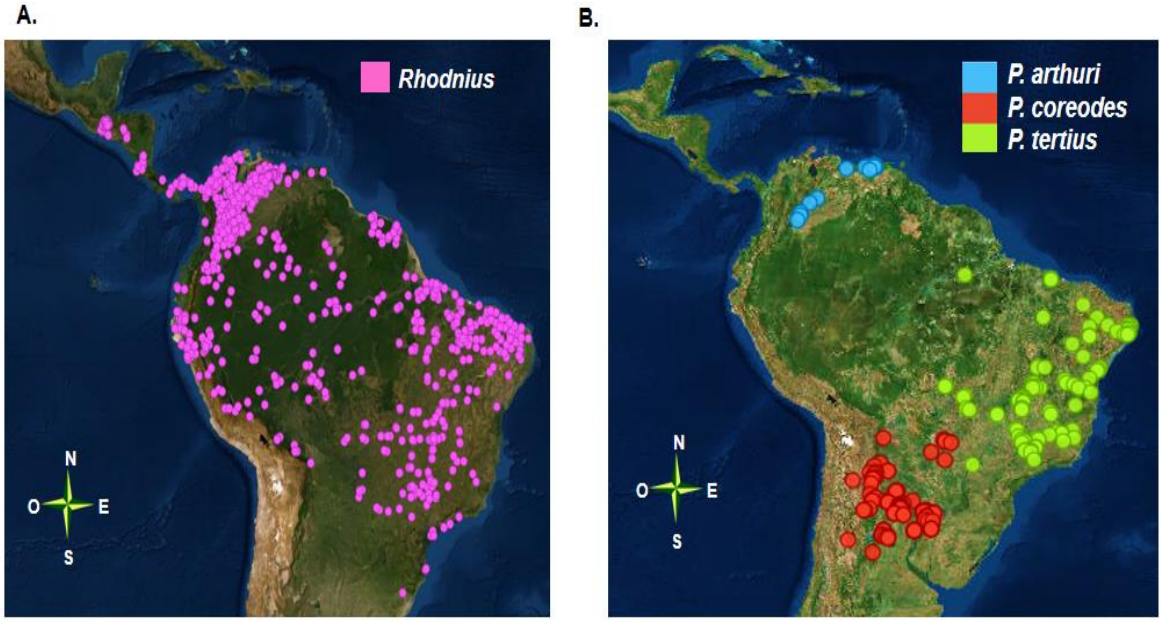

Figure 1. Geographical distribution of the Rhodniini tribe (**A**) Geographical distribution of the genus *Rhodnius* (**B**) Geographical distribution of species within the genus *Psammolestes*. Each point corresponds to geographical locations where individuals of each species were identified, these points were reported in several studies and compilated in a database by Ceccarelli and colleagues. This database was used to reconstruct the maps in Orange software (v.3.24.1) [42].

The genus *Psammolestes* has an interesting geographical distribution, and each of the three species with this genus has a distinct distribution (Figure 1B). Members of this genus are found mainly in bird nests (Furnariidae and Psittacidae), although they have also been identified in palm trees [43,44]. *Psammolestes arthuri* is distributed in Colombia and Venezuela. In Colombia, it has been described in the departments of the Orinoco region and in Venezuela it has been described in 15 different states, mainly in the plains region [42,45]. The range of *P. tertius* extends widely in Brazil, mainly in the Caatinga and much of the Cerrado [44]. *Psammolestes coreodes* is distributed in the Gran Chaco, and is widely distributed throughout 11 provinces in Argentina as well as in Paraguay, Bolivia, and the state of Mato Grosso do Sul, in the Brazilian Cerrado [46].

The geographical distribution of the genus *Rhodnius* is wider, covering part of Central America and a large part of South America. Geographical distribution is one of the criteria used for classification of the previously mentioned groups: species of the group *pallescens* (trans-Andean) are distributed to the west of the Andes mountain range (Figure 2A), while species of the groups *prolixus* and *pictipes* (cis-Andean) are distributed east of the Andes and the Amazon region (Figure 2B,C) [8].

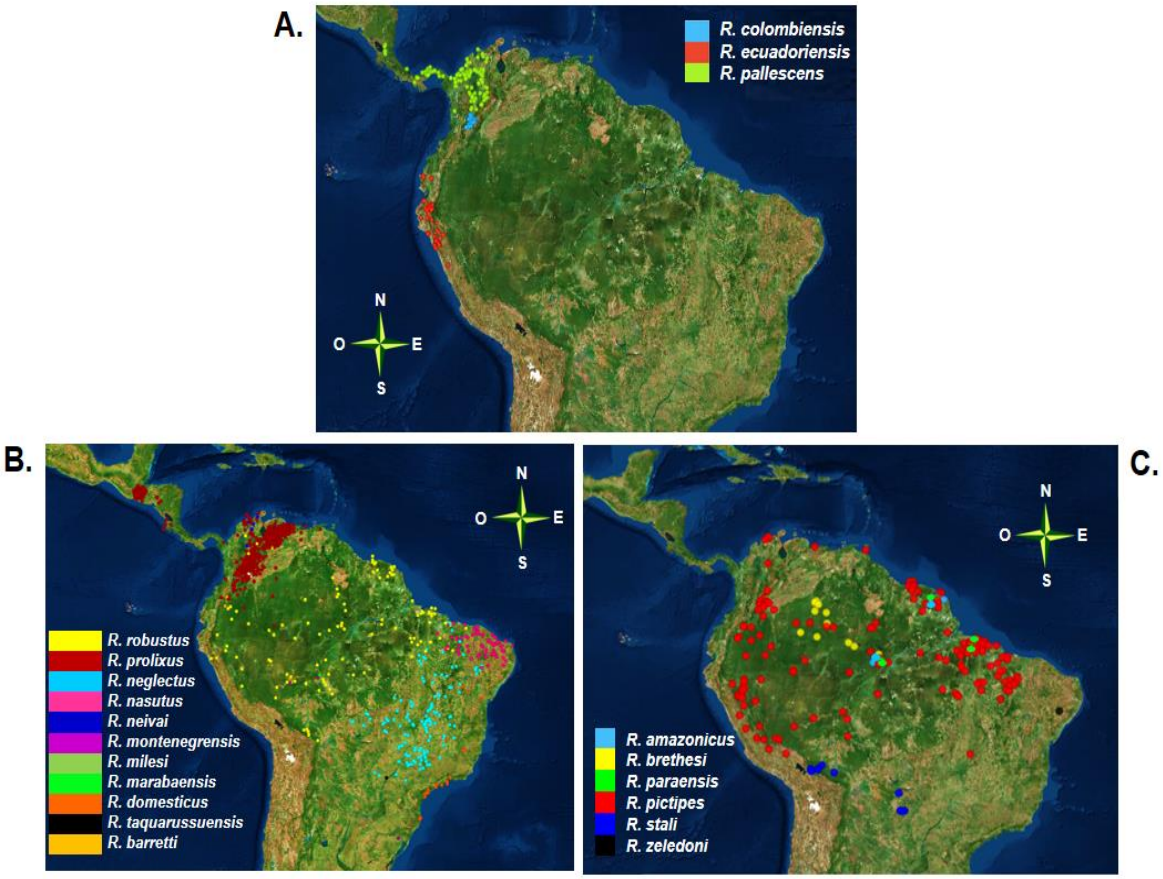

Figure 2. Distribution of species within the genus Rhodnius (**A**) Geographical distribution of species of the trans-*pallescens* group (**B**) Geographical distribution of species of the cis-*prolixus* group (**C**) Geographical distribution of species of the cis-*pictipes* group. Each point corresponds to geographical locations where individuals of each species were identified, these points were reported in several studies and compiled in a database by Ceccarelli and colleagues. This database was used to reconstruct the maps in Orange software (v.3.24.1) [42].

In the trans-*pallescens* group, all species are associated mainly with palms. The species with the widest geographical distribution in this group is *R. pallescens*, whose range extends into western Colombia, Panama, Costa Rica, Belize, and Nicaragua. *R. pallescens* is mainly associated with wine palms (*Attalea butyracea*) and oil (*Elaeis oleífera*) and has also been found in human dwellings, mainly in Panama [47,48]. *R. ecuadoriensis* is distributed in Ecuador and northern Peru and is strongly associated with human dwellings and with the palm *Phytelephas aequatorialis* in northern Ecuador, although it has also been identified in squirrel nests [28,47,49]. Finally, *Rhodnius colombiensis* is found in Colombia in the departments of Cundinamarca and Tolima, where it is also associated with the wine palm *A. butyracea* [10,46,47,50,51].

The cis-*prolixus* group has the largest number of species and the largest geographical distribution of the three groups. Among the species of this group, *R. prolixus* is the best studied given its epidemiological importance and has been reported in countries of Central America as well as in Colombia and Venezuela [12,43,46,52]. As a major vector, *R. prolixus* is mainly found in domestic habitats, although jungle populations associated with palms have also been identified in Colombia and Venezuela [22,52]. Control programs in Central America have achieved both the interruption of *T. cruzi* transmission and the direct elimination of *R. prolixus*, drastically decreasing its distribution in Mesoamerican countries [40,53–56]. However, the presence of *R. prolixus* has been reported again in Mexico, in 2019 [57]. Additionally, some reports of *R. prolixus* in Bolivia, Brazil, Ecuador, Guyana,

French Guiana, Panama, and Suriname were erroneous, probably due to confusion with *R. robustus* and in central Brazil with *R. neglectus* [10,58–62].

Another species within the *prolixus* group with a wide geographical distribution is *R. robustus*, whose range extends through Bolivia, Brazil, Colombia, Ecuador, French Guiana, Peru, Venezuela, and Suriname [10,46,60,61]. Five cryptic species (I–V) have been reported within this species: *R. robustus* I is found in Venezuela, while *R. robustus* II, III, and IV are distributed in the Amazon region spanned by the previously mentioned countries [61]. There are reports of species of the *prolixus* group outside of Brazil with more restricted ranges such as *R. barretti* and *R. dalessandroi*. *R. barretti* has been identified from different palms in the Napo ecoregion in the western Amazon, the region that encompasses the lowlands of eastern Ecuador and the adjacent areas of southern Colombia (south of the Caguán River), and the north from Peru [63]. *Rhodnius dalessandroi* has only been reported once in the department of Meta, Colombia [46,56].

The other species of the *prolixus* group are exclusively Brazilian. *R. neglectus*, *R. nasutus*, and *R. domesticus* are associated with the Brazilian ecoregions, *R. neglectus* is dispersed in the Cerrado and São Paulo state, *R. nasutus* in the arid regions of the Caatinga, and *R. domesticus* in the Atlantic forest. The first two species are found in palm trees and bird nests, while *R. domesticus* has been identified in bromeliads [7,40,46,56,64]. *Rhodnius montegrensis* has been found in two states of Brazil (Acre and Rondonia), while the ranges of *R. milesi* and *R. marabaensis* are limited to the state of Pará [65–67]. *Rhodnius taquarussuensis* n.sp. was initially proposed as a new species in Taquarussu (Mato Grosso do Sul), but is currently considered a phenotypic form of *R. neglectus* [68,69].

Finally, the cis-*pictipes* group also has a wide geographic distribution. Within this group, *R. pictipes* has the widest geographical distribution of the entire Rhodniini tribe, extending throughout the Amazon basin (north and northwest of South America) in association with different palms. There have been reports of this species in Belize, Bolivia, Brazil, Colombia, Ecuador, Guyana, French Guiana, Peru, Suriname, Trinidad, and Venezuela (Figure 2C) [10,40,46,60]. The species with the next-widest distribution is *R. brethesi*, which is characterized by its association with the palm *Leopoldina piassaba*. The geographical distribution of this species extends to the Amazon basin in Colombia and Venezuela and the states of Para and Amazonas in Brazil [10,40,46,47,52,56,70].

Rhodnius stali is distributed in Brazil (Matto Grosso and Acre) and in several provinces of Bolivia, where it is associated with *A. phalerata* and is also found in human dwellings, primarily in Alto Bení (Bolivia) [40,43,44,46,71]. *Rhodnius neivai* is found on fallen tree trunks and in the crowns of the llanera palm (*Copernicia tectorium*) in Colombia and Venezuela [47,56]. *Rhodnius amazonicus* and *R. paraensis* are distributed in French Guiana and Brazil, where *R. paraensis* has been found in nests of arboreal rodents of the genus *Echimys* [11,56,58]. Finally, *R. zeledoni* was identified in a single report in the northeast of Brazil in the state of Sergipe [72].

4. Taxonomic and Phylogenetic Studies of the Rhodniini Tribe

The taxonomy and systematics of the Rhodniini tribe are complex. Efforts to classify the species of this tribe date back to the middle of the 18th century, at which time *R. prolixus*, *R. pictipes*, and *R. nasutus* were first described. The most studied species is *R. prolixus*. Since its life cycle is relatively short compared with other triatomines, it has been used as a biological model for studies of the physiology and biology of the Triatominae [40]. In addition to morphological studies, cytogenetic, isoenzymatic, and molecular studies have all been applied to members of the Rhodniini tribe, providing different levels of resolution in the study of their specific relationships [40]. However, it should be noted that many of these studies have focused only on the differentiation or analysis of selected species and few attempted to clarify the relationships among all members of the tribe.

4.1. Morphological Studies

Taxonomic classification of the Rhodniini tribe was initially based on morphological similarities and differences between species and included biogeographical aspects. However, one of the main limitations,

especially for the Rhodniini tribe, was low morphological variability between species [8,41,73]. The characters that have been classically used for classification of the Rhodniini tribe were generally size, color, or patterns of coloration in some parts of the insect, and aspects of the cuticle. Additionally, various characters are used at the level of the head, thorax, abdomen, and legs [41,74]. New characters have been proposed for identification of triatomines and have been applied to the Rhodniini tribe. These include the spermatheca, geometric morphometry of the wings, morphology of abdominal segments IX and X, coloration of the salivary glands, and morphology of the genitalia. Use of some of these new characters has enabled differentiation at the level of the tribe presence of nitrophorins that confer red coloration to the salivary glands of the Rhodniini tribe and the morphometry of the wings as well as at the intra-specific level of the genus *Rhodnius* (Form of the female genitalia) [74–78].

The inclusion of the genera *Rhodnius* and *Psammolestes* within the Rhodniini tribe was based on their mainly arboreal behavior and the presence of post-ocular tuberosities in members of both genera. The latter feature is exclusive to both genera [41]. Between the two genera, differences can be observed in the morphology of the head and the shape of the femurs. However, these characters were insufficient to accurately reconstruct a cladogram and investigate relationships between the genera (Figure 3) [8,40,41].

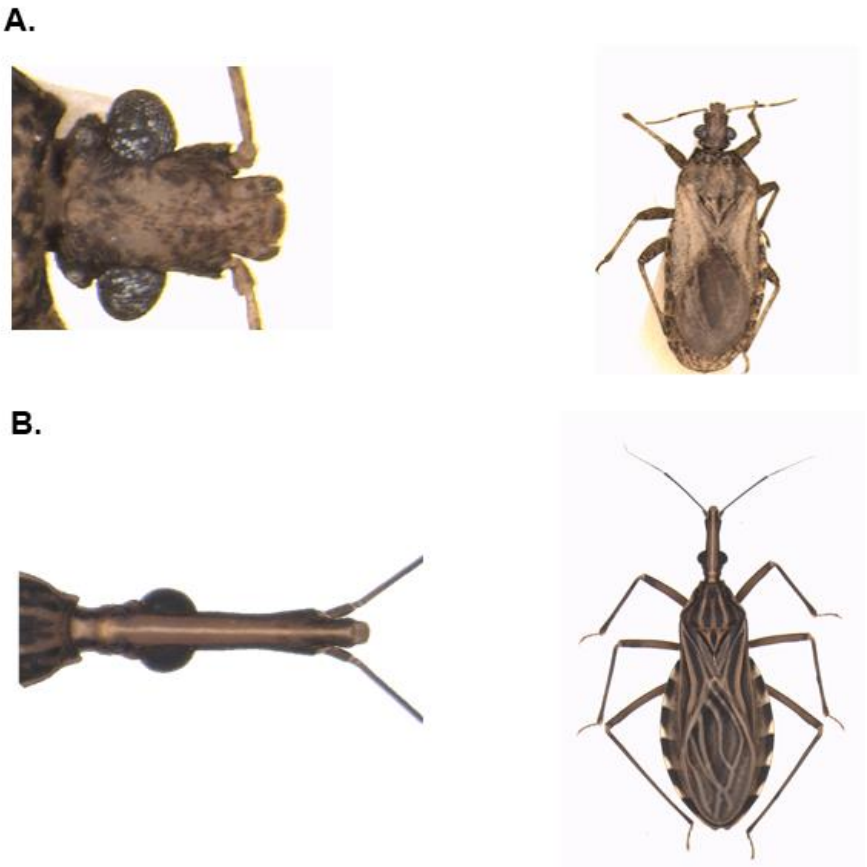

Figure 3. Morphology of the head and complete body of genera of the Rhodniini tribe (**A**) Morphology of the genus *Psammolestes* (*P. tertius*) (**B**) Morphology of the genus *Rhodnius (R. prolixus*). The images were supplied by João Aristeu da Rosa (Co-author of this manuscript).

In the genus *Psammolestes*, the morphology of the head, antennae, pronotum, genitalia of the males, and their phallosoma represent characters enabling identification of the three described species [24]. In the case of the genus *Rhodnius*, morphological characters enabling species differentiation are very few and can be affected by phenotypic variability. These characters also vary in association

with environmental changes yielding minor changes, which have been postulated to contribute to misclassification of species [12,38,40,46].

The cis-*prolixus* group presents very little morphological variability (Figure 4A). Thus, the feasibility of species identification using morphological characters has been questioned. There have even been mistakes in distinguishing *R. prolixus* and *R. robustus*, because the most useful difference between them for classification lies in the coloration of the hind tibiae of nymphs IV and V [41,61,79,80]. Additionally, the identity of some species within the *prolixus* group has been questioned: (i) *R. milesi* and *R. taquarussuensis* due to their similarity with *R. neglectus;* (ii) *R. montenegrensis* and *R. marabaensis* due to similarities with the cryptic species *R. robustus* II and III, respectively and (iii) *R. dalessandroi* due to morphological similarities with *R. brethesi* (group *pictipes*) and *R. robustus* [38,41,56,68,69]. With respect to coloration patterns, *R. neivai* differs from other species in the group by its dark coloration, and a dark black morphotype of *R. nasutus* has been identified with size variations in accordance with the environment and colonized palm trees [41,81,82].

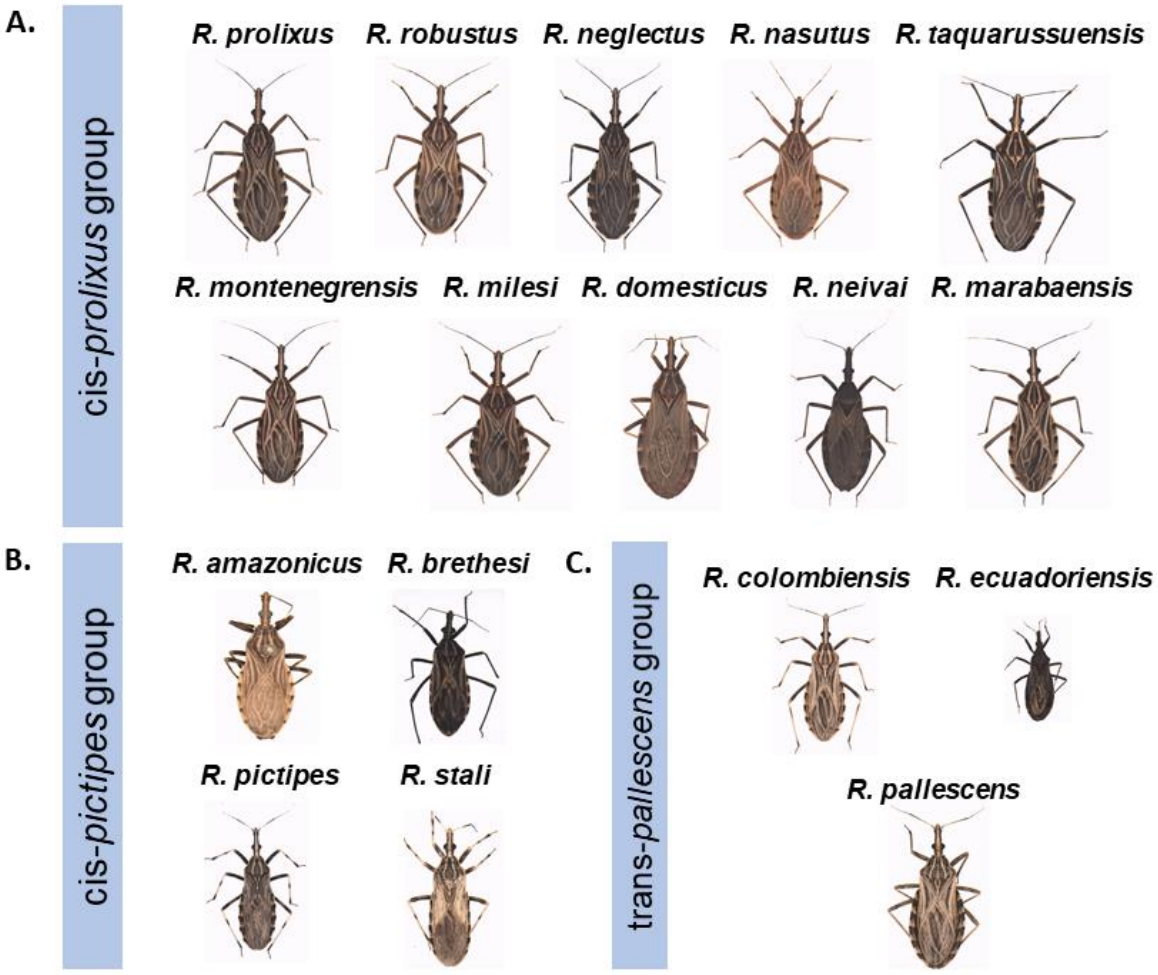

Figure 4. Morphology of species within the genus *Rhodnius*. (**A**) cis-*prolixus* group species, *R. taquarussuensis* is currently considered a phenotypic form of *R. neglectus*, (*) indicates that *R. nasutus* has two isoforms (**B**) cis-*pictipes* group species (**C**) trans-*pallescens* group species. The images were obtained by João Aristeu da Rosa. The morphology of *R. barreti* can be observed in Abad-Franch et al., 2013 [63] and *R. dalessandroi*, *R. paraensis* and *R. zeledoni* can be found in Jurberg et al., 2014 [83].

More morphological differences are observed between members of the group *pictipes* (Figure 4B). The validity of the species *R. amazonicus* has been questioned because it was documented in only a single original report and differences with respect to *R. pictipes* were not evident [41]. However,

subsequent reports in French Guiana and Breves, Pará, Brazil reaffirmed the characteristics observed in the original report [11,58,84]. *Rhodnius zeledoni* was also described in only a single report and is thought to be more similar morphologically to *R. domesticus* (group *prolixus*) than to members of the group *pictipes* and correspond to a poorly-preserved adult male therefore, currently it is difficult to validate its identity as a species [8,72]. *Rhodnius brethesi* differs from the remainder of the tribe by the size of the second antenna segment, which is longer than the third and the red or orange spots on the connexivum [41]. Finally, the *pallescens* group is composed of three morphologically very similar species. However, *R. colombiensis* was initially confused with *R. prolixus* of wild origin due to morphological similarities [56]. *Rhodnius ecuadoriensis* is characterized by its smaller size (Figure 4C). However, it has been proposed that this species should be grouped with *R. pictipes* due to the similarities of their antennal sensilla [56,85].

4.2. Cytogenetic Studies

In the Rhodniini tribe, the number of chromosomes is homogeneous in all of the 17 species analyzed so far (14 species of *Rhodnius* and three of *Psammolestes*), which were representative of the two genera and the three groups of the genus *Rhodnius*. Thus, the diploid chromosome number of the Rhodniini tribe is made up of 20 autosomes and the XY sex chromosomes; variations are observed between the Triatomini and Bolboderini tribes in the number of autosomes and sex chromosomes present in species and complexes [40,86–89]. Nucleolar persistence has also been observed during meiosis, as reflected by the presence of nucleoli or nucleolar corpuscles during the meiotic metaphase in 15 members of the tribe (*P. tertius*, *R. brethesi*, *R. colombiensis*, *R. domesticus*, *R. ecuadoriensis*, *R. milesi*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. pallescens*, *R. pictipes*, *R. prolixus*, *R. robustus*, and *R. stali*) [90,91]. Flow cytometry measurements of genome size have been performed in four species of the tribe (*R. ecuadoriensis*, *R. colombiensis*, *R. pallescens*, and *R. prolixus*) and indicated sizes of 0.72, 0.58, 0.73, and 0.75 picograms of haploid DNA, respectively, smaller than all other triatomines [87,92,93].

Studies at the genus level in *Psammolestes* have shown that in all three species, heterochromatin and AT repeats are present in the Y chromosome. The chromocenter is formed by the sex chromosomes and autosomal heterochromatin is absent [40,44,87]. Studies of the genus *Rhodnius* have demonstrated the presence of autosomal heterochromatin in *R. pallescens*, *R. colombiensis*, *R. domesticus*, *R. nasutus*, *R. taquarussuensis*, and its absence in *R. brethesi*, *R. ecuadoriensis*, *R. pictipes*, *R. neglectus*, *R. prolixus*, *R. robustus*, and *R. montenegrensis*. In species that have constitutive heterochromatin, usually very tiny C-bands are observed [40,87,89,92,94]. However, in spite of the homogeneity of cytogenetic characteristics observed in the tribe, intra- and inter-specific differences exist in the chromosomal location of ribosomal genes. Two patterns of location have been observed: one on both sex chromosomes X and Y (*P. tertius*, *R. domesticus*, *R. neglectus*, *R. neivai*, *R. milesi*, *R. pictipes*, *R. pallescens*, and *R. stali*) and another only on the X chromosome (*R. nasutus*, *R. prolixus*, *R. robustus*, *R. colombiensis*, and *R. ecuadoriensis*). In *R. ecuadoriensis*, both patterns were observed in populations from Peru and Ecuador. Analysis of ribosomal genes using cytogenetic techniques can be a useful marker of recent divergence of species or populations and allowed the formulation of two hypotheses regarding the evolution of ribosomal gene patterns, which may be due to: (i) loss of the loci in the Y chromosome, with the XY pattern representing the ancestral state; or (ii) partial transfer of genes from the X to the Y chromosome through mechanisms of transposition or ectopic recombination between sex chromosomes [95,96].

There is clear utility in analyses of heterochromatin C at the intraspecific level (e.g., for *Triatoma infestans* and *Panstrongylus geniculatus*) [40,97,98]. Cytogenetic analyses have been applied to different specimens of *R. pallescens* obtained from different geographical locations in Colombia and Panama, and revealed intraspecific variability: two cytotypes were described differing in terms of size, number and distribution of heterochromatin C during mitosis and meiosis. The frequencies of cytotypes varied in relation to the ecological and geographical characteristics of collection sites. Additionally, the cytotypes were consistent with morphological differences in size, morphometry of the wings and characters of the head [92,99]. Finally, cytogenetic characteristics (number and size of chromosomes, autosomal

heterochromatin, and sex chromosomes) have also been used to evaluate intraspecific variability of different populations of *R. prolixus* and *R. neglectus* and no variation in cytotypes was observed with respect to the ecological and geographical characteristics of the populations analyzed [100].

4.3. Isoenzymatic Studies

Isoenzyme analyses have also been applied to species of the Rhodniini tribe, although few cross-sectional studies have examined all species at once. The first phylogenetic study addressing in several species of the Rhodniini tribe (*R. brethesi*, *R. ecuadoriensis*, *R. nasutus*, *R. neglectus*, *R. pallescens*, *R. prolixus*, *R. pictipes*, and *R. stali*) analyzed 12 enzymes, and the results of the analysis of distances and genetic variability allowed the grouping of species into the three groups currently known, in agreement with their geographical distribution [101]. Differently, one study proposed that *Rhodnius* species should be classified into only two groups: *prolixus* and *pictipes*, with the *pictipes* group including members of the group *pallescens* [80]. In addition, other isoenzymatic studies also showed that: (i) *R. stali* and *R. pictipes* despite their morphological similarities and geographical proximity are different species (ii) *R. nasutus* and *R. neglectus* are very closely related, indicating that their speciation probably occurred very recently, and that (ii) wild specimens erroneously identified as *R. prolixus* in Tolima (Colombia) were placed within the group *pallescens* and not with *R. prolixus*, and that they therefore could represent a new species that is known today as *R. colombiensis* [101,102]. Subsequently, two isoenzyme studies were published and included the species *P. coreodes* [103] and *P. tertius* [80] along with species of the three groups of the genus *Rhodnius*. In these studies, it was not possible to define the species status of *Psamolestes* in the tribe, because these species showed paraphyly with Rhodnius [80,104]. Another isoenzyme studies, addressing in species of the genus *Rhodnius*, showed that *R. prolixus* and *R. robustus* had identical electrophoretic patterns and were not reproductively isolated, however by using salivary, heme proteins was possible to differentiate these species [40,80,104–106]. The species status of *R. neglectus* was confirmed by genetic distance analysis and reproductive isolation although only one locus allowed its differentiation with *R. nasutus* [104,106].

4.4. Random Amplification of Polymorphic DNA (RAPD)

With the advent of molecular techniques based on DNA, the use of high resolution markers, such as random amplification of polymorphic DNA (RAPD), became possible. This technique was used mainly for differentiating species of the tribe where morphological and isoenzyme differentiation was not possible (e.g., *R. prolixus* vs. *R. robustus*, *R. ecuadoriensis* vs. *R. pictipes*, and *R. nasutus* vs. *R. neglectus*). The electrophoretic patterns by RAPD allowed differentiation of all six species [107]. Additionally, RAPD was used to compare populations of domestic *R. prolixus* from Central America (Honduras) and South America (Colombia). RAPD electrophoretic patterns differed according to geographic location and showed that genetic variability was greater in specimens obtained from Colombia. Thus, it can be deduced that the Central American specimens were derived from those in South America; this conclusion was reached not only from the results of RAPD but also based on differences in morphology, because the specimens from Honduras were smaller. Finally, an isoenzymatic analysis showed no differences between the two types of specimens, suggesting a common origin [108]. However, RAPD studies also showed that *R. prolixus* (domestic) and *R. colombiensis* (wild) have different electrophoretic patterns, suggesting no genetic flow and that the effective migration rate between the species is insufficient to maintain genetic homogeneity in the two species [109]. RAPD has also been used to evaluate intraspecific genetic variability of populations of *P. tertius* of differing geographic origins in Brazil. One study found differences in electrophoretic patterns in association with geographic origin, and these results were supported by differences in isoenzyme patterns and morphological features [110].

4.5. Microsatellites

Microsatellite markers have also been used in the Rhodniini tribe, mainly with the aim of analyzing intraspecific variation given the high resolution of these markers. A scheme of 10 microsatellite loci was designed based on DNA sequences of *R. prolixus* and its amplification was tested in 10 species of the genus *Rhodnius*. Amplification of all loci was successful in *R. robustus* and in 6–9 of the species of the *prolixus* group, while in species of the groups *pallescens* and *pictipes*, amplification of more than three loci was not achieved [111]. Subsequent studies have used microsatellites to analyze intraspecific genetic structure in different populations of *R. pallescens* [111,112], *R. nasutus* [36], *R. ecuadoriensis* [113], and *R. prolixus* [22]. In populations of *R. pallescens*, field and laboratory specimens have been compared, and laboratory colonies of *R. pallescens* showed a different genetic structure than their wild relatives [112]. Studies of microsatellite markers in *R. nasutus* and *R. ecuadoriensis* showed that populations of these species from different geographic locations had genetic differences. For *R. nasutus*, four differentiated groups were revealed in eight geographic localities of the Brazilian Caatinga [36], and for *R. ecuadoriensis*, specimens in two biogeographically distinct localities of Ecuador had distinct genetic structures in association with other phenotypic differences [113]. Given its epidemiological importance, microsatellite markers were analyzed in wild and domestic populations of *R. prolixus* from Venezuela. No genetic differences were found, reflecting a high risk of wild populations easily invading human habitations [22].

4.6. Sequencing of Mitochondrial and Nuclear Markers

Improvements in sequencing technologies have enabled the analysis of mitochondrial and nuclear DNA markers. Analysis of these markers has been applied in several species of the tribe, both for intra- and inter-species analysis and together with other species of the Triatominae subfamily. Some of these studies have been carried out in combination with other molecular markers [8,38]. Nuclear and mitochondrial marker sequencing studies (26 conducted to date) have explored the phylogenetic relationships among the species of the tribe. Twelve studies explored species of the Rhodniini tribe and other tribes. The following mitochondrial markers have been evaluated: 16S rRNA (16S ribosomal RNA), 12S rRNA (12S ribosomal RNA), ND4 (4 subunit NADH dehydrogenase), Cytb (Cytochrome b), COI (Cytochrome oxidase I), and COII (Cytochrome Oxidase II). The following nuclear markers have been evaluated: 18S (18S ribosomal RNA), ITS-2 (internal transcribed spacer of ribosomal DNA 2), EF-1α (elongation factor 1 alpha), Wg (Wingless), and 28S rRNA (RNA ribosomal 28S) [8,38,40]. The sequences of the Cytb gene were searched in Genbank using the following Entrez line: "esearch -db nucleotide -query "<organism> CYTB"|efetch -format fasta" that yielded 226 records corresponding to 13 species of *Rhodnius* (Table S1) and *Triatoma infestans* sequence was used as outgroup. The alignment was made using MUSCLE, correct by hand and the sequence were translated to proteins in order to verify for stop codons in Mesquite 3.04. A maximum likelihood topology was obtained with the evolution model HKY + F + I + G4 (BIC score: 6711.067753) in IQ-TREE and node support was calculated with 1000 ultrafast bootstrap replicates. (Figure 5). In relation to the other tribes (mostly the Triatomini tribe), the Rhodniini tribe is of monophyletic origin [114–119]. In studies in which specimens of the Cavernicolini and Borbodelini tribes were included, these tribes were grouped with the Rhodniini and not with the Triatomini, although all tribes preserved their monophyletic status. However, it should be noted that some of these studies only included one or two species of the Rhodniini tribe, given that their focus was not on the Rhodniini tribe but on the subfamily Triatominae and other Reduviidae [120–125].

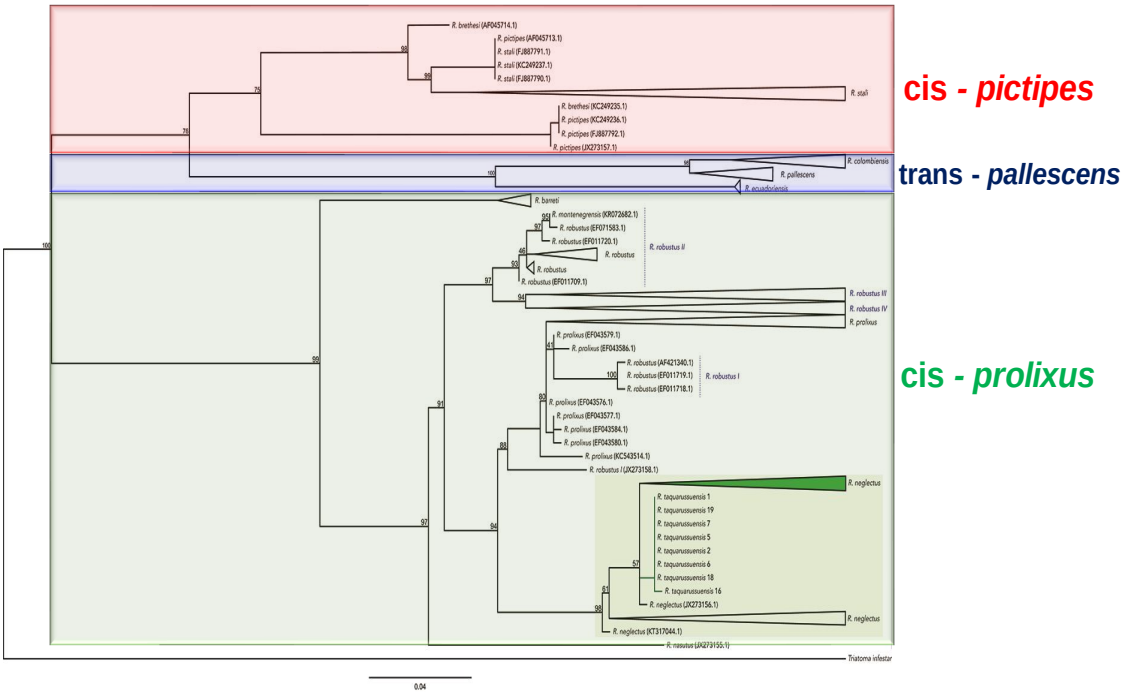

Figure 5. Maximum likelihood tree for *Rhodnius* based on Cytb sequences. The tree was reconstructed using sequences of *Rhodnius* species from GenBank.

The presence of two groupings within the *pictipes* group (Table 2) has led some authors to propose that the genus *Rhodnius* should be composed of clades or lineages. Some authors have proposed that the lineage *prolixus* be formed only by species of the group *prolixus* (named *robustus* lineage for other authors) and that another lineage should be formed by the species of the *pallescens* and *pictipes* groups called lineage *pictipes* [7,79,80,126]. Other authors proposed that the *prolixus* clade should be formed by species of the group *prolixus* and the group *pictipes* [122].

The remaining 14 studies focused on evaluating the phylogenetic relationships of Rhodniini species only and the grouping of species in particular. In these studies, the number of markers was limited to five: two mitochondrial (Cytb and ND4) and three nuclear (28S, AMPg, and ITS-2) [8]. Regarding phylogenetic relationships within the tribe, different issues have been identified that have been controversial with respect to previous studies: (i) paraphyly of the two genera of the tribe and grouping of the genus *Psammolestes* with members of the group *prolixus* were observed; (ii) the grouping of lineages within the genus *Rhodnius* was incongruent as well as the assignment of some species within groups; and (iii) the validity of some species was questionable due to inconsistencies in some phylogenies. These controversial aspects arose from the fact that many studies did not address all species of the tribe: most studies were intraspecific and used a limited number of markers. The objectives of most studies were limited to describing lineages but did not attempt the accurate delineation of all species nor the understanding the evolutionary processes that led to their formation [8,38].

Table 2. Characteristics of phylogenetic studies that have evaluated the relationships between groups of the genus *Rhodnius*.

Grouping	Nuclear Marker	Mitochondrial Marker	Taxa	Reconstruction Method	Reference
pictipes + *pallescens*		16S	4	MP	Stothard et al., 1998
		16S, Cyt b	8	NJ	Lyman et al., 1999
		16S, Cyt b	15		Schofield and Dujardin, 1999 *±
	28S	16S, Cyt b	13	MP, NJ	Monteiro et al., 2000
		Cyt b	18	NJ	Monteiro et al., 2018 ±
	28S	Cyt b	9	NJ	Marquez et al., 2011
		Cyt b	12	MP	Maia da Silva et al., 2007
		Cyt b	5	ML, BI	Da Rosa et al., 2012
pictipes + *prolixus*		16S, 12S	14	MP, NJ	Hypsa et al., 2002
		16S	14	MP, ML	De Paula et al., 2007 ±
		16S	14	MP, ML	De Paula et al., 2005
	18S, 28S	16S, Cyt b, COI, COII	10	ML, BI	Justi et al., 2014
	18S, 28S, Wg	16S	11	ML, BI	Justi et al., 2016

* In this study, several markers were consolidated: RAPD, isoenzymes, morphometry and marker sequencing. NJ: Neighbor-Joining, MP: Maximum parsimony, ML: Maximum likelihood, BI: Bayesian inference. ± In this study, the sequences were collected from previous studies.

Several primarily morphological studies established that the Rhodniini tribe is made up of two monophyletic genera [41,46,56]. However, in nine studies both nuclear [ITS-2, 28S] and mitochondrial (Cyt b, 18S, 12S, and 16S) molecular markers have indicated the paraphyly of *Psammolestes* and *Rhodnius* [38,79,114,115,117,118,122,123,127]. Some studies showed that *P. coreodes* [79,114,115,117, 122,127], *P. tertius* [79,114,115,117,122,127] and *P. arthuri* [123] were grouped with the species of the *prolixus* group of the genus *Rhodnius*, while a neighbor-joining analysis of Cytb sequences indicated the paraphyly of *Psammolestes* but not its grouping with the *prolixus* group [38,123].

Sequencing of molecular markers has detected groupings within the genus that have been postulated using other markers. However, sequencing did not confirm that the three groups were monophyletic nor the expected basal location of the *pictipes* group; these results have been controversial [40,117]. Some studies have found that the *pictipes* group is more closely related to the *pallescens* group [38,56,79,118,119,126,128], while other studies described topologies in which the *pictipes* group is more closely associated with the *prolixus* group [117,121,122,127]. These differences in the associations between groups may be due to variability in study characteristics (Table 2): the number of taxa may be an important aspect that provides greater precision to phylogenetic reconstructions [121,129].

Within the genus *Rhodnius*, there are also controversies regarding groupings of species. One example consists of the associations among species of the group *pallescens*, since after the identification of *R. colombiensis* it was grouped in some studies with *R. ecuadoriensis* [79,122] and in others with *R. pallescens* [38,117,127,130]. The position of *R. neivai* within the group *prolixus* has also been questioned, because some studies have proposed that it should belong to the group *pictipes* [56] and others that it should belong to the *prolixus* group [79,117,127]. In other cases, a clear location was not identified in any of the groups [38,80]. The validity of the species *R. robustus* has been strongly questioned and for that reason phylogenetic studies have been carried out to explore its associations with *R. prolixus*, mainly due to the important epidemiological implications of confusing the two species due to their morphological similarity. Monteiro et al., 2000, conducted a first study using specimens

of both species and sequenced 28S, 16S, and Cytb. These analyses showed discordances between morphological identification and phylogenetic grouping of individuals of both species [79].

These confusions in classification of these species led to a second study in which 26 different specimens of the two species from seven countries of Central and South America were used. Mitochondrial Cytb sequences were analyzed and the results showed that all specimens of *R. prolixus*—both from Central and South America—were grouped into a monophyletic and homogeneous cluster. By contrast, *R. robustus* showed a paraphyletic assembly composed of four clades (I–IV) from two different geographic regions: specimens of *R. robustus* clade I came from Venezuela and clades II, III, and IV came from French Guyana and different subregions of the Brazilian Amazon. Phylogenetic and distance analyses showed two main groups, one formed by *R. prolixus* and *R. robustus* I and another formed by the three remaining clades of *R. robustus*. This drew attention to the genetic distance between *R. robustus* clade I and *R. robustus* clades II, III, and IV, and to the distance between *R. robustus* I and *R. prolixus* [61]. These groupings and the topology of the two species were replicated in a study that included 551 specimens from Orinoco and Amazonia [22]. Subsequently, *R. robustus* clade V was discovered in the central-north zone of the Amazon and grouped with *R. robustus* clade I and *R. prolixus* [38,131]. Subsequent studies used the term cryptic species for *R. robustus* instead of clades or genotypes I–IV and confirmed the paraphyly of this species with *R. prolixus*. In these studies, two techniques were used to differentiate the two species: band size of PCR amplicons derived from Cytb [62] or sequencing the nuclear marker AMPg (a region located in the fourth intron of transmembrane protein 165) [132]. Other arguments such as loss of fertility (number of eggs per female) in interspecific crosses favored the differentiation of the two species; however, no overall loss of fertility was observed (number of females that lay eggs) in these crosses [133,134].

Another study revealed that specimens similar to *R. robustus* (identified by classical morphometry) collected in Puerto Asis, Colombia, corresponded according to sequence analysis of Cytb and 28S a new cryptic species of *R. robustus*, previously reported in Ecuador (Abad-Franch 2005,111). These specimens did not group with any other cryptic species of *R. robustus* and were located as a basal clade of the *prolixus* group [126]. However, more recent analyses of Cytb and morphometry indicated that the previously reported *R. robustus* is located as a basal clade only of *R. robustus* clade I and *R. prolixus*, and that these specimens do not correspond to *R. robustus* but rather represent a new species called *R. barretti* that is basal to the *prolixus* group [38,63].

The analysis of Cytb and ITS-2 markers performed by Monteiro et al. has been applied in some species whose validity has been questioned. However, in some species the number of specimens analyzed was very limited. The first was *R. amazonicus*, which was questioned as a potential variant of *R. pictipes*; the study showed that this species had an independent origin and did not group with specimens of *R. pictipes*. Other species questioned by marker sequencing were *R. milesi* (because of its monophyly with specimens of *R. neglectus*) and *R. montenegrensis* (because of its location within *R. robustus* clade II). However, another study using Cytb sequences identified *R. montenegrensis* as an independent species of *R. robustus*, supported by morphological characters and PCR restriction fragment length polymorphism analysis of ITS-2 [135]. Several species have also been questioned due to a lack of information regarding molecular markers, including *R. marabaensis*, *R. taquarusuensis*, *R. zeledoni*, and *R. dalessandroi*. Sequencing of Cytb has also enabled identification of species such as *R. stali* in *A. phalerata* palms in Alto Beni, Bolivia, and *R. prolixus* in *A. butyracea* palms in Casanare, Colombia [38].

Finally, Cytb sequencing has also been used to assess intraspecific variability of several species of the tribe including *R. prolixus*. The results showed very low genetic diversity and that wild and domestic specimens shared 7/18 haplotypes found in all analyzed specimens [22]. In 157 specimens of *R. nasutus* collected in eight localities of the Brazilian Caatinga, 16 haplotypes of Cytb were detected, of which two major haplotypes were shared by specimens from five localities with low levels of diversity observed [36]. In the case of *R. ecuadoriensis*, 174 specimens from two provinces of Ecuador (Loja and Manabí) were analyzed and 34 haplotypes were identified, of which only three were shared between the

two provinces. High haplotype diversity was observed and the model of isolation by distance applied to the two populations, showing that the populations of the two provinces were highly differentiated at the genetic level [113]. In *R. pallescens*, analysis of the Cytb, ND4 and 28S markers in populations from Panama and Colombia indicated the presence of two evolutionary lineages with genetic and morphological differences associated with their biogeographical and ecological distribution. It was even possible to observe genetic variation within lineages. In this manner, the Colombian lineage *R. pallescens* clade I could be subdivided into two populations, one from the north and one from southern Colombia and the Central American lineage *R. pallescens* clade II, which is composed of populations collected in western Colombia and different areas of Panama [92,130].

4.7. The Advent of Genomic Data: Genomics and Transcriptomics in the Rhodninii

Recently, additional sequencing tools have been applied to some members of the Triatominae subfamily and specifically to the Rhodniini tribe to obtain genome and transcriptome data. So far, the only member of the subfamily whose genome has been sequenced is *R. prolixus*; its genome was sequenced with 8× coverage using Sanger and 454 technologies and assembled using the CABOG program [120]. The genome was composed of 16,537 scaffolds with an N50 of 1.08 Mb, had an estimated size of 733 Mb and achieved an assembly that was 95% complete, corresponding to 706 Mb without chromosome mapping. The most updated version (October 2017) includes annotation of 15,738 genes and 15,752 transcripts (Rpro version C3.3 in VectorBase) (https://www.vectorbase.org/organisms/rhodnius-prolixus/cdc/rproc33).

This important approach to the genome of *R. prolixus* showed that 5.6% of the genome corresponded to transposable elements and demonstrated the presence of transcriptionally active genes transferred horizontally from *Wolbachia*. Exploration of the *R. prolixus* genome and knockdown experiments confirmed the presence of genes from different immune pathways (Toll and Imd) and the expansion of defensins, which function to control the intestinal microbiota but are unrelated to *T. cruzi* infection. Finally, comparative analysis of proteins revealed tandem expansions of genes families related to chemoreception, feeding, and digestion that potentially contributed to the evolution of a blood-feeding lifestyle [136].

The genome of *R. prolixus* has been used to search for satellite DNA sequences previously described in *T. infestans* using BLAST. The two species share four families of satellite DNA sequences of the 42 that are present in *T. infestans*, suggesting that their genomes are highly differentiated. Through hybridization experiments, it was demonstrated that these shared sequences were found at the autosomal level and the X chromosome in both species. By contrast, these sequences were absent in the Y chromosome in *T. infestans* and present in *R. prolixus*, suggesting a possible origin and evolution of the independent Y chromosome [137].

Recently, mitochondrial genomes of three triatomine species (*R. pictipes*, *Triatoma migrans*, and *Panstrongylus rufotuberculatus*) were sequenced and compared with the mitochondrial genomes of *Triatoma rubrofasciata*, *T. infestans*, and *Triatoma dimidiata* to explore evolutionary relationships with the other Reduviidae species and between the Rhodniini and Triatomini tribes. The results showed that mitochondrial genes had different rates of molecular evolution and six genes (ND1, ND2, ND4L, ND5, ND6, and ATP8) had higher rates than other protein-coding genes (PCGs) in all species. *R. pictipes* showed differences in the start codons of three PCGs (ND4L, ND6, and ND1) and in the stop codons of two PCGs (ATP6 and ND1) compared with other *Triatoma* species. Additionally, the sister relationship between Stenopodainae and Triatominae subfamilies was strongly supported, and the Triatomini species formed a sister group to *R. pictipes* [138].

Several transcriptomes of *R. prolixus* have been analyzed. The first was derived from ovarian follicle tissue in order to explore the role of gene expression in insect reproductive processes. This study showed expression of some genes that promote oogenesis and development of the embryo, suggesting that they may be important in insect control processes [139]. The transcriptome of the digestive system has also been described, which enabled exploration and differentiation of transcripts present in the three

segments of the intestine. The results showed that increased levels of some transcripts were related to the processes of digestion, detoxification, and transport of proteins through the digestive tract [140]. The transcriptome of the antennas has been described in all larval stages in order to characterize the expression of genes involved with the sensory functions of the insect. The results showed increased expression of genes related to chemoreceptors mainly in adult stages, as well as high expression of odorant binding proteins and chemosensory proteins in all stages [141]. Transcriptomes of *T. dimidiata*, *T. infestans*, and *T. pallidipennis* and the genome of *R. prolixus* have also been compared to evaluate the expression of gene families related to resistance to insecticides. In the case of *R. prolixus*, these studies revealed the expansion of two families, CYP4 (cytochrome P450-4) and CCE (carboxyl-Cholinesterases), related to pyrethroid resistance, odor processing, and degradation of hormones and pheromones [142].

A recent study analyzed and compared the transcriptomes of the head and salivary glands of *R. robustus* and *R. montenegrensis*; the validity of the latter species has been questioned due to its limited morphological differences compared with the former. The authors used RNA-Seq and detected 3055 single nucleotide polymorphisms (SNPs) distinguishing the two species and 216 transcripts with high levels of divergence. Several SNPs were detected in the same contig, suggesting that the two species were highly differentiated with possibly an extended time of divergence. In addition, the authors suggested that some of the genes studied could be subsequently tested for use in the identification and differentiation of these two species [143]. Finally, the most recent study combined the analysis of the previously reported transcriptomes of *R. robustus* and *R. montenegrensis* with the analysis of three molecular markers: Cytb, 28S, and ITS-2. In this study, it was concluded that *R. montenegrensis* and *R. robustus* clade II are in all likelihood the same species [127]. However, the repeatome and proteomic analyses detected high differentiation between *R. prolixus*, *R. montenegrensis*, and *R. marabaensis*, showing they are different species [144,145].

5. Biogeographical Hypotheses Pertaining to the Rhodninii Tribe

Studies of biogeography in the Rhodniini tribe are limited in number but revealed high complexity, including theories of possible vicariance, duplications (sympatry), dispersion, and extinction events. These are related to several geological events such as: (i) the elevation of the Central Andes during the Miocene; (ii) the branching of the Andes into three separate mountain ranges (eastern, central, and western) during the Plio-Pleistocene; (iii) the formation of a land corridor connecting South and North America during the Pliocene; and (iv) the elevation of the Serra do Mar and the mountain systems of the Serra da Mantiqueira between the Oligocene and the Pleistocene [56,127]. Because morphometric, cytogenetic, and molecular markers have yielded contradictory results in several systematic and phylogenetic studies of the Rhodniini tribe, there are several contradictory hypotheses regarding the origin and diversification of the species within this tribe.

5.1. Monophyletic Groups Hypothesis

The first hypothesis was formulated by Schofield and Dujardin in 1999. It assumes that the ancestor of the Rhodniini was closest to *R. pictipes* species and originated during the Quaternary Period in arboreal habitats of the Amazon-Orinoco and tropical forests dispersed towards the south of the Brazilian Amazon and the northeast of Bolivia. In turn, the ancestor gave rise to *R. stali* in palms and invaded homes. This ancestor underwent other more specific dispersions, giving rise to the remaining species of the *pictipes* group: (i) towards the north of Colombia and central Venezuela, to *R. neivai* which is found in *Copernicia tectorum* palms; (ii) towards the border area between Brazil, Colombia and Venezuela, to *R. brethesi* which is found in palms of *Leopoldinia piassaba*; (iii) towards the Colombian Orinoquia, to *R. dalessandroi* which is morphologically very similar to *R. brethesi* but is found in other palms; and (iv) towards the state of Pará, Brazil, to *R. paraenesis* which is found in nests of *Echymis crysurus* (Figure 6A). The ancestor *pictipes* also gave rise to the group *pallescens*. In this case, the ancestor *R. pictipes* was dispersed towards the northwest of Colombia and through a bottleneck in the Sierra Nevada de Santa Marta and the northern tip of the Cordillera de los Andes, gave rise to *R. pallescens*,

which is associated with *Attalea butyracea* palms and whose range extends to Central America and northwestern Colombia. The ancestor then dispersed through the Magdalena valley giving rise to *R. colombiensis* and into eastern Ecuador and Peru, giving rise to *R. ecuadoriensis* in Ecuador and northern Peru, possibly due to adaptation to the palm *Phytolepas aequatorialis* (Figure 6B) [56].

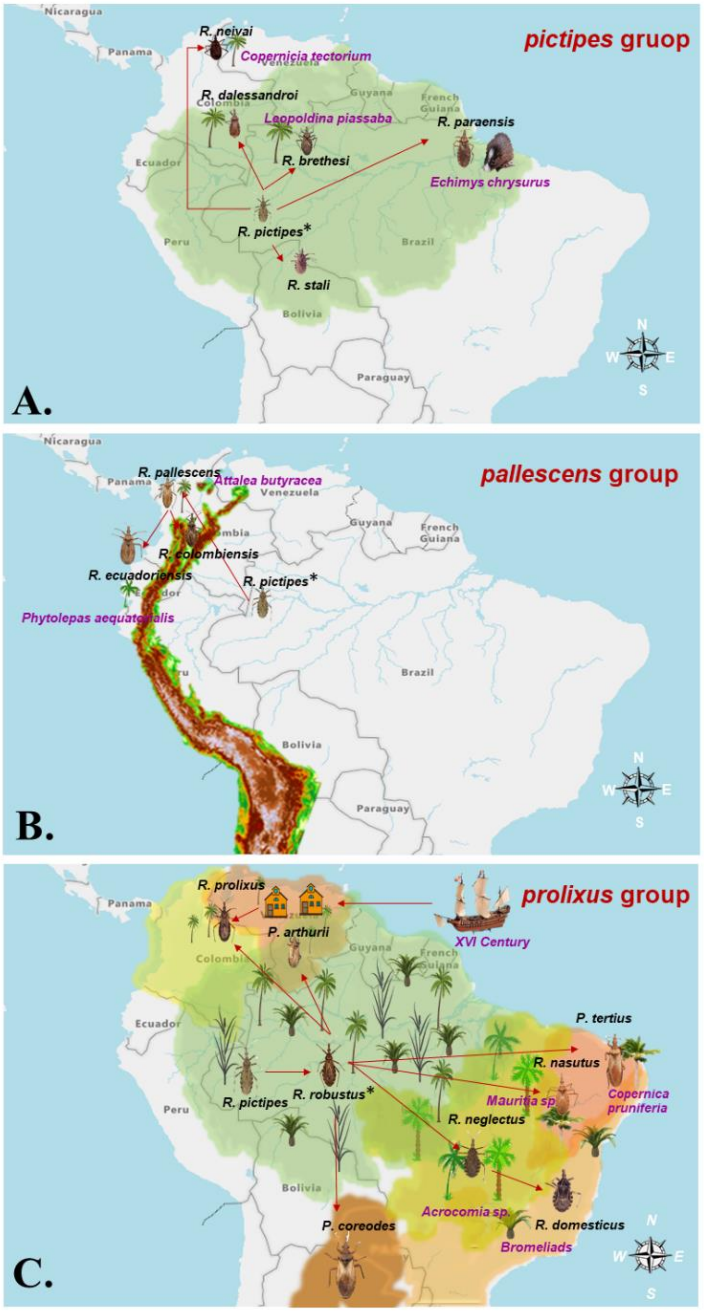

Figure 6. Monophyletic groups biogeographic hypothesis explaining speciation within the Rhodninii tribe. (**A**). Speciation and dispersion of *pictipes* group species (**B**). Speciation and dispersion of *pallescens* group species (**C**). Speciation and dispersion of *prolixus* group species. The asterisk (*) indicates the species closest to the most recent common ancestor (MRCA) of Rhodniini tribe by each hypothesis.

The ancestor *R. pictipes* gave rise to *R. robustus* which was scattered throughout the Amazon in two forms: (i) a first form dispersed to the north reaching Venezuela where it gives rise to wild *R. prolixus* and *P. arthuri*; and (ii) a second form dispersed towards the south reaching the Brazilian Cerrado, where

it gave rise to *R. neglectus* (associated with *Mauritia* and *Acrocomia* palms) and *P. tertius*. The latter and the southern form of *R. robustus* are dispersed towards the Caatinga where *R. robustus* gave rise to *R. nasutus* associated with the palm *Copernicia prunifera*. Additionally, *R. neglectus* is dispersed towards the Atlantic Forest where it gave rise to *R. domesticus* associated with Bromelia. Finally, this hypothesis proposes that the Spanish colonization of Venezuela triggered the domiciliation of wild *R. prolixus* and that the presence of *R. prolixus* domiciled in Central American countries may be due to the transport of some collections from Venezuela to El Salvador followed by their accidental release in rural homes. Subsequently, they dispersed and adapted to homes of several Central American countries. This last hypothesis is supported by morphometric analysis, isoenzyme analysis, and RAPD (Figure 6C) [56,108].

5.2. Lineages Hypothesis

Conflicting with these hypotheses, analysis of molecular markers did not support the idea that *R. pictipes* was the species closest to the ancestor of the three groups of *Rhodnius*. Instead, two topologies were generated, one in which *R. pictipes* was a sister group to the *pallescens* group and another in which it was a sister group to the *prolixus* group (Table 2). In addition, some studies grouped *R. colombiensis* as a sister species of *R. pallescens* and others as a sister speices of *R. ecuadoriensis*. Thus, two different hypotheses supported the two types of topologies and groupings observed [7,122,127].

The second hypothesis, based on morphometric studies, mitochondrial markers and isoenzyme analysis, was proposed by Abad-Franch et al., 2009 and Díaz et al., 2016. Under this hypothesis, the genus *Rhodnius* is classified into two lineages (*pictipes* and *robustus*). The first gave rise to the transandean (*pallescens*) group and the Amazonian species (*pictipes*), while the second diversified into the Amazon (group *robustus*) and underwent radiation in nearby ecoregions (Orinoco, Chaco, Caatinga, Cerrado, and Mata Atlântica) allowing the formation of the cis-*prolixus*. Under this hypothesis it is assumed that *R. colombiensis* and *R. pallescens* are sister species. Thus, the ancestor of the *pictipes* lineage was dispersed to the northern part of the then low eastern mountain range of Colombia in the late Miocene (11–6 Ma) [7,131] or the mid-Miocene (16–11 Ma) [116]. Thus, the diversification of the *pallescens* and *pictipes* could be explained by a vicariance, either caused by the formation of the Pebas system [130] or by the elevation of the Andes mountain range during the Pliocene (5 Ma) [7,131]. Regarding the diversification of the ancestral *pallescens*, two hypotheses have been proposed: (i) the divergence of the ancestor of *R. pallescens*/*R. colombiensis* and *R. ecuadoriensis* could have occurred during the late Miocene, a period during which the Northern Andes had not reached more than half of its modern elevation (11–7 Ma), and the diversification of the ancestor of *R. pallescens*/*R. colombiensis* occurred with the elevation of the northern Andes and the separation of the *R. pallescens* lineages with the formation of the isthmus of Panama during the early Pliocene [130]; and (ii) diversification of the ancestral group *pallescens* occurred during the Pliocene with the elevation of the then-low eastern mountain range of Colombia, which divided the population into two main clades, a northern group comprising the ancestral forms of *R. pallescens* and *R. colombiensis*, and an isolated southern group which adapted to the new ecotopes and eventually gave rise to *R. ecuadoriensis* [7,131]. The radiation of the ancestor of *pictipes* in the Amazon is postulated to have occurred during the Pliocene and Pleistocene, according to the specific adaptations described under the first hypothesis, with the exception of *R. neivai* that was included in the *robustus* lineage [7].

However, the radiation of the taxa of the *robustus* lineage (*R. domesticus*, *R. neivai*, *R. robustus*, and *R. nasutus*) in the ecoregions mentioned above can be attributed to another possible vicariance event at the end of the Miocene and subsequent radiation during the Pliocene. The division of the *robustus-neglectus-prolixus* group can be explained by more recent cladogenic events during the Pliocene and Pleistocene. Additionally, it was proposed that the lineages of *R. robustus* were potentially generated by parapatric speciation during the Pleistocene, and *R. robustus* in Venezuela does not appear to be a product of gene flow between *R. prolixus* and *R. robustus* from Amazon basin, but rather *R. prolixus* and *R. robustus* I seem to share a (relatively young) MRCA (most recent common ancestor) [7,61].

5.3. Clades Hypothesis

Finally, the third hypothesis proposed by Justi et al., 2016 using nuclear and mitochondrial markers proposes the formation of the *prolixus* clade (cis-andean) and locates the *pictipes* and *prolixus* groups as siblings, suggesting that the separation of the *prolixus* (cis-andean) group from the *pictipes* group (cis-andean) occurred by means of a vicariance during the formation of the Pebas system (23–11 Ma). Thus, the ancestor of the *prolixus* group was isolated in subregions of Brazil and that of the *pictipes* in the Chaco subregion and then the Amazon. Subsequently, the formation of the Acre system (10–7 Ma) allowed the ancestor of *R. neivai*/*R. domesticus* to diversify towards the north, giving rise to *R. neivai*, and towards the south, giving rise to *R. domesticus*. With respect to the group *pallescens*, this hypothesis proposes that divergence from the clade *prolixus* occurred during the late Miocene via the formation of the Pebas and that the separation of the three species occurred during the Pliocene. However unlike the second hypothesis, it proposes that separation occurred between *R. pallescens* and an ancestor *R. colombiensis*/*R. ecuadoriensis*, probably induced by the expansion of *R. pallescens* in Central America [122].

6. Future Perspectives

In the Andean countries, the main triatomines responsible for vector-mediated transmission of *T. cruzi* to people are those of the genus *Rhodnius*; thus, control measures are directed to species of this genus. Therefore, further studies of the evolution, phylogeny, biogeography, ecology, physiology, and behavior of *Rhodnius* species are needed to help improve existing Chagas disease control programs [8,10,38]. One of the primary steps in any control program is the accurate identification of vector species and detailed understanding of their genetic and population structures.

Therefore, future studies should be based on integrative taxonomy approaches, taking into account the delimitations of species, the synthesis of morphological characteristics with information obtained from molecular genetic studies, biogeography, phylogeography, behavior, ecology, and development [146]. The incongruities shown so far between morphological and genetic studies are due to several intrinsic limitations of several techniques. Studies to date have focused on describing groupings of species within the tribe but have not delimited these groups within the tribe, which has generated systematic problems.

One of the most important limitations of current studies lies in the limited morphological variability between different species of the tribe; therefore, there are few synapomorphic characters. Added to this, identification of some species of the tribe (*R. prolixus* vs. *R. robustus*) relies on few characters with complex interpretation, resulting in inconsistent results between morphological studies and errors in morphological identification. These problems arose because few cladistic studies or studies based on morphological characters of all species of the Rhodniini tribe have been conducted [8,74,147].

Some characters used for identification of species are affected by phenotypic variability and morphological plasticity in association with environmental changes. The latter generates minor morphological changes between populations of the same species and can therefore lead to erroneous identification, such as the presence of color morphotypes in *R. nasutus*. These changes can occur before genetic barriers are present between species and therefore will generate inconsistencies between morphological and genetic studies. Phenotypic variability and morphological plasticity can also lead to morphological convergence between species that are genetically distinct but are adapted to the same ecological niche, as is the case for species of the *prolixus* group [38,40]. Due to these complicating factors, future studies should focus on identifying morphological synapomorphic characters that facilitate differentiation and identification of appropriate species, and are complemented by genetic, biogeographic, behavioral, and ecological analyses. This would help in laying out a rational approach to the systematics of the tribe.

Complexities in delineation of species of the Rhodniini tribe could also be addressed through crosses in the laboratory: these would allow evaluation of reproductive isolation and the potential presence of hybrids between species. Although several studies have been carried out in the Triatomini tribe, few reports have documented a cross between members of the Rhodniini tribe (*R. prolixus*–*R.*

robustus and *R. prolixus–R. nasutus*) [134,148]. This reflects the complex morphological identification of species of the tribe and the difficulties associated with identification of hybrids, because these usually correspond to morphological intermediates between the parental species. Thus, it is important that future studies carry out interspecific crosses to verify if members of the tribe meet the biological definition of species. These crosses should be evaluated not only at the level of morphological characters, but also using genetic characters such as molecular markers. Analyses of nuclear DNA and mitochondrial DNA can both be used for appropriate identification of species involved in crosses as well as for analysis of the progeny of crosses: the nuclear and mitochondrial DNA sequences allow identification of introgression and the latter allows identification of hybridization processes [149].

Finally, genetic studies in the tribe have been limited, and the majority of them have focused on studying relationships between the Rhodniini tribe and other members of the Triatominae or Reduviidae. Additionally, studies that have exclusively examined species of the tribe have focused on analysis of epidemiologically important species or been focused at the intra-specific level. In addition, different studies have examined different species and are thus not directly comparable. There is only one study in which Cytb sequences from most of the tribe's species were included [38]. Therefore, it is necessary to carry out studies that include a large number of species of the Rhodniini tribe to attempt relevant delineation of tribe members.

In *Anopheles* and several species of the Triatomini tribe, nuclear ribosomal sequences present intragenomic variability due to their high copy number. Thus, data obtained from these specimens should be analyzed with caution because errors of interpretation may occur. Multilocus approaches will enable better resolution in phylogenetic analyses and also allow identification of introgression and potential hybridization events. Therefore, attempts to design molecular markers for multilocus studies are required; these markers need to be sequenced in a representative number of species to elucidate their phylogenetic relationships and to provide useful tools for integrative taxonomy [149].

Finally, genomic and transcriptomic resources in the tribe are scarce. To date, only the mutation rates of Cytb sequences in species of the genus *Triatoma* and of mitochondrial loci in *R. pictipes* have been examined. This information may be used for coalescence analyses and phylogeographic reconstructions in future studies. However, the genomic mutation rate of at least one representative species of the Rhodniini tribe remains to be determined. The genome of *R. prolixus* has low coverage and has not yet been assembled at a chromosomal level. Therefore, future studies could improve genomic resources, especially because the resolution of phylogenies drastically improves when genomic data are used, as previously documented in plants and in a recent phylogenomic analysis of the Hemiptera [150,151].

7. Conclusions

In spite of the valuable contributions of studies carried out to date on species of the Rhodniini tribe, limitations in our understanding have led to inconsistencies between and within morphological and phylogenetic studies. In turn, this has created three problems in the systematics of the Rhodniini tribe: (i) paraphyly of two genera in the tribe, (ii) different types of classification and grouping of the species of the genus *Rhodnius*, and (iii) difficulties in identification and adequate delineation of some species. These issues have resulted in generations of conflicting hypotheses regarding the origin, evolution, and dispersion of the species of the Rhodniini tribe. This information is of great importance because of the biologic role of several species of the tribe in transmission of *T. cruzi*. Thus, it is necessary to conduct further genetic, ecological, morphological, and biogeographical studies of the tribe to provide an integrative approach that can address the current systematic and taxonomic inconsistencies; in turn, these studies will provide the information necessary to develop improved vector control strategies for Chagas disease.

Supplementary Materials: The following are available online at http://www.mdpi.com/1424-2818/12/3/97/s1, Table S1: Table S1_Cytb_Rhodnius_Sequences.

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