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Taxonomic study of the *Phyllosoma* complex and other triatomine (Insecta: Hemiptera: Reduviidae) species of epidemiological importance in the transmission of Chagas disease: Using ITS-2 and mtCytB sequences

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Abstract

The purpose of this work was to clarify the taxonomy and phylogenetic relationship of the *Phyllosoma* complex and other important vectors in Mexico. The internal transcribed spacer 2 (ITS-2) of rDNA and the cytochrome *B* gene of mtDNA (mtCytB) were analyzed for the following species of triatomine: *Triatoma bassolsae*, *T. longipennis*, *T. mazzottii*, *T. mexicana*, *T. pallidipennis*, *T. picturata*, and *T. phyllosoma* belonging to the *Phyllosoma* complex, as well as *T. dimidiata*, *T. rubida*, *T. infestans*, and *Rhodnius prolixus*. The results obtained with the analysis of the ITS-2 sequences showed that the *Phyllosoma* complex species could not be phylogenetically separated, since *T. bassolsae* and *T. pallidipennis*, as well as *T. phyllosoma* and *T. mazzottii* were indistinguishable. In contrast, the mtCytB gene separates each one of these triatomine species. The results support the proximity of all seven species currently included in the *Phyllosoma* complex as well as the exclusion of *T. dimidiata*. For the first time *T. lecticularia* and *T. rubida* were analyzed and were also shown to be related to the *Phyllosoma* complex.

Keywords: *Phyllosoma* complex; Triatomine; Vectors of Chagas disease

1. Introduction

The Triatominae subfamily includes more than 130 species of insects that feed on the blood of vertebrates. In this group are the vectors that transmit the hemoflagellate protozoan *Trypanosoma cruzi*, the causal agent of Chagas disease or American Trypanosomiasis. This protozoan is mainly transmitted to humans and other animals by insects of the genera *Triatoma*, *Panstrongylus*, and *Rhodnius* (WHO, 1991). The genus *Triatoma* is the most widely distributed with approximately 70 species (Panzera et al., 1997).

In Mexico, the presence of a total of 32 species of the Triatominae subfamily has been reported. These species are distributed in seven genera: *Dipetalogaster*, *Eratyrus*, *Paratriatoma*, *Panstrongylus*, *Belminus*, *Triatoma*, and *Rhodnius*. Twenty-eight of these species are endemic, and 23 have been reported to be naturally infected with *T. cruzi* (Zárate and Zárate, 1985). Of these species, the following have been considered of epidemiological importance: *T. barberi* (Zárate and Zárate, 1985; Guzmán-Bracho, 2001), *T. dimidiata* (Vidal-Acosta et al., 2000; Dumontiel et al., 2002),

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T. longipennis, *T. pallidipennis*, *T. picturata* (Martínez-Ibarra, 1992; Cortés-Jiménez et al., 1996), *T. rubida*, and *T. gerstaeckeri* (Paredes et al., 2001).

The species of the *Phyllosoma* complex (*T. phyllosoma*, *T. picturata*, *T. pallidipennis*, *T. longipennis*, *T. mazzottii*, and *T. bassolsae*) are primarily distributed in the central regions of Mexico. Variable percentages of infection with *T. cruzi* have been reported: i.e., from 9.1% to 66.7% in *T. phyllosoma* and 8% to 66% in *T. longipennis* (Ramsey et al., 2000; Vidal-Acosta et al., 2000; Martínez-Ibarra et al., 2001). In the Yucatan peninsula (states of Yucatan, Campeche, and Quintana Roo), *T. dimidiata* is also important due to its wide distribution and predominance in this region, and for the infestation and infection indexes that they present (Guzmán-Marín et al., 1990; Dumontiel et al., 2002).

The classification of these insects is based primarily on morphological characteristics. However, this type of classification offers several problems mainly concerning the species grouped in complexes. Species of the *Triatoma* genus have been morphologically grouped in nine complexes (Lent and Wygodzinsky, 1979) among which the *Phyllosoma* is the most important complex in Mexico. This group presents several classification problems that have not been approached in detail. For example, some species such as *T. brailovsky*, *T. bolivari*, *T. dimidiata*, *T. hegneri*, *T. mexicana*, and *T. ryckmani* have been tentatively included in this group. Nevertheless, these inclusions remain controversial (Lent and Wygodzinsky, 1979; Schofield, 1994; Flores et al., 2001; Bustamante et al., 2004). Recently, it has been proposed that the species of the *Phyllosoma* complex could be transferred to genus *Meccus* (Carcavallo et al., 2000; Galvão et al., 2003). Furthermore, species from the *Rubrofasciata* and *Lecticularia* complexes, in particular *T. rubida* and *T. lecticularia*, are important vectors in northern Mexico and southern United States. However, they have not been analyzed in detail with molecular markers, and their relationships with the *Phyllosoma* complex are not clear.

The phylogenetic relationship between the Triatominae subfamily has been discussed by various authors. The first studies were based only on morphological characters. Some autapomorphic characters have created controversy with respect to the possible monophyletic origin of this group (Lent and Wygodzinsky, 1979). Later biogeographic and ecological information was used to propose a polyphyletic origin of the triatomine (Schofield, 2000). But recently, the use of molecular markers has contributed more data for the understanding of the classifications and the phylogenetic and phylogeographic relationships of this important group of vectors.

Preliminary reports have helped to clarify the phylogeny among several triatomine (Lyman et al., 1999; García et al., 2001; Marcilla et al., 2001; Bargues et al., 2002). These reports mainly analyzed South American species using mitochondrial DNA markers such as the ribosomal subunits 12S and 16S, cytochrome oxidase I and mtCytB, and DNA markers of nuclear origin, such as the internal transcribed spacer 2 (ITS-2).

In contrast, the use of molecular markers for the study of the triatomine distributed in the northern part of the American continent has been limited, and only a few species have been analyzed (Hypsa et al., 2002). It is also interesting to note that in the studies that analyzed ITS-2, it has been possible to identify microsatellite sequences. These can be an additional tool for the classification of the organism (Marcilla et al., 2001). The phylogenetic studies will help to explain population structure, possible existence of cryptic species, and the evolutionary history of these important vectors. The aim of this study is to clarify the taxonomic position of important vector species within their complex, in particular the *Phyllosoma*, *Rubrofasciata*, and *Lecticularia* complexes. Thus, the present study employed two molecular markers, mtCytB and ITS-2, for the taxonomic analysis of triatomine species present in Mexico and southern United States, some of them not previously analyzed by these markers.

2. Materials and methods

2.1. Taxon sampling

All triatomine used in this study were male in the adult stage. They were maintained in laboratory colonies, except for *T. dimidiata*, which was collected in Campeche, Mexico. The specimen identification was based on the Lent and Wygodzinsky (1979). To determine the levels of intraspecific variation, up to four specimens were sequenced per species. Similar specimens to those used in this study are deposited in the collection belonging to Laboratorio de Entomología del Departamento de Parasitología de la Escuela Nacional de Ciencias Biológicas del Instituto Politecnico Nacional. Data on their geographical origin, percentage of TA of the analyzed sequences, and GenBank accession numbers are shown in Table 1.

2.2. DNA extraction

Total genomic DNA was extracted from the six legs of each insect. Briefly, the tissue was frozen with liquid nitrogen and macerated until pulverized. It was resuspended in 1 mL of lysis solution (50 mM Tris–HCl, 50 mM EDTA, pH 8, 50 mM NaCl, 1% SDS and 20 μg/mL RNase) and incubated at 37 °C overnight. The phenol chloroform technique was used to extract DNA (Sambrook et al., 1989). The material was kept at 4 °C until used.

2.3. Oligonucleotides

A previously described polymorphic mtCytB fragment was amplified. This polymorphic sequence has been successfully used to analyze the phylogenetic relationships among triatomine (Lyman et al., 1999). New forward and reverse oligonucleotides used for the amplification of mtCytB were based on the consensus sequences of highly conserved regions for triatomine species reported in

Table 1
Analyzed species from the Triatominae subfamily

Species	Origin	GenBank Accession number[c]		Microsatellite of the ITS-2 sequences	% TA mtCytB/ITS-2
		mtCytB	ITS-2		
Infestans complex[a]					
T. infestans Klug, 1934.	Brazil	DQ118975	AY860387, AY860388	$AT_{(7)}$ T $AT_{(1)}$ AA $AT_{(8)}$ $AT_{(6)}$ T $AT_{(1)}$ AA $AT_{(6)}$	65.5/78.34
Lecticularia complex[a]					
T. lecticularia Stål, 1859.	Nuevo León (Mex)	AY859414	AY860405, AY860406, AY860407	$AT_{(2)}$ TTTTGCAATGT $AT_{(3)}$	63.9 / 77.5
Phyllosoma complex[a]					
T. bassolsae Alejandre-Aguilar, 1999.	Puebla (Mex)	AY859410	AY860394, AY860400, AY860401, AY864002	$AT_{(4)}$ TTT $AT_{(6)}$	64.2 / 77.4
T. dimidiata[b] Latreille, 1811.	Campeche (Mex)	AY859417, AY859418			62.9 / 75.93
T. longipennis Usinger, 1939.	Nayarit (Mex)	AY859412	AY860397, AY860398	$AT_{(4)}$ TTT $AT_{(6)}$	63.9 / 77.14
T. mazzottii Usinger, 1941.	Guerrero (Mex)	AY859421, AY859422	AY860392, AY860393	$AT_{(4)}$ TTT $AT_{(5)}$ $AT_{(4)}$ TTT $AT_{(6)}$	63.9 / 76.93
T. mexicana[b] Herrich-Schaeffer, 1848.	Hidalgo (Mex)	DQ118976			62.8
T. pallidipennis Stål, 1872.	Morelos (Mex)	AY859419, AY859420	AY860395, AY860403	$AT_{(4)}$ TTT $AT_{(6)}$	63.9 / 77.45
T. phyllosoma Burmeister, 1835.	Oaxaca (Mex)	AY859411			62 / 77
T. picturata Usinger, 1939.	Nayarit (Mex)	AY859413	AY860396, AY860399, AY860404	$AT_{(4)}$ TTT $AT_{(6)}$	65.5 / 77.4
Protracta complex[a]					
T. barberi Usinger, 1939.	Oaxaca (Mex)	AY830137			62.9 / 75.9
Prolixus complex[a]					
R. prolixus Stål, 1859.	Chiapas (Mex)	DQ118977	DQ118977	None	55 / 77.64
Rubrofasciata complex[a]					
T. rubida Klug, 1859.	Sonora (Mex)	AY859415, AY859416	AY860389, AY860390, AY860391	$AT_{(3)}$ TTT $AT_{(1)}$ AA $AT_{(4)}$	65.5 / 77.3

[a] Complexes determinates by Lent and Wygodzinsky (1979).
[b] Species tentatively assigned to these complexes.
[c] Species with two or more GenBank accession number correspond to individual variants.

GenBank. The designed forward primer was mtCytBF01 5′-CGAATTAGTTAAATGATTRTGRGG-3′ and the reverse primer was mtCytBR02 5′-TATGCRAATAGGA ARTATCATTC-3′ which amplified one region of approximately 313 bp. The oligonucleotides employed to amplify the ITS-2 sequence were reported by Marcilla et al. (2001).

2.4. DNA amplification, cloning, and sequencing

Approximately 200 ng of genomic DNA was amplified by PCR (polymerase chain reaction). The amplification reaction was performed in 25 μL of the following mixture: 100 pmol of each oligonucleotide, 1× of PCR buffer (8 mM Tris–HCl, pH 8, 20 mM KCl, 1 mM $MgCl_2$), 0.8 mM of dNTPs, and one unit of Platinum *Taq* DNA polymerase (Invitrogen). After the first denaturalization step at 94 °C for 1 min, the amplification parameters of the subsequent 35 cycles were: 94 °C for 1 min (denaturalization), 55 °C for 1 min (annealing), 72 °C for 1 min (extension). The fragments obtained by amplification were subcloned in the cloning vector pCR 2.1 (Invitrogen) and sequenced using the vector primers (T7 promoter and M13 reverse). Sequencing was performed for both chains in the ABI prism sequencer (Model Perkin-Elmer 310).

2.5. Sequence alignment and phylogenetic analysis

Besides the 22 sequences amplified in this work, several accessed mtCytB and ITS-2 sequences were analyzed (see paragraph below). Multiple alignments were performed with the CLUSTAL W program, version 1.8 (Thompson et al., 1994; Hall, 1999). Phylogenetic trees were constructed with Neighbor-Joining (NJ) analysis, using the distance model by Tamura-Nei with the MEGA program, version

1.02 (Kimura, 1980; Kumar et al., 2004) with 1000 bootstrap repetitions. The program Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the appropriate model of molecular evolution. The mtCytB sequences were employed for two phylogenetic analyses, each one with a different evolution model. The first analysis was done with the Hasegawa-Kishino-Yano model with gamma distribution (HKY + G). The second analysis was a combination of the data obtained in this study and the sequences reported in GenBank employing the General Time Reversible with gamma distribution and invariable sites (GTR + G + I). For ITS-2 sequences it was GTR + G. The phylogenetic reconstruction using Bayesian inference was performed with the program MrBayes 3.1.2 (Holder and Lewis, 2003; Huelsenbeck et al., 2001; Ronquis and Huelsenbeck, 2003). The analysis was run for 1 million generations, sampling trees every 1000 generations. The mtCytB data were treated comprising three separate partitions based on codon positions. Trees with scores lower than those at stationery (burn-in) were discarded from the analysis. The trees sampled that reached the stationary phase (after 1,00,000 generations) were collected and used to build a tree majority consensus.

The following mtCytB sequences were taken from GenBank (Lyman et al., 1999): *Dipetalogaster maximus* AF045728; *T. brasiliensis* AY336527; *T. dimidiata* AF301594, AY062152; *T. infestans* AF045721; *T. nitida* AF045723; *T. pallidipennis* AF045724; *T. protracta* AF045727; *T. sanguisuga* AF045725; *T. sordida* AF045730; *Panstrongylus megistus* AF045722; *Psammolestes coreodes* AF045719; *Rhodnius brethesi* AF045714; *R. ecuadoriensis* AF045715; *R. neglectus* AF045716; *R. pallescens* AF045720; *R. pictipes* AF045713; *R. prolixus* AF421339; *R. robustus* AF045717; *Philaenus spumarius* AY630340 (Stewart and Beckenback, 2005), and *Arilus cristatus* AF045729. The accessed ITS-2 sequences (Marcilla et al., 2001) were: *T. barberi* AJ293590; *T. dimidiata* AJ286877, AJ286879; *T. phyllosoma* AJ286881.

3. Results

3.1. Sequence variations

Ten triatomine species were sequenced for ITS-2 and 13 for mtCytB, and all data were deposited in GenBank (Table 1). The size of the amplified mtCytB gene fragment was 313 bp, while for ITS-2 it ranged from 454 to 493 bp in species of the *Triatoma* genus and 703 bp in *R. prolixus*.

Analysis of the variations of the two sequences revealed that the mtCyB gene showed a larger number of variables (44%) and informative sites (38.7%) than ITS-2 (35.7% and 17.4%, respectively). The average percentage of nucleotide A + T composition was 77% for the sequences obtained with ITS-2, while for mtCytB it was 64%.

3.1.1. Identification of microsatellite sequences

Microsatellite sequences were detected within ITS-2 sequences in all species from the *Triatoma* genus analyzed in this study (sixth column of Table 1). The majority of the species pertaining to the *Phyllosoma* complex showed the sequence $AT_{(4)}$ TTT $AT_{(6)}$, with the exception of *T. mazzottii*, in which two microsatellite sequences were identified. In addition, *T. lecticularia*, *T. rubida*, and *T. infestans* showed microsatellites differing in AT repetitions and in the internal section of the microsatellite. In contrast, in *R. prolixus* no microsatellite sequences were found.

3.1.2. Analysis of genetic distances

The genetic distances were calculated according to Tamura-Nei. Species pertaining to the *Phyllosoma* complex showed very small distances between each other with respect to other species. The distances were between 0.002 and 0.017 in the case of ITS-2 and 0.034 and 0.172 in the case of the mtCytB gene fragment (Table 2). For the species included in this complex that have been only studied with morphological markers, *T. bassolsae*, showed a range between 0.002 and 0.017 when compared with the rest of the individuals of the *Phyllosoma* complex with ITS-2 and 0.034 to 0.064 with mtCytB. In the case of *T. mexicana*, it was found from 0.084 to 0.151 with mtCytB (analysis with ITS-2 was not done).

In respect to the specie *T. dimidiata*, the following distances were observed: 0.033–0.046 with ITS-2 and 0.116 – 0.195 with mtCytB. As expected for the species of other complexes analyzed, larger distances were found with respect to the *Phyllosoma* complex (Table 2).

3.2. Phylogenetic analysis

For the phylogenetic analysis two methodologies were used: the Bayesian and the Neighbor-Joining (data not shown). The data obtained by both analyses demonstrated similar topology and established a particular clade for the *Phyllosoma* complex. In the particular case of the Bayesian inference the posterior probability values were 94% for mtCytB sequences and 82% for ITS-2 sequences. In the case of the ITS-2 generated sequences, the following polytomies were observed: *T. bassolsae* with *T. pallidipennis*, and *T. phyllosoma* with *T. mazzottii*. However, this lack of resolution of the tree was not observed for the remaining species, since most could be differentiated in separate branches (*T. longipennis*, *T. picturata*, *T. dimidiata*, *T. lecticularia*, *T. rubida*, *T. infestans*, and *R. prolixus*). With respect to *T. dimidiata*, it was outside the main group of species of the *Phyllosma* complex. On the other hand, using the mtCytB gene sequences, it was possible to identify each of the species analyzed. A strong phylogenetic relationship was observed between *T. bassolsae* and *T. mexicana* with the remaining species of the *Phyllosoma* complex. *T. dimidiata* proved to be outside of the branch that contained all the rest of the *Phyllosoma* species, but it was very close to them. Similar topologies were observed with the two markers regarding species distribution and their phylogenetic relationship to other complexes, i.e. *T. lecticularia* and *T. barberi* were closer to the *Phyllosoma* complex, and

Table 2
Genetic Tamura-Nei distances of triatomine

	1	2	3	4	5	6	7	8	9	10	11	12	13
1.- *T. bassolsae*	—	0.249	0.129–0.134	0.212	0.259	0.149	0.141–0.151	0.148	0.034–0.041	0.126	0.149–0.164	0.199–0.204	0.314
2.- *T. barberi*	0.089–0.098[a]	—	0.236–0.241	0.251	0.248	0.258	0.260–0.270	0.245	0.235–0.239	0.270	0.258–0.265	0.230–0.235	0.328
3.- *T. dimidiata*	0.035–0.041	0.098	—	0.206–0.211	0.253–0.259	0.177–0.182	0.134–0.158	0.195–0.201	0.116–0.138	0.146–0.151	0.144–0.162	0.192–0.201	0.308–0.314
4.- *T. infestans*	0.164–0.177	0.186–0.189	0.176–0.179	—	0.264	0.205	0.193–0.229	0.200	0.194–0.198	0.199	0.200–0.232	0.186–0.190	0.215
5.- *T. lecticularia*	0.078–0.089	0.067–0.73	0.086–0.092	0.180–0.183	—	0.254	0.241–0.256	0.255	0.254–0.259	0.227	0.247–0.264	0.232–0.237	0.274
6.- *T. longipennis*	0.002–0.01	0.092–0.095	0.033–0.035	0.167–0.173	0.075–0.083	—	0.110–0.157	0.133	0.163–0.172	0.154	0.134–0.141	0.187–0.191	0.348
7.- *T. mazzottii*	0.005–0.017	0.098–0.101	0.041–0.046	0.174–0.177	0.083–0.095	0.007–0.015	—	0.084–0.108	0.127–0.141	0.121–0.133	0.124–0.142	0.191–0.241	0.289–0.312
8.- *T. mexicana*	NA	NA	NA	NA	NA	NA	NA	—	0.142–0.151	0.134	0.145–0.151	0.259–0.263	0.365
9.- *T. pallidipennis*	0.002–0.012	0.092	0.033–0.38	0.167–0.170	0.075–0.086	0.002–0.007	0.007–0.017	NA	—	0.118–0.126	0.142–0.169	0.196–0.205	0.286–0.297
10.- *T. phyllosoma*	0.010–0.012	0.098	0.041	0.174–0.177	0.083–0.089	0.007–0.010	0.000–0.005	NA	0.007–0.012	—	0.156–0.161	0.225–0.230	0.276
11.- *T. picturata*	0.002–0.010	0.089–0.092	0.033–0.35	0.164–0.170	0.075–0.083	0.000–0.005	0.007–0.012	NA	0.002–0.005	0.007–0.010	—	0.186–0.191	0.254–0.269
12.- *T. rubida*	0.107–0.112	0.132	0.127	0.224–0.228	0.127–0.130	0.109	0.112–0.118	NA	0.109–0.110	0.112	0.107–0.109	—	0.292–0.297
13.- *R. prolixus*	0.394–0.399	0.409	0.424–0.28	0.481–0.486	0.419–0.437	0.403–0.404	0.390–0.394	NA	0.403–0.404	0.394	0.339–0.403	0.439	—

Genetic Tamura-Nei distances of triatomine using two molecular markers, above the diagonal the distances calculated with mtCytB and below the diagonal the distances calculated with ITS-2.
NA, not analyzed.
[a] Two values indicate the range found between the different individual analyzed.

T. rubida was more distant. *T. infestans* and *R. prolixus*, of Central and South American distribution were even farther (Fig. 1).

A third phylogenetic tree was built with the triatomine sequences reported in this work and the mtCytB gene sequences for the species reported in GenBank. In this analysis, very clear groups can be identified, such as the *Rhodnius* and the *Triatoma* genera. Moreover, inside the *Triatoma* group, the clear separation of the two groups was confirmed. The first group corresponded to the North and Central American triatomine including the complexes: *Protracta*, *Lecticularia*, *Phyllosoma*, *Rubrofasciata*, and the genus *D. maximus*. A second group, corresponding to the South American triatomine, was also observed (Fig. 2). It was again observed in this tree that *T. dimidiata* is outside the *Phyllosoma* complex but in close relationship with it. Furthermore, *T. lecticularia* and *T. barberi* were localized in independent clades with some relationship with the *Phyllosoma* complex. Finally, *T. rubida* was localized out of the main clade of *Phyllosoma*, *Protracta*, and *Lecticularia* complexes.

4. Discussion

The present taxonomic analysis of different species from the Triatominae subfamily endemic to Mexico and Central America, includes some species that had not been previously studied by ITS-2 or mtCytB. Among these species are the following: *T. bassolsae*, a recently described species (Alejandre-Aguilar et al., 1999), which, by its morphological characteristics and isoenzymatic marker analysis, was included in the *Phyllosoma* complex (Martínez et al., 2005); *T. mexicana* reported in several states of Mexico, for example, Guanajuato, Hidalgo, Querétaro and San Luis Potosí, and which has been consistently included in the *Phyllosoma* complex only by its morphological characteristics (Lent and Wygodzinsky, 1979; Bustamante et al., 2004); also *T. lecticularia*, which belongs to the *Lecticularia* complex and *T. rubida*, to the *Rubrofasciata* complex, that are epidemiologically important species of the northern regions of Mexico (Zárate and Zárate, 1985; Martínez-Ibarra, 1992; Paredes et al., 2001).

Very few studies have been conducted in which the North American species of triatomine have been analyzed. Only three studies showed partial information about a few individuals of the triatomine groups from Mexico and the United States (Lyman et al., 1999; Marcilla et al., 2001; Hypsa et al., 2002). More recently, an isoenzymatic analysis of the *Phyllosoma* complex was published (Martínez et al., 2005). In the present study several groups are analyzed, allowing a better understanding of the phylogenetic relationships between the triatomine groups.

With ITS-2 sequences, the presence of microsatellites in different species was found. Microsatellite could be an auxiliary tool to determine the complex or group to which a given species belongs. The present analysis showed specific sequences of microsatellites for the *Phyllosoma* complex ($AT_{(4)}$ TTT $AT_{(6)}$), although specimens of the *T. mazzottii*

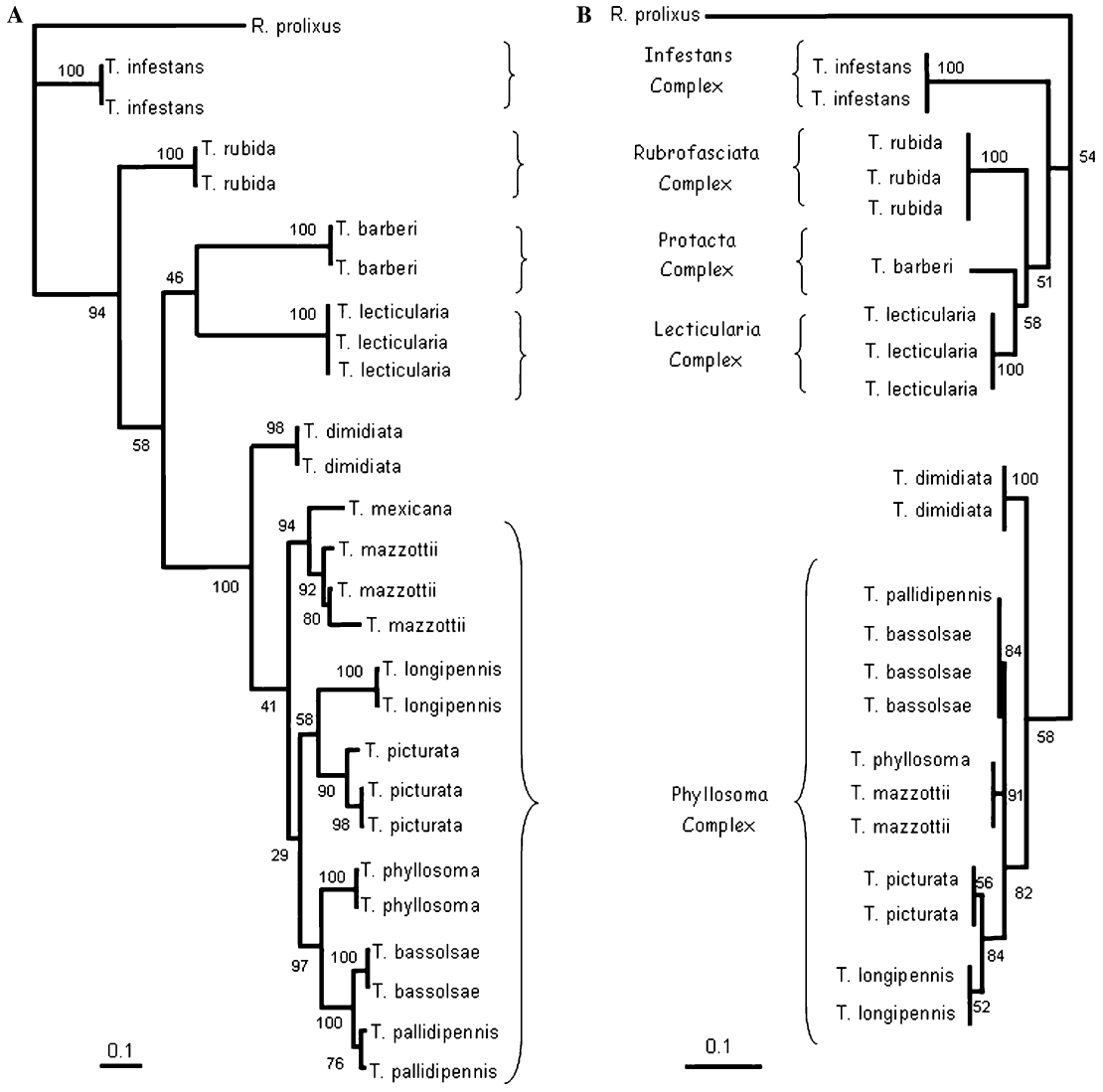

Fig. 1. Bayesian phylogenetic trees of triatomine insects with mtCytB and ITS-2 sequences. (A) Majority rule phylogram resulting from Bayesian analysis with the mtCytB data set under the HKY + G model of evolution; (B) majority rule phylogram resulting from Bayesian analysis with the ITS-2 data set under the GTR + G model of evolution. The values of the nodes indicate the percentage of the posterior probability.

species showed two types of variants. One of them was equal to the microsatellite sequence of the rest of the species of the *Phyllosoma* complex and the other with less repetitions of AT ($AT_{(4)}$ TTT $AT_{(5)}$). This could constitute an intraspecific variation of these species. Species that had not been analyzed before showed microsatellite sequences, which could help to identify the complex to which they belong, i.e., *T. bassolsae* to the *Phyllosoma* complex, *T. lecticularia* to the *Lecticularia* group and *T. rubida* to the *Rubrofasciata* group.

Rodnius does not present microsatellite. This finding, combined with the differences found in nucleotide sequences and in the ITS-2 length, support the idea of the probable polyphyletic origin of the Rhodninii and Triatomini tribes, (Monteiro et al., 2001; Marcilla et al., 2001, 2002; Hypsa et al., 2002).

Inter-specific variations at sequence level using the two mentioned markers inside the *Phyllosoma* complex species were small. This suggests a close relationship between them, showing Tamura-Nei distance values smaller than 0.017 for ITS-2 and 0.172 for mtCyt B.

The trees obtained separately for either ITS-2 or mtCytB exhibited the same topology, i.e., the distribution of the different groups of species was similar. Analysis of these trees distinguish a clade that separates the *Phyllosoma* complex from the other complexes. However, not all the species within the *Phyllosoma* complex could be phylogenetically distinguished with the use of ITS-2, for example *T. pallidipennis* and *T. bassolsae* as well as *T. mazzottii* and *T. phyllosoma*. In contrast, with mtCytB, the species of this complex were clearly differentiated into specific clades, although very short branches separate them. This, and the minimal differences in nucleotides, suggest that the *Phyllosoma* complex is in an early process of divergence. This can be the reason why classification with morphological criteria or only one molecular marker is difficult.

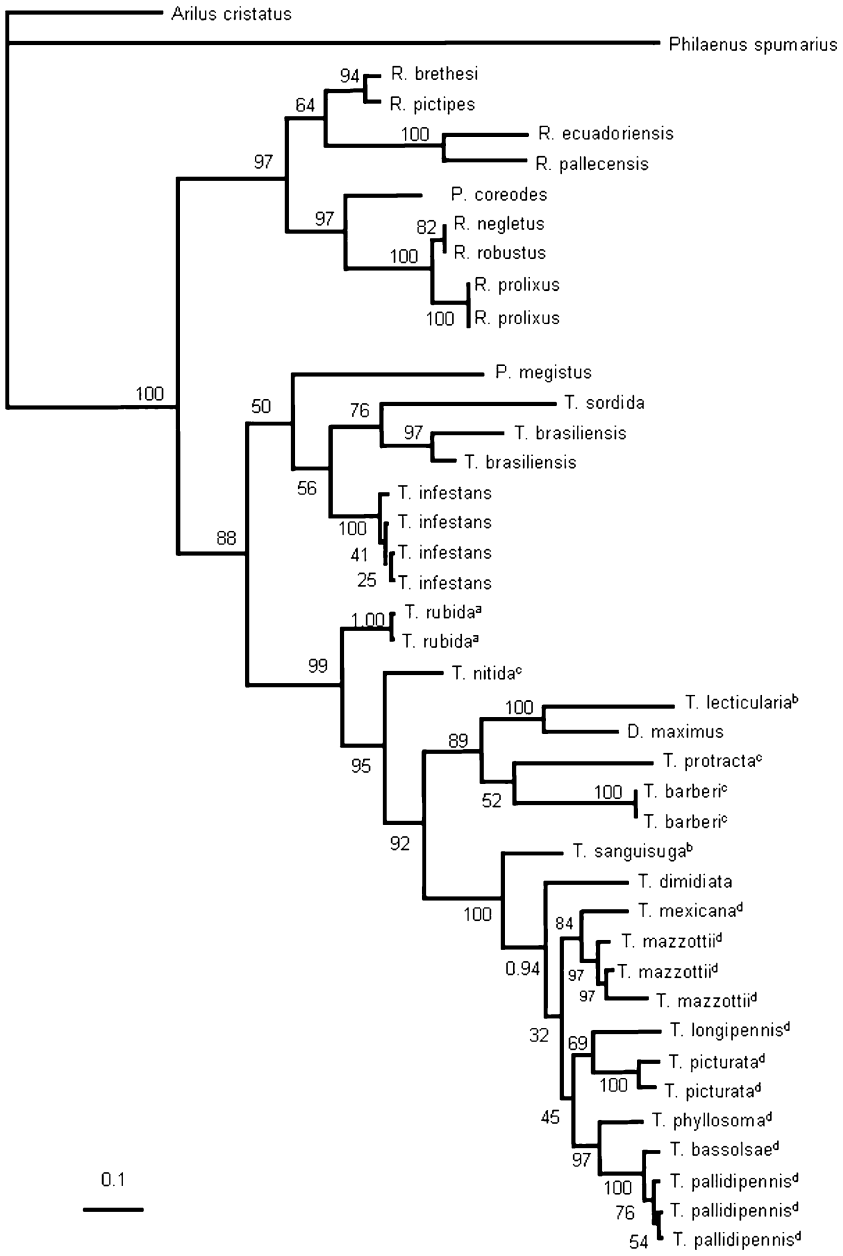

Fig. 2. Bayesian phylogenetic tree of triatomine insects using the sequences obtained in this work and sequences retrieved from GenBank for the mtCytB sequences. (a) *Rubrofasciata* complex; (b) *Lecticularia* complex; (c) *Protracta* complex; (d) *Phyllosoma* complex. Majority rule phylogram resulting from Bayesian analysis with the mtCytB data set under the GTR + G + I model of evolution, the values of the nodes indicate the percentage of the posterior probability.

Our results with both markers (ITS-2 and mtCytB) and the studies that indicated the presence of fertile hybrids from some crosses between species from the *Phyllosoma* complex (Mazzotti and Osorio, 1940) support the idea, suggested by Usinger (1944), of the subspecies level of *Phyllosoma* complex members.

On the other hand, the close genetic distances and phylogenetic relationship observed with the two markers in the species *T. bassolsae* and *T. mexicana* with the *Phyllosoma* complex, corroborate the inclusion of them in this complex. It was also found that *T. dimidiata* is in close relationship with this complex but probably in process of divergence.

When the general mtCytB tree was analyzed in this study with a more representative number of species from North America (Fig. 2), the triatomine group showed a separation in two main groups, the first corresponding to the North and Central American triatomine and the second group corresponding to the South American triatomine. This scenario supports the hypothesis of genetic divergence within the *Triatoma* genus, (Marcilla et al., 2001; Hypsa et al., 2002).

For the species that belong to other complexes such as *T. barberi*, *T. lecticularia*, *T. rubida*, *T. infestans*, and *R. prolixus*, the present study confirms their grouping and evolu-

tionary relationship, i.e., *T. barberi* is closely related to *T. protracta*, and both belong to the *Protracta* complex.

In contrast, our study suggests that the species *T. nitida*, that is also included within the *Protracta* complex, was more related to *T. rubida* (*Rubrofasciata* complex). Also, the species *T. sanguisuga* and *T. lecticularia*, both from the *Lecticularia* complex, are in separate clades. Our study indicates that the species *T. lecticularia* is more related to *D. maximus*, *and T. sanguisuga* was close to *T. dimidiata* and the species of the *Phyllosoma* complex. From our results, the group of *T. protracta* (including the *Protracta* complex and the *Lecticularia* complex), as well as, *D. maximus* are closely related to the *Phyllosoma* complex (Fig. 2). These results show the discrepancies found between morphological and genetic analysis and support the paraphyletic origin of this group.

It was possible to observe that *R. prolixus*, of Mexican origin, presented 100% similarity with *R. prolixus* from South America, and was grouped with the remaining *Prolixus* complex species reported in GenBank.

The classification obtained by this analysis agrees with that obtained by the isoenzymatic analysis of the same species (Martínez et al., 2005), in which *T. lecticularia* is the species most closely related to the *Phyllosoma* complex, followed by *T. rubida*, and then *T. infestans*. These results should be confirmed by further analysis with other markers to clarify the relationship between *T. lecticularia* and the *Phyllosoma* complex, since there is little information about the *Lecticularia* complex.

It can be concluded that ITS-2 is an effective marker to differentiate triatomine species at a supraspecific level; however, it is unable to determine the phylogenetic relationships within the *Phyllosoma* complex, whereas mtCytB showed greater accuracy at this level.

In summary, this work suggests the following: (1) the possibility that the groups of the *Phyllosoma* complex are subspecies rather than species; (2) the exclusion of *T. dimidiata* from the *Phyllosoma* complex, as well as, the inclusion of *T. bassolsae* and *T. mexicana* within this group; (3) the need for a reevaluation of the grouping category proposed by Lent and Wygodzinsky (1979), in particular the *Protracta* group; and (4) that mtCytB gives better resolution in the identification of cryptic species than the ITS-2 marker.

Since each gene tells a different evolutionary history it would be worthwhile to perform a general analysis using various genes with different origins, thus obtaining a more precise evolutionary history of these important groups of vector transmitters of one of the major parasitic diseases present on the American continent.

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