

Genetic Variation of North American Triatomines (Insecta: Hemiptera: Reduviidae): Initial Divergence between Species and Populations of Chagas Disease Vector

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Abstract. The triatomines vectors of *Trypanosoma cruzi* are principal factors in acquiring Chagas disease. For this reason, increased knowledge of domestic transmission of *T. cruzi* and control of its insect vectors is necessary. To contribute to genetic knowledge of North America Triatominae species, we studied genetic variations and conducted phylogenetic analysis of different triatomines species of epidemiologic importance. Our analysis showed high genetic variations between different geographic populations of *Triatoma mexicana*, *Meccus longipennis*, *M. mazzottii*, *M. picturatus*, and *T. dimidiata* species, suggested initial divergence, hybridation, or classifications problems. In contrast, *T. gerstaeckeri*, *T. bolivari*, and *M. pallidipennis* populations showed few genetics variations. Analysis using cytochrome B and internal transcribed spacer 2 gene sequences indicated that *T. bolivari* is closely related to the *Rubrofasciata* complex and not to *T. dimidiata*. *Triatoma brailovskyi* and *T. gerstaeckeri* showed a close relationship with *Dimidiata* and *Phyllosoma* complexes.

INTRODUCTION

The subfamily Triatominae includes species that are important vectors in the transmission of the protozoan parasite *Trypanosoma cruzi*, the causal agent of Chagas disease. Although more than 33 species of triatomines have been reported in Mexico,^{1,2} epidemiologic, biologic, and genetic data for these insects in the literature is scarce. These data are crucial for establishing an appropriate strategy of Chagas disease control. Currently, vector control is the only way to control this infection because of limited efficacy of treatment during the chronic infectious phase and the absence of a vaccine.^{3,4}

Phylogenetic and genetic relationships between *Triatoma bolivari*, *T. brailovskyi*, *T. mexicana*, *T. gerstaeckeri*, *T. recurva*, and *T. protracta* have not been documented. *Triatoma brailovskyi*, *T. bolivari*, and *T. mexicana* are species that are endemic to Mexico and considered as part of the *Phyllosoma* complex species (*Meccus bassolsae*, *M. longipennis*, *M. mazzottii*, *M. pallidipennis*, *M. picturatus*, and *M. phyllosomus*).

Triatoma brailovskyi and *T. bolivari* have been found in the states of Colima, Nayarit, Oaxaca, and Jalisco.^{5–8} Their epidemiologic importance as potential vectors of *T. cruzi* is unknown. *Triatoma mexicana* has been found in the states of Guanajuato, Hidalgo, Queretaro, and San Luis Potosi, where the rate of *T. cruzi* infections is less than 4%.^{1,9,10} *Triatoma gerstaeckeri* has been found in the states of Chihuahua, Coahuila, Nuevo Leon, San Luis Potosi, Sonora, Hidalgo, Tamaulipas, and Veracruz^{2,10–13} and in the United States in Texas.¹⁴ In some of these states, this species was associated with human houses and *T. cruzi* infections rates of 0% to 63%.^{10,15–18} *Triatoma recurva* has been reported in the states of Baja California, Baja California Sur, Chihuahua, Guerrero, Nayarit, Sinaloa and Sonora, and a high level of

T. cruzi infection greater than 90%,^{1,19} and has been reported in the United States in Arizona. *Triatoma protracta* has been reported in the states of Baja California Norte, Chihuahua, Coahuila, Durango, Nuevo Leon, Tamaulipas, Sinaloa, Sonora, San Luis Potosi, and Zacatecas and in several states in the southwestern United States (Arizona, California, Nevada, Texas, New Mexico, and Utah), with *T. cruzi* infections less than 19%.^{1,9,10,20,21}

Triatoma brailovskyi has been proposed to be a member of a specific complex *Dimidiata* by using morphologic marker analysis; this complex includes *T. dimidiata*, *T. hegneri*, *T. ryckmani* and *T. gomeznunezi*.²² On the basis of internal transcribed spacer 2 (ITS-2) sequences, the species *T. gerstaeckeri* and *T. mexicana* have been included in the *Phyllosoma* complex, and *T. bolivari* has been included in the *Rubrofasciata* complex.²³ *Triatoma protracta* is included in the *Protracta* complex, and *T. recurva* was proposed to be included in the *Phyllosoma* complex by using cytochrome B (*cytB*) sequences, although this last species reportedly shares intermediate characteristics with the genus *Dipetalogaster*.^{22,24}

In North America, genetic population studies of triatomines by using nucleotide sequences are scarce. Most of this research focuses on *T. dimidiata*.^{25–27} In some cases, large hybrid zones exist between sympatric sibling species.²⁸ *Triatoma recurva* and *T. rubida* showed significant genetic differences when *cytB* and cytochrome oxidase I sequences were compared in populations from widely separated geographic localities.²⁴ With respect to the *Phyllosoma* complex species, the principal studies have focused on taxonomic relationships. However, genetic population analyses are scarce. These types of studies are important because taxonomic status of these species would provide useful information.

The present study used *cytB* and ITS-2 sequences and taxonomic analysis to examine genetic variation between the *T. brailovskyi*, *T. bolivari*, *T. gerstaeckeri*, *T. mexicana*, *T. protracta*, and *T. recurva*, and with species of their respective complexes (*Phyllosoma* and *Protracta*) and other sequences of triatomines of epidemiologic importance in North America.

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MATERIALS AND METHODS

Taxon sampling. Adults of the species *T. bolivari*, *T. brailovskyi*, *T. gerstaeckeri*, *T. mexicana*, *T. protracta*, and *T. recurva* were collected in different states in Mexico (Table 1). Specimens were identified morphologically by using keys of Lent and Wygodzinsky,⁹ Martínez and others,⁵ and Carcavallo and others.⁶ Similar specimens are deposited in the collections of the Laboratorio de Tripanosomiasis of Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México and Centro Universitario del Sur, Universidad de Guadalajara.

DNA extraction, amplification, cloning, and sequencing.

Genomic DNA was extracted from one leg of each insect by using the extraction method of Martínez and others.⁵ The oligonucleotides used for the amplification of *cytB* and ITS-2 sequences have been described.^{29,30} Fragments obtained by amplification were subcloned into the cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced by using universal vector primers (T7 promoter and M13 reverse). Sequencing for both chains was performed by using the 310 ABI prism sequencer (Applied Biosystems, Foster City, CA).

Population genetic analysis. The percentage of polymorphic sites, nucleotide diversity (π), and haplotype polymorphism

TABLE 1
GenBank accession numbers and genetic diversity of species of triatomines analyzed, Mexico*

Species	Origin	<i>cytB</i> (no.)	ITS-2 (no.)
<i>Meccus bassolsae</i> (<i>Triatoma bassolsae</i>)	Puebla	AY859410 ²⁹ (1)	AY860394, ²⁹ AY860400-2 ³⁰ AM286724 ²³ (5)
<i>T. barberi</i>	Oaxaca	AY830137 ²⁹ (1)	AJ293590 ³⁰ (1)
<i>T. bolivari</i>	Jalisco	JQ282718-9† (2)	JQ282701-3† (3)
	Oaxaca		AM286725 ²³ (1)
<i>T. brailovskyi</i>	Jalisco	JQ282720-2† (3)	JQ282704-6† (3)
		$\pi = 0.006$, $h = 2$, $\theta = 0.67$, $S = 99\%$	$\pi = 0$, $h = 1$, $\theta = 0$, $S = 100\%$
<i>T. gerstaeckeri</i>	Chihuahua	JQ282723-5† (3)	JQ282707-9† (3)
	San Luis Potosi		AM286734 ²³ (1)
<i>T. lecticularia</i>	Nuevo Leon	AY859414 ²⁹ (1)	AY860405-7 ²⁹ (3)
		$\pi = 0.004$, $h = 3$, $\theta = 1$, $S = 99.4\%$	$\pi = 0.007$, $h = 3$, $\theta = 1$, $S = 99\%$
<i>Meccus longipennis</i> (<i>Triatoma longipennis</i>)	Nayarit	AY859412 ²⁹ (1)	AY860397-8 ²⁹ (2)
	Zacatecas	DQ198815 ²⁴ (1)	AJ286883 ⁴⁵ (1)
<i>T. mexicana</i>	Guanajuato	JQ282726-7† (2)	JQ282710-2† (3)
		$\pi = 0.058$, $h = 3$, $\theta = 1$, $S = 92\%$	$\pi = 0.02$, $h = 3$, $\theta = 0.8$, $S = 96\%$
	Hidalgo	DQ118976 ²⁹ (1)	AM286728 ²³ (1)
<i>T. nitida</i>	Guatemala	AF045723 ³¹ (1)	AM286733 ²³ (1)
<i>T. protracta</i>	? Sonora	AF045727 ³¹ (1)	JQ282713† (1)
		$\pi = 0.038$, $h = 3$, $\theta = 1$, $S = 94\%$	$\pi = 0.02$, $h = 2$, $\theta = 0.7$, $S = 99\%$
	Chihuahua	JQ282728-9† (2)	JQ282714-5† (2)
<i>M. pallidipennis</i> (<i>Triatoma pallidipennis</i>)	? Morelos	AF045724 ³¹ (1), AY859419-20, ²⁹ DQ198814 ²⁴ (3)	AJ286882, ⁴⁵ AM286729-30, ²³ AY860395, ²⁹ AY860403 ²⁹ (5)
		$\pi = 0.015$, $h = 4$, $\theta = 1$, $S = 97\%$, $T = -0.15$	$\pi = 0.003$, $h = 3$, $\theta = 0.8$, $S = 99\%$
<i>M. picturatus</i> (<i>T. picturata</i>)	Nayarit	AY859413 ²⁹ (1)	AY860396, ²⁹ AY860399, ³⁰ AY860404 ²⁹ (3)
		$\pi = 0.14$, $h = 2$, $\theta = 1$, $S = 86\%$	$\pi = 0.002$, $h = 3$, $\theta = 0.8$, $S = 99\%$
	Jalisco	DQ198817 ²⁴ (1)	AJ286884 ⁴⁶ (1)
<i>M. phyllosomus</i> (<i>Triatoma phyllosoma</i>)	Oaxaca	AY859411, ²⁹ DQ198818, ²⁴ HQ185139-64 ⁴⁶ (28)	AJ286881, ⁴⁵ AY860403, ²⁹ HQ185165-90 ⁴⁶ (28)
		$\pi = 0.016$, $h = 10$, $\theta = 0.6$, $S = 94\%$, $T = -1.7$	$\pi = 0.004$, $h = 11$, $\theta = 0.7$, $S = 96\%$, $T = -2.4$
<i>T. mazzottii</i> (<i>Meccus mazzottii</i>)	Guerrero	AY859421-22 ²⁹ (2),	AY860392-3 ²⁹ (2)
		$\pi = 0.09$, $h = 3$, $\theta = 1$, $S = 87\%$	$\pi = 0.003$, $h = 2$, $\theta = 0.7$, $S = 99.5\%$
	Oaxaca	DQ198816 ²⁴ (1)	AJ286885 ⁴⁵ (1)
<i>T. dimidiata</i>	Several places	AY859417-8, ²⁹ AF301594, ⁴⁴ AY062149-64 ^b , FJ197154-9, ²⁵ FN641804-18 ⁴² (40)	AJ286875-80, ⁴⁵ AM286693-723, ²³ AY860408-17, ²⁶ DQ871354-56, ⁴³ EF383122-29, ²⁶ FJ197146-53, ²⁵ GQ214508-13 ⁴² (72)
		$\pi = 0.08$, $h = 35$, $\theta = 0.94$, $S = 87\%$, $FST_{A-2} = 0.89$, $FST_{B-2} = 0.81$, $FST_{A-B} = 0.46$, $\pi_1 = 0.04$, $\pi_2 = 0.01$, $\pi_A = 0.02$, $\pi_B = 0.04$.	$\pi = 0.02$, $h = 25$, $\theta = 0.9$, $S = 93\%$, $FST_{A-2} = 0.84$, $FST_{B-2} = 0.85$, $FST_{A-B} = 0.47$, $\pi_1 = 0.008$, $\pi_2 = 0.002$, $\pi_A = 0.004$, $\pi_B = 0.005$
<i>T. recurva</i>	Sonora	DQ198812, ²⁴ JQ282730-1†	JQ282716-7† (2)
		$\pi = 0.009$, $h = 3$, $\theta = 1$, $S = 99\%$, $T = 0.04$	$\pi = 0$, $h = 1$, $\theta = 0$, $S = 100\%$
	Arizona, USA	DQ198813 ²⁴ (4)	
<i>T. rubida</i>	Nayarit	DQ198810, ²⁴	AM286735 ²³ (1)
	Sonora	AY859415-16 ³⁰ (3)	AY860389-91 ²⁹ (3)
	Baja California Sur	DQ198811 ²⁴ (1)	
	Arizona, USA	DQ198808-09 ²⁴ (1)	
		$\pi = 0.05$, $h = 5$, $\theta = 1$, $S = 89\%$, $T = 0.04$	$\pi = 0$, $h = 1$, $\theta = 0$, $S = 100\%$

*Numbers in superscript indicate article that referred to the GenBank accession no. of the sequence analyzed in this study. *cytB* = cytochrome B; ITS-2 = internal transcribed spacer 2; ? = unknown location; π = nucleotide diversity; h = no. haplotypes; θ = haplotype diversity; S = similarity percentage; T = Tajima's D test; FST_{1-2} = coancestry coefficient (CC) between group 1 and 2; FST_{A-B} = CC between subgroup A and group 2; FST_{B-2} = CC between subgroup B and group 2; π_1 = nucleotide diversity group 1; π_2 = ND group 2; π_A = ND subgroup A; π_B = ND subgroup B. †Sequences obtained in this study.

(θ) were calculated within populations of the same species. When the samples size was sufficient, the coancestry coefficient statistics (FST) and Tajima's D test were evaluated between populations. All tests were estimated using DnaSP version 4 software (www.ub.edu/dnasp/).

Sequence alignment and phylogenetic analysis. Multiple alignments were made by using MEGA version 5 software (www.megasoftware.net/). Modeltest version 3.7 software www.molecularrevolution.org/software/phylogenetics/modeltest was used to determine the appropriate model of molecular evolution. For *cytB* sequences, the best model was the Hasegawa Kishino Yano model with gamma distribution and invariable sites. For ITS-2 sequences and both markers (*cytB* and ITS-2), the best model was the general time reversible model with gamma distribution. The phylogenetic reconstruction using Bayesian inference was performed with the Mr. Bayes 3.1.2 program (<http://mrbayes.sourceforge.net/>). The analysis ran for 10 million generations, sampling trees every 100 generations. Trees with scores lower than those at stationery (burn-in) were discarded from the analysis. The sampled trees that reached the stationary phase were collected and used to build majority consensus trees. Other species used in this study were *Dipetalogaster maximus* AF045728; *T. infestans* AF045721, DQ118975; *T. sanguisuga* AF045725; *Rhodnius prolixus* AF045718, DQ118977 with *cytB* and *D. maximus* AJ286887; *T. infestans* AY860387-8; and *R. prolixus* DQ118978 with ITS-2 sequences.²⁹⁻³¹

Phylogenetic inferences based on RNA secondary structure. RNA secondary structure of ITS-2 sequences from *M. bassolsae*, *T. bolivari*, *T. brailovskyi*, *T. dimidiata*, *T. gerstaeckeri*, *M. mazzottii*, *T. mexicana*, *M. pallidipennis*, *M. phyllosomus*, and *T. recurva* were predicted by using consensus alignment and singles sequences in three different web software: RNAalifold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi>), RNA Folding form (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>), and RNA secondary structure prediction (www.genebee.msu.edu/services/rna2_reduced.html). Structural domains in ITS-2 initially were predicted according to the frequency of bases and mutation information in the multiple alignments and then identified based on the structure logo program RNA Structure Logo (www.cbs.dtu.dk/~gorodkin/appl/slogo.html).^{32,33}

RESULTS

Sequence analysis. Thirty-one sequences with both markers were obtained and deposited in GenBank (Table 1). The size of the amplified *cytB* gene fragment was 313 basepairs; for ITS-2, it ranged from 454 to 584 basepairs. The *cytB* sequences showed a larger number of variables (49.8%) and informative sites (43.5%) than ITS-2 (39.9% and 34.8%, respectively). As expected for a *cytB* protein-coding gene, the third codon positions had the most variation, (75%), followed by the first (19.6%) and second (5.4%) positions.

Intraspecific variation in *cytB* of the species in the *Phyllosoma* complex (*T. mexicana*, *M. mazzottii*, *M. picturatus*, *M. longipennis*, and *M. pallidipennis*) from different geographic areas was analyzed. Sequences of *T. mexicana* from Hidalgo and Guanajuato showed 92% similarity and $\pi = 0.058$ between them; *M. mazzottii* from Guerrero and Oaxaca showed 87% similarity and $\pi = 0.09$; *M. picturatus* from Nayarit and Jalisco showed 86% similarity (43 of 313)

and $\pi = 0.14$; *M. longipennis* from Nayarit and Zacatecas showed 91% similarity (29 of 313) and $\pi = 0.093$; and *M. pallidipennis* from Jalisco and other regions in Morelos showed 97% similarity (9 of 313) and $\pi = 0.015$. *Triatoma dimidiata* had two groups (groups 1 and 2), and group 1 had two subgroups (subgroups A and B) (Figure 1). The percentage of nucleotides variability between subgroups was 85% (47 of 313) with $FST_{A-B} = 0.46$, and the variation between subgroups A and B compared with group 2 was 79% (67 of 313) and $FST_{A-2} = 0.89$ and 80% (64 of 313) and $FST_{B-2} = 0.81$, respectively. For *T. protracta* from Chihuahua and unknown regions of Mexico, we observed 94% similarity (18 of 313) and $\pi = 0.038$.

Using the ITS-2 marker, we found that *T. bolivari*, *T. gerstaeckeri*, *M. pallidipennis*, *M. picturatus*, *M. mazzottii*, *T. recurva*, and *T. rubida* populations showed high values of similarity ($\geq 99\%$) and low diversity values ($\pi \leq 0.005$, $\theta \geq 0.7$), in contrast with populations of *T. protracta* and *T. mexicana*, which showed high genetic differentiation (Table 1). As with *cytB*, *T. dimidiata* showed a similar grouping. The percentage of variable nucleotides between the subgroups (A and B) was 98.5% (7 of 459) and $FST_{A-B} = 0.47$. In contrast with the second group, which was integrated with 21 sequences, we observed 94% similarity (28 of 459) and $FST_{A-2} = 0.84$ and 97.7% similarity (11 of 459) with $FST_{B-2} = 0.85$ between subgroups A and B, respectively.

Microsatellite sequences. *Triatoma brailovskyi* showed microsatellites identical to those of some species of the *Phyllosoma* complex ((AT)₄TTT(AT)₅). For *T. bolivari*, their microsatellites ((AT)₄TTTAAA(AT)₅) were more similar to those of *T. rubida* ((AT)₄TTTATT(AT)₅), a species that belongs to the *Rubrofasciata* complex. *Triatoma mexicana* had three microsatellites, two from the samples collected in Guanajuato ((AT)₃TTTATA(AT)₄ and (AT)₅T(AT)₆) and one from Hidalgo ((AT)₅TTT(AT)₆). *Triatoma protracta* had two microsatellites sequences, one from Sonora ((AT)₃TTTTATAA(AT)₄) and one from Chihuahua ((AT)₃TTTATAA(AT)₄). *T. recurva* microsatellites sequences (AT)₄TTTATT(AT)₅ were more similar to *T. bolivari* microsatellites; only two mutations were observed.

Phylogenetic analysis. Similarity between phylogenetic reconstructions using Bayesian inference with the *cytB* (Figure 1) and ITS-2 sequences (Figure 2) was observed. Phylogenetic analyses of *Phyllosoma* and *Dimidiata* complex species and *T. brailovskyi*, *T. gerstaeckeri*, and *T. recurva* showed a strong clade with 0.99 of posterior probability. In this major clade, there are four subgroups: *T. dimidiata* group 1 (with two subgroups), *T. dimidiata* group 2, and *Phyllosoma* complex groups 1 and 2.

Triatoma bolivari showed a clear relationship with *T. rubida*, with posterior probability values of 77% for *cytB* and 100% for ITS-2. *T. protracta* and *T. barberi* showed high similarity with *T. bolivari*.

To increase support for clades of the separate trees and to obtain a better evolutionary correlation, a third phylogenetic tree was built to align *cytB* and ITS-2 for each species. Only sequences that had similar numbers of basepairs and geographic origins for both markers were used. A total of 1,053 characteristics were analyzed, with approximately 20% informative sites. *Meccus longipennis*, *T. mexicana*, and *M. picturatus* from different geographic origins were distributed into separate clades. Among the other species,

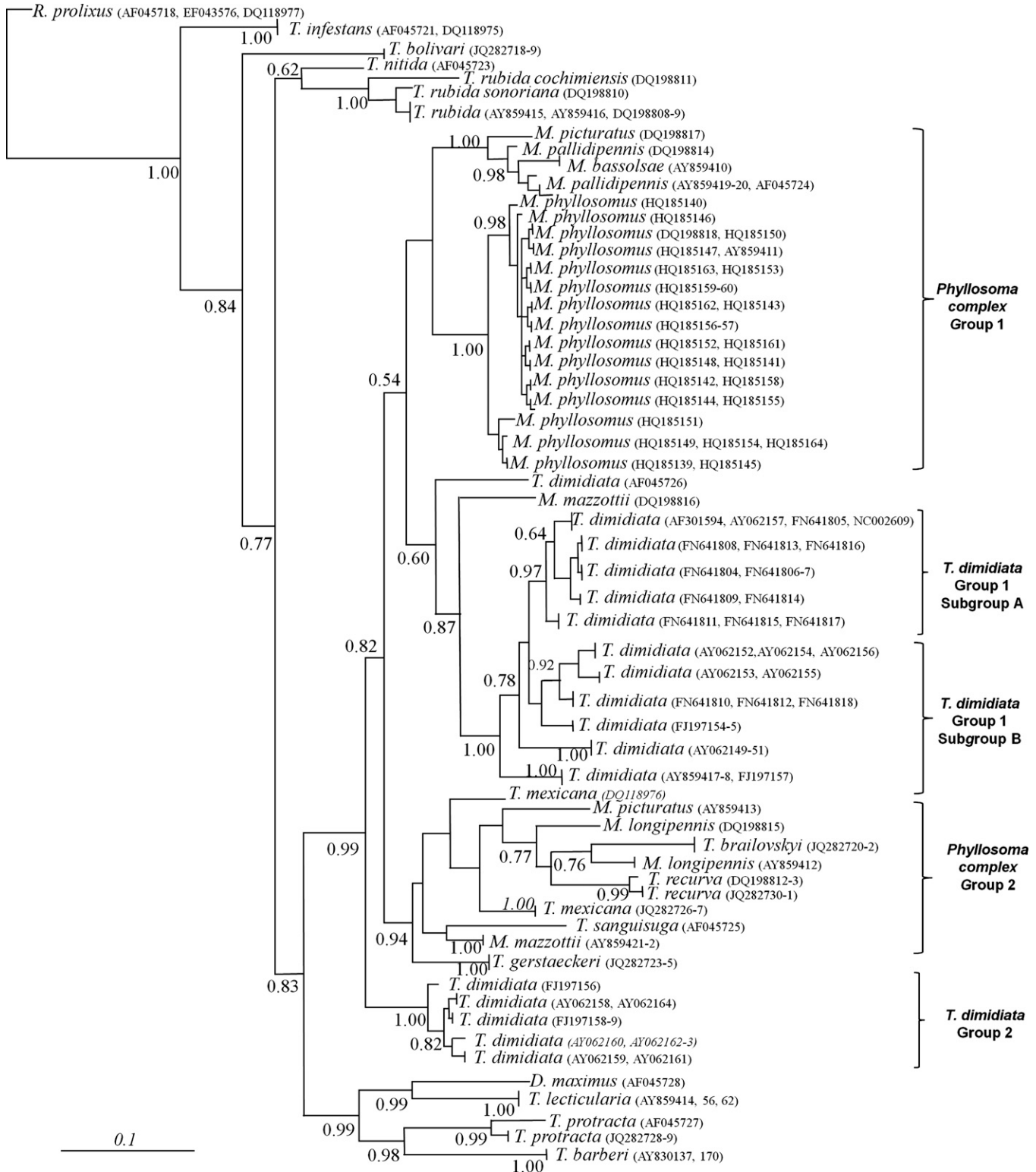


FIGURE 1. Bayesian phylogenetic trees of triatomine insects with cytochrome B (*cytB*) sequences. Majority rule phylogram resulting from Bayesian analysis with the *cytB* data set under the Hasegawa, Kishino, and Yano + gamma distribution + proportion invariate model of evolution. Scale bar indicates nucleotide substitutions per site.

the genetic relationships were similar to the topology for particular trees (Figure 3).

RNA secondary structure. The ITS-2 RNA secondary structures obtained showed the lowest free energy structures, with values less than -184 kcal/mol denoting highly stable models.

Four preserved domains were identified (I, II, III, and IV) by using consensus alignment and logo structure. All analyzed sequences had four domains and some modifications between hairpins and bulges in each domain. *Meccus bassolsae*, *M. longipennis*, *M. mazzottii*, *M. pallidipennis*, *M. phyllosomus*,

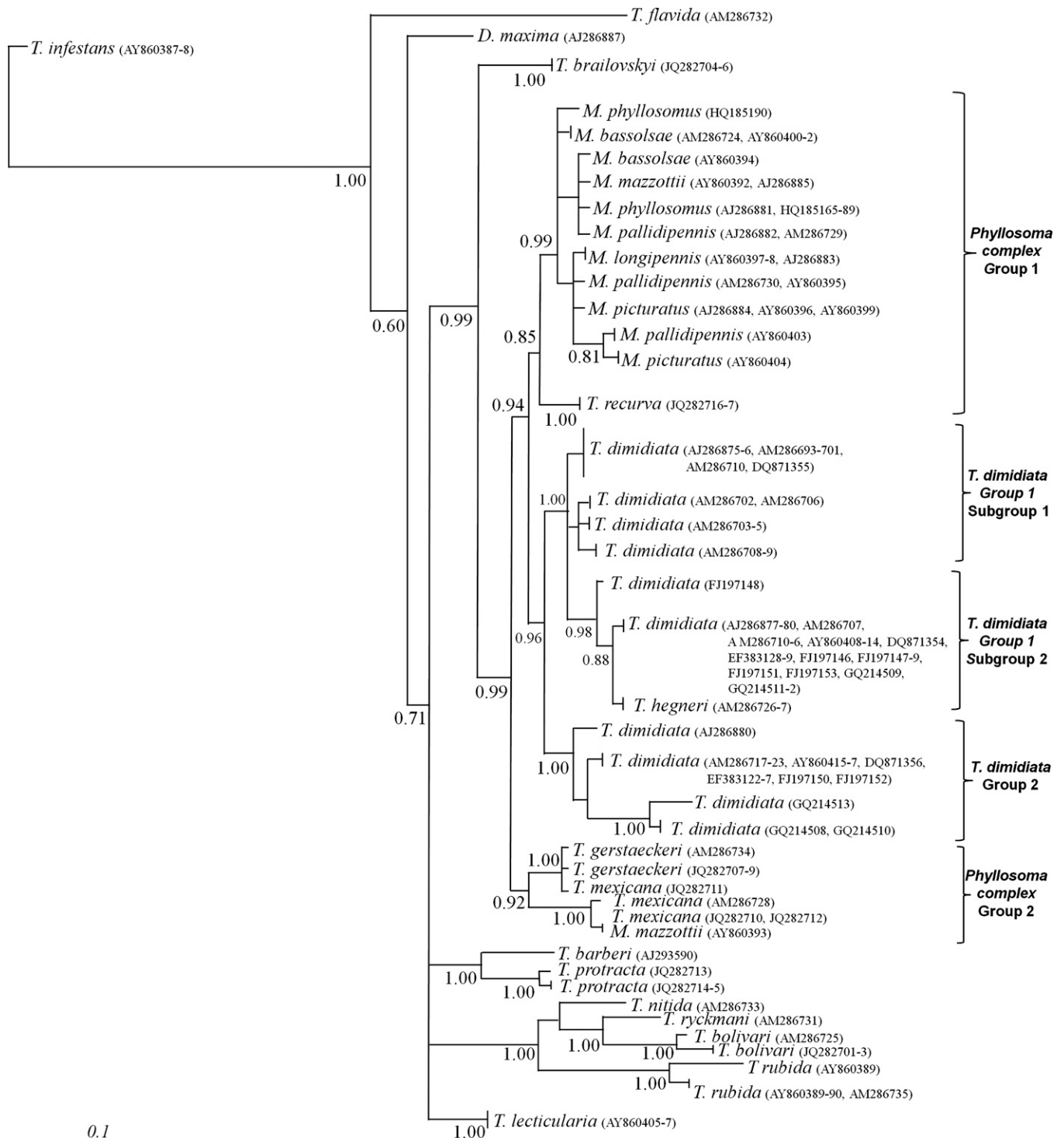


FIGURE 2. Bayesian phylogenetic trees of triatomine insects with internal transcribed spacer (ITS-2). Majority rule phylogram resulting from Bayesian analysis with the ITS-2 data set under the general time reversible +gamma distribution model of evolution. Values at nodes indicate percentage of the posterior probability. Scale bar indicates nucleotide substitutions per site.

M. picturatus, and *T. gerstaeckeri* had similar structures and few modifications in domains I and II. *Triatoma mexicana* showed high similarity between domains I, II and IV and modifications in domain III, and *T. recurva* showed similarity only in domain IV with the above mentioned species.

Triatoma dimidiata RNA secondary structures were constructed according to the sequences that the ITS-2 tree used

for each group or subgroup: *T. dimidiata* subgroup A was constructed with 15 sequences, *T. dimidiata* subgroup B with 20 sequences, and *T. dimidiata* group 2 with 16 sequences. These groups showed three structures with modifications in domains I, II, and III and were similar in domain IV. *Triatoma bolivari* and *T. rubida* showed similarity within domains I and IV; *T. barberi* and *T. protracta* shared only

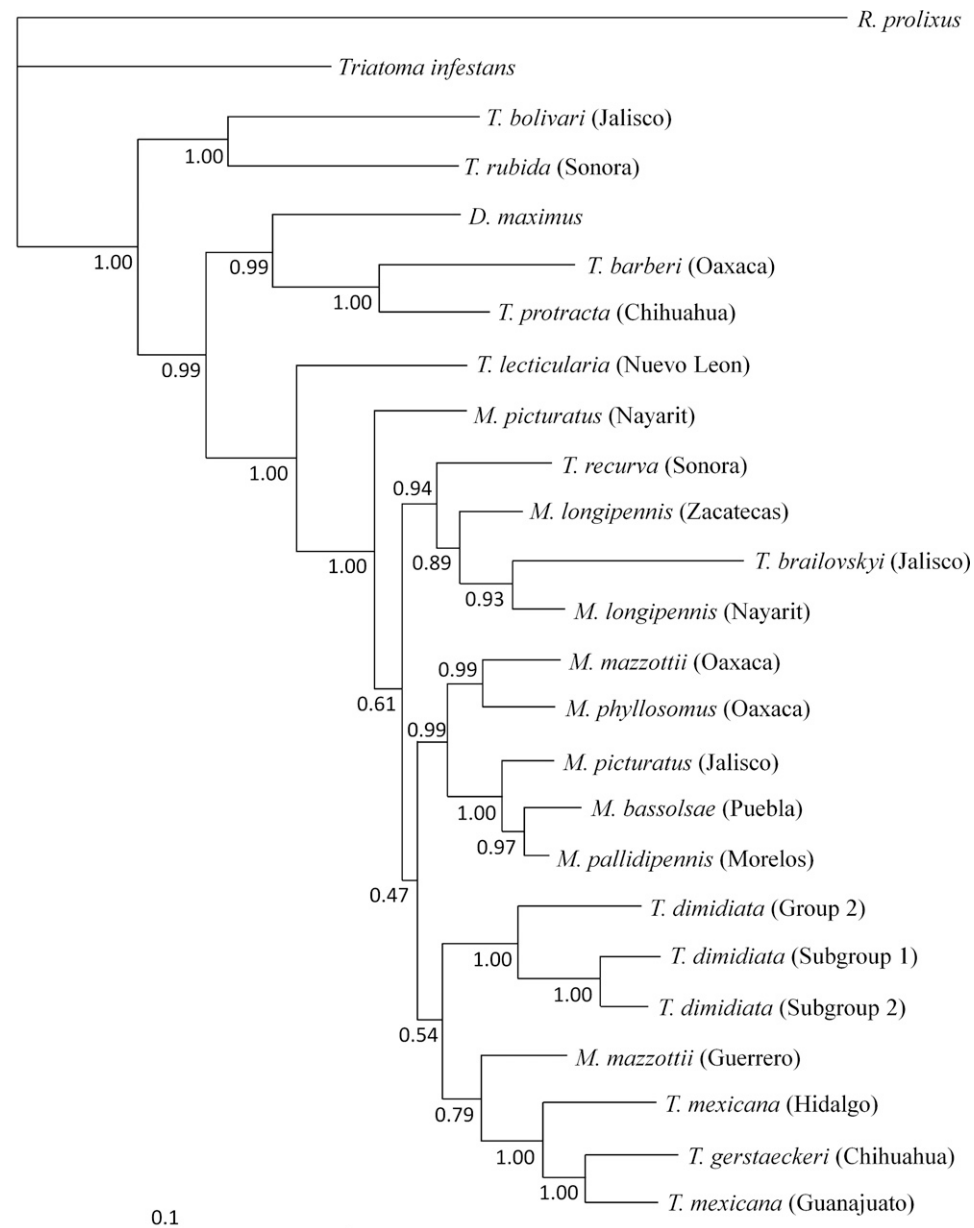


FIGURE 3. Bayesian phylogenetic trees of triatomine insects using *cytochrome B* (*cytB*) and internal transcribed spacer 2 (ITS-2) sequences. Majority rule phylogram resulting from Bayesian analysis with *cytB* and ITS-2 data set under the general time reversible + gamma distribution + proportion invariate model of evolution. Values at nodes indicate percentage of the posterior probability. Scale bar indicates nucleotide substitutions per site.

domain I. In general, domains II and IV were the most conserved (Figure 4).

DISCUSSION

This study analyzed five species of *Triatoma* from Mexico and the United States for which little genetic and epidemiologic data are available: *T. brailovskyi*, *T. bolivari*, *T. gerstaeckeri*, *T. recurva*, and *T. protracta*. *Triatoma gerstaeckeri* and *T. recurva* have been associated with human dwellings and high rates of *T. cruzi* infections (90%). In contrast, *T. bolivari*, *T. brailovskyi*, and *T. protracta* are considered to be sylvatic species.^{5-7,10,23,34}

The aim of this study was to determine genetic variation in populations and perform taxonomic analysis by using

ITS-2 and *cytB* sequences of some triatomine species from North America, particularly in *Phyllosoma*, *Dimidiata*, and *Rubrofasciata* complexes. Although several studies have also focused on these aspects, we expanded our analysis to include important phenotypic characteristics such RNA secondary structures, which commonly determine similarity and evolutionary relationships between species. The secondary structures of ITS-1 and ITS-2 regions of RNA reportedly are involved in RNA ribosome maturation processing. Therefore, structures that retain a highly conserved configuration may be useful tools for analyzing this cryptic species.³⁵

Using *cytB* sequences, we detected low values of intra-specific similarity (86–91%) and high values of nucleotide

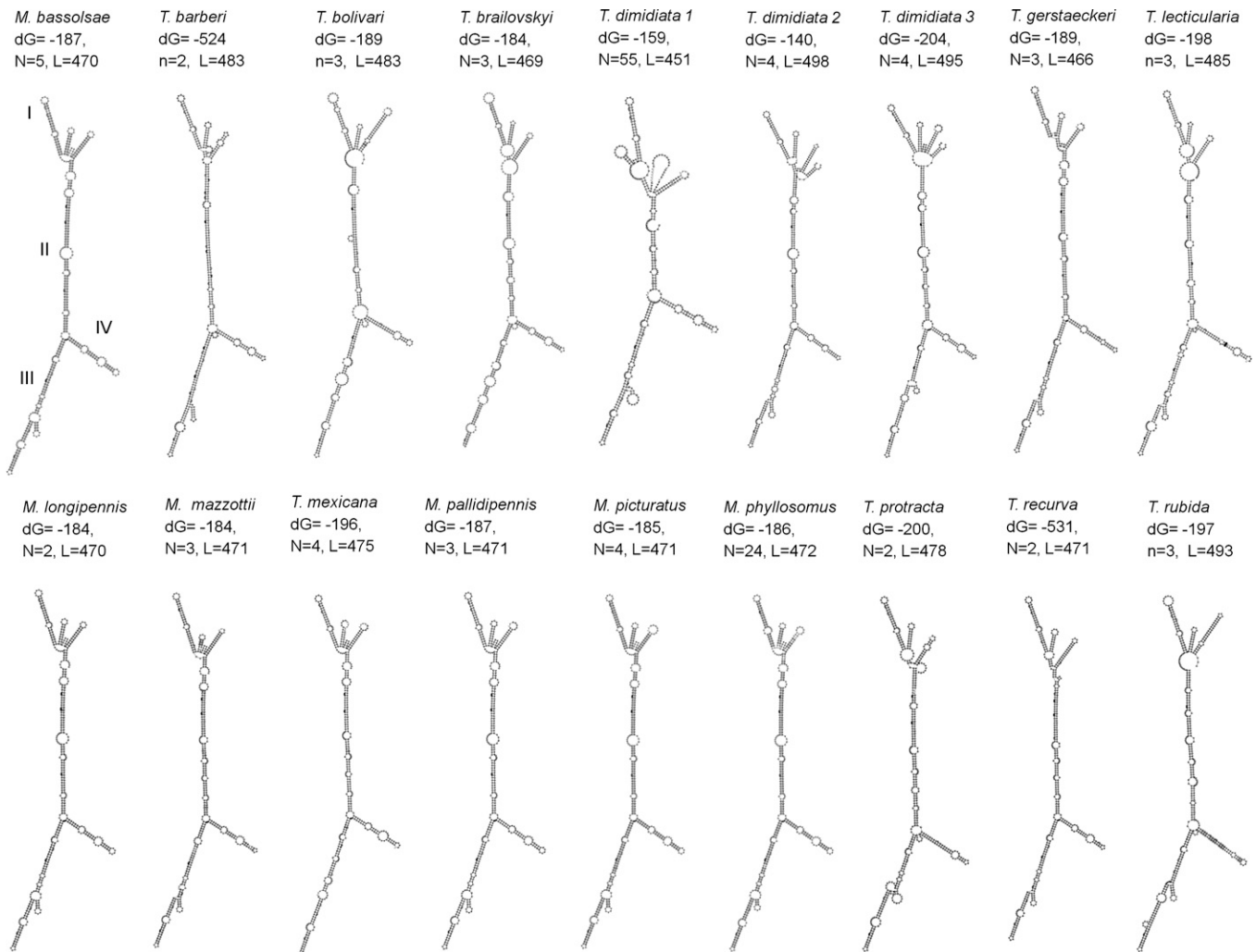


FIGURE 4. RNA secondary structures of internal transcribed spacer 2 for species of triatomines. dG = entropy energy; n = no. sequences used; L = no. basepairs.

diversity (0.09–0.14) between the *Phyllosoma* complex species *M. longipennis*, *M. mazzottii*, *T. mexicana*, and *M. picturatus* from different geographic areas. For example, divergence levels for several *T. infestans* populations in South America did not exceed 2%³⁶; Within *T. rubida* populations from Mexico and the United States, the percentage similarity was 87%, and within the *T. recurva* population, the percentage similarity was 92%.²⁴ In contrast, *T. brasiliensis* populations showed high divergence.³⁷

A species complex was proposed for *T. rubida*, *T. recurva*, and *T. brasiliensis*. Analysis of our data from the *Phyllosoma* complex suggests two hypotheses. The first hypothesis is that geographic fragmentation of these populations generated genetic variants that could indicate either a subspecies or a speciation process. The second hypothesis is that these differences are caused by classification problems related to morphologic plasticity or hybridization of different triatomines species, resulting in genetic introgression. Both hypotheses are feasible. For example, inconsistency in morphologic and genetic characteristics between sympatric species as a result of natural hybridization has been reported, and re-validation of the subspecies in this complex has been proposed.^{38–41} In areas with sympatric speciation, classification problems could

be common, and occasionally morphologic variants are not genetically differentiated.

Unlike previous analyzes of *T. dimidiata* that focused on genetic grouping and correlation with geographic origin or subspecies of *T. dimidiata*,^{23,25} our intraspecific variation analysis formed two groups based on habits: the first group corresponds to insects with domestic and peridomestic habits and the second group corresponds to sylvatic insects. Data for both genetic markers analyzed were similar, and we observed the greatest variation between these two groups. According to the fixation index, the observed null interaction between the two groups and both markers ($F_{ST} \geq 0.81$) suggests limited migrations. Similar results were reported by Tamay-Segovia and others, who analyzed two groups in a tropical area (sylvatic) and a mainly coastal distribution (peridomestic and domestic).²⁶

Triatoma protracta also showed high variation between the two populations. In this regard, Ryckman proposed five subspecies,²⁰ and Lent and Wygodzinsky proposed synonymy.⁹ No previous genetic studies have analyzed these populations. However, these types of studies are important because *T. protracta* populations have high variation, and genetic analyses can verify the taxonomic status of these populations.

We conducted few ITS-2 sequence-structure analyses because no comparative geographic data are available for some species. *Triatoma bolivari*, *T. gerstaeckeri*, and *M. pallidipennis* showed low intraspecific similitude between populations (99.3–99.4%), indicating homogeneity that is independent of their geographic distributions. In this instance, data suggested that natural selection pressures are stable and do not favor the new variant generation. However, we suggest that these data should be confirmed by extensive population genetic studies using several markers and other genes.

The *cytB* sequences, RNA secondary structures of ITS-2 (with similarity between domains II, III and domain IV) and microsatellites sequences ((AT)₄TTT(AT)₇) grouped insects in *T. dimidiata* group 2 with species of the *Phyllosoma* complex. *Triatoma dimidiata* group 2 from sylvatic areas could be the origin of the entire *Phyllosoma* complex species and *T. dimidiata* group 1. The domestication process could be an important source of genetic variation between groups. Insects in the *Phyllosoma* complex from sylvatic areas should be analyzed to determinate this relationship. An additional important consideration is the taxonomic controversy, particularly in the inclusion or separation of *T. dimidiata* from the *Phyllosoma* complex, which could be complicated by genetic similarity between these groups (*T. dimidiata* group 2 and *Phyllosoma* complex group 2).

Previous taxonomic classification of *T. brailovskyi* morphologically related this species to the *Dimidiata* complex (*T. dimidiata*, *T. hegneri*, and *T. gomeznunezi*).²² We observed that *T. brailovskyi* was related to the *Phyllosoma* complex on the basis of analogous microsatellites ((AT)₄TTT(AT)₅) and a strong phylogenetic relationship with *M. longipennis* and *T. mexicana* as demonstrated by *cytB* and ITS-2 trees. However, *T. brailovskyi* had a different ITS-2 secondary structure and greater similarities with *T. lecticularia* (*Lecticularia* complex) in domain I, *T. protracta* (*Protracta* complex) in domain 2, and the *Phyllosoma* complex in domains III and IV. The phylogenetic tree consensus (*cytB* and ITS-2) and RNA structure suggest that *T. brailovskyi* could be related to *T. recurva* and *M. longipennis* from Zacatecas.

Our study is the first to demonstrate that *Phyllosoma* complex species have genetic differences between *T. mexicana*, *M. longipennis*, and *M. picturatus*; these species have at least two geographically differentiated variants. This finding is important because genetic diversity or phylogenetic relationships with other species or genera could also be different. Therefore, we propose that the *Phyllosoma* complex is divided into two paraphyletic genetic groups: group 1 is composed of *M. bassolsae*, *M. longipennis*, *M. mazzottii*, *M. pallidipennis*, *M. phyllosomus*, and *M. picturatus*, and group 2 is composed of *T. brailovskyi*, *T. mexicana*, *T. gerstaeckeri*, *T. recurva*, and other variants of *M. mazzottii* and *M. picturatus*. Phylogenetic, taxonomic, and paraphyletic characteristics of this complex need to be carefully considered because of large genetic variations in some species such as *M. mazzottii*, *M. longipennis*, and *M. picturatus*.

RNA secondary structure analyzed in species of the *Phyllosoma* complex (*T. mexicana*, *M. bassolsae*, *T. gerstaeckeri*, *M. longipennis*, *M. pallidipennis*, *M. picturatus*, *M. mazzottii*, *M. phyllosomus*, and *T. recurva*) generally showed four domains with similar morphology and minimal variation in domains I, II, and IV. Domain IV seems to be characteristic of *M. bassolsae*, *M. longipennis*, *M. mazzottii*, *M. pallidipennis*, *M. phyllosomus*, and *M. picturatus*. The ITS-2 secondary

structure correlation with minimal changes observed and results of phylogenetic analysis suggested close evolutionary relationships in the *Phyllosoma* complex.

Pfeiler and others used *cytB* and the cytochrome oxidase I gene to include *T. recurva* in the *Phyllosoma* complex.²⁴ Our data for *cytB* and ITS-2 corroborates this inclusion. Moreover, *T. recurva* showed greater similarity to *M. longipennis* when *cytB* sequences were compared and with *M. mazzottii* when ITS-2 secondary structure was analyzed.

Triatoma species have been related morphologically to the *Rubrofasciata* complex (*T. rubida* and *T. rubrofasciata*) because of similar topology for ITS-2 and *cytB* and similarities in secondary structure of domains I and IV of RNA in *T. bolivari* and *T. rubida*.^{22,23} We have confirmed this relationship. Additional morphologic and genetic studies are needed to determine potential relationships between others species.

Finally, there is little data for triatomine groupings. Our analysis reflects the difficulty in separating these insects because of their high genetic variability. The continuous domestication process and fragmentation of habitats could be an important variation source. Therefore, grouping proposals demand careful consideration. These considerations are useful for establishing adequate vector control and for increasing biologic, taxonomic, and phylogenetic information for this these important species.

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