Am. J. Trop. Med. Hyg., 82(1), 2010, pp. 74–82
doi:10.4269/ajtmh.2010.09-0272

Natural Crossbreeding between Sympatric Species of the *Phyllosoma* Complex (Insecta: Hemiptera: Reduviidae) Indicate the Existence of Only One Species with Morphologic and Genetic Variations

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Abstract. The nucleotide sequences of the cytochrome B gene and the antennal phenotypes were analyzed for the following triatomine species: *Triatoma longipennis, Triatoma pallidipennis,* and *Triatoma picturata*, which belong to the *Phyllosoma* complex. These species inhabit sympatric areas from Talpa de Allende, Autlan de Navarro, and Teocuitatlan de Corona in Jalisco, Mexico. Molecular marker analysis showed that the sympatric individuals are the natural crossbred descendents of different individuals living in close proximity in these natural areas that resulted in mixed populations. The antennal phenotype results are coincident with these genetic findings, which point to the high similitude between all *Phyllosoma* complex populations analyzed. These data support the hypothesis that these species are morphotypes with chromatic and genetic varieties, which preserves the possibility of natural breeding with fertile descent. In conclusion, our results strongly support the hypothesis that *T. pallidipennis, T. longipennis,* and *T. picturata* are subspecies of the *Phyllosoma* complex.

INTRODUCTION

The Triatominae (Insect: Hemiptera: Reduviidae) are the vectors of the protozoan parasite *Trypanosoma cruzi* that is responsible for Chagas disease or American trypanosomiasis, which is a disease that affects between 10 and 18 million people on the American Continent.[1,2] Approximately 137 triatomine species have been reported worldwide. More than 33 species have been identified in Mexico, and many of them are endemic.[3–5] Among the endemic species present in Mexico are those that belong to the *Phyllosoma* complex: *Triatoma pallidipennis*, *Triatoma longipennis*, *Triatoma bassolsae*, *Triatoma phyllosoma*, *Triatoma mazzottii, Triatoma mexicana,* and *Triatoma picturata*.[6] This complex is principally distributed in the center and southern part of the country. Because of their parasitic infection and colonization indexes, these vectors are of epidemiologic importance. Variable percentages of *T. cruzi* infection have been reported, ranging from 7.4% to 52% in *T. picturata*; 14% to 88% in *T. pallidipennis*; and 18% to 55% in *T. longipennis*.[7–12]

Originally, the members of this complex had been considered subspecies of the *T. phyllosoma* species.[13] Hybrid forms have been observed in laboratory crossbreeding experiments between some of the species that integrate this complex.[14,15] Nevertheless, Lent and Wygodzinsky,[16] using qualitative morphologic characters, classified them as a defined species apparently because the typical specimen of every species could be recognized.

The proposal of the subspecies classification of the members of the *Phyllosoma* complex has again been considered because after their molecular analysis a high phylogenetic similarity was shown to exist between the species that integrate the complex. For example, genetic proximity and little variation between the species *T. longipennis* and *T. picturata* or *T. pallidipennis* and *T. bassolsae* were found using Cyt B and ITS-2 markers (Kimura distances between 0.002 and 0.017 in the case of ITS-2 and 0.034 and 0.172 in the case of the Cyt B).[17] Furthermore, the observations that the *Phyllosoma* complex species are included in compact clades suggest the subspecies status with the existence of morphologic varieties within one species.[6,18,19]

Species recognition traditionally follows the morphologic qualitative characteristics. Nevertheless, the phenotypic plasticity of the triatomines and the similarity of the nymphal state limit the appropriate recognition of the species and their interbreeding descendants.[20] More than 25 years ago, Lent and Wygodzinsky[16] pointed out the need to develop intensive collection activity over the entire *Phyllosoma* complex distribution area with rigorously planned and executed rearing and crossbreeding experiments to provide the definitive answer regarding the taxonomic rank of the group components. Zárate and Zárate[3] agreed and pointed out the need to develop an exhaustive collection of the complex in the wild to establish continuity between currently disjointed populations and to detect those areas with overlapping distributions.

Even though several researchers have sampled overlapping areas, little recent information about individuals with mixed characteristics is available.[8,10,21,22] Apart from Mazzotti and others,[14] there have been only three observations of the presence of individuals who present morphologic characteristics of two species; these were located in sympatric areas for some species of the *Phyllosoma* complex.[15,23,24] In the later work, Martínez-Ibarra and others conducted laboratory hybridization studies between *T. longipennis* and *T. picturata* and found individuals with fertile offspring, showing a low reproductive isolation between them.

The use of molecular and morphologic markers has generated important information about the phylogenetic and genealogical relationships between species of triatomine that present taxonomic problems. In the case of molecular markers, the Cyt B sequences of triatomine species present in Mexico have been analyzed, separating the members of the

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Phyllosoma complex from other species and defining the phylogenetic relationships of this and other complexes.[17]

The analysis of the antennal phenotype is a powerful quantitative tool for Triatominae study, allowing characterization of genera, species, and populations.[25] Catalá and others[26] showed high similarities among the antennal phenotypes of species of the *Phyllosoma* complex: *T. pallidipennis*, *T. phyllosoma*, and *T. longipennis* with a better differentiation of *T. pallidipennis* from Morelos.

This work analyzed individuals of the *Phyllosoma* complex collected in sympatric areas and determined possible crossbreeding using Cyt B sequence analysis. Additionally, the antennal phenotype of these individuals was also analyzed. Natural crossbreeding between sympatric species of the *Phyllosoma* complex with fertile progeny would be a determining factor to understand the taxonomic and phylogenetics problems of one of the more important triatomine complexes in Mexico.

MATERIALS AND METHODS

Study area. The study took place in three areas of Mexico: Talpa de Allende (20°22′ N, 104°54′ W) at an altitude of 1,134 meters above sea level (MASL), Autlán de Navarro (19° 46′ N, 104° 22′ W) at an altitude of 900 MASL, and finally the Teocuitatlán de Corona (20° 1′ N, 103° 1′ W) at an altitude of 1,375 MASL. All these localities belong to the Jalisco State in the central west part of Mexico (Figure 1) where *T. pallidipennis, T. picturata*, and *T. longipennis* species have been reported.[8,11,12,27] The average annual temperature ranges from 21.3 to 23.5°C and average annual precipitation ranges from 719.8 to 1,029.7 mm.

Species. The triatomine used in this study were adult females, collected according to Martínez-Ibarra and others.[24] All specimens were identified morphologically as *T. longipennis*, *T. pallidipennis,* and *T. picturata* with the Lent and Wygodzinsky[16] key. *Triatoma picturata* have an overall dark brown corium with one large, subtriangular basal coloration, and yellow or orange-red subapical coloration with short, slightly decumbent setae, and are not more than 0.2 mm long. They have pronotum with an extensively orange-yellow posterior lobe and a yellowish connexivum with a subrectangular black spot (Figure 2A). *Triatoma pallidipennis* have a mostly yellowish white corium and an extensively black connexivum with subrectangular orange spots (Figure 2B). *Triatoma longipennis* have a largely black corium, a black connexivun with yellow or orange-red markings basally and subapically with short, slightly decumbent setae, and are not more than 0.3 mm long. They have pronotum with an entirely black posterior lobe (Figure 2C). Other female specimens presented morphological characteristics with an intermediate phenotype between *T. picturata* and *T. pallidipennis*: a largely yellowish white corium, pronotum with extensively orange-yellow rounded humeral angles, a length of more than 25 mm, strongly widened abdomen, abundant pilosity, the first antennal segment attaining or surpassing clypeus apex, and spongy fossulae absent in both sexes. These specimens were designated *Triatoma* sp. (Figure 2D). They were similar to those named "hybrids" described by Mazzotti and Osorio[14] and Martinez-Ibarra and others.[15,24] Other individuals of the species *T. longipennis, T. pallidipennis*, and *T. picturata* were collected in areas where only one species has been reported. *Triatoma phyllosoma* (*Phyllosoma* complex), *Triatoma dimidiata*, and

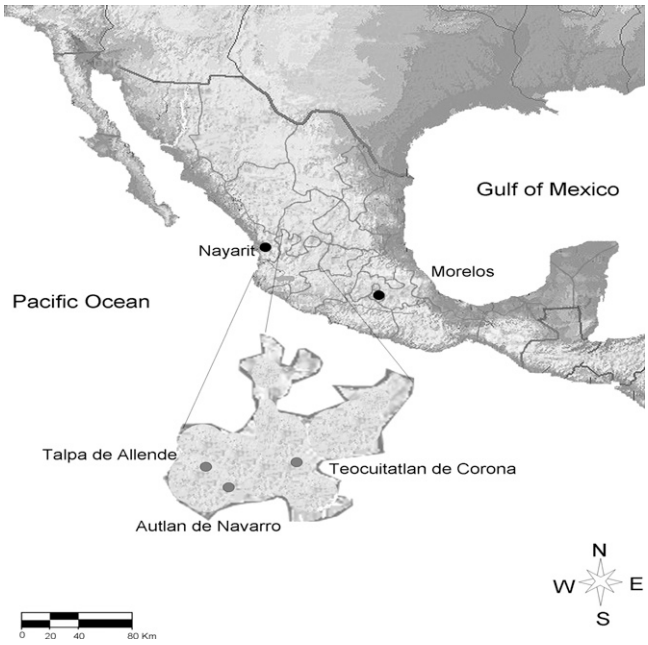

FIGURE 1. Map of the Mexican Republic showing the geographic location of the *Phyllosoma* complex species capture sites in the state of Jalisco (Autlán de Navarro, Talpa de Allende, and Teocuitatlán de Corona), Morelos and Nayarit, Mexico.

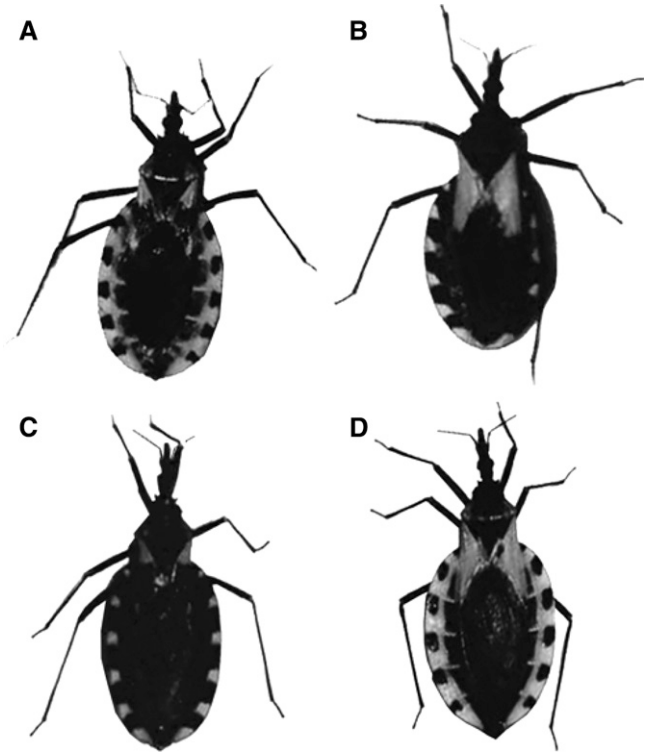

FIGURE 2. Morphologic phenotypes of the triatomines collected in a sympatric area. **A**, *Triatoma picturata*; **B**, *Triatoma pallidipennis*; **C**, *Triatoma longipennis*; and **D**, *Triatoma* sp. showing morphological characteristics with an intermediate phenotype between *T. picturata* and *T. pallidipennis*: largely yellowish white corium, pronotum with extensively orange-yellow rounded humeral angles, length more than 25 mm, strongly widened abdomen, abundant pilosity.

Triatoma barberi were also included as outgroups in the phylogenetic analysis. They were a denominated-type species and showed the characteristics reported by Lent and Wygodzinsky.[16] The origin and number of the analyzed individuals are described in Table 1. All of these insects were identified by the same specialist (Dr. Martínez-Ibarra).

DNA extraction and amplification. Genomic DNA was extracted from one leg of each insect. The extraction was carried out according to Martínez and others.[17] The oligonucleotides used for the amplification of Cyt B sequences were previously described.[17] Approximately 200 ng of genomic DNA was amplified by polymerase chain reaction (PCR).

Cloning and sequencing. 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).

Sequence alignment and phylogenetic analysis. In addition to the 28 sequences amplified in this work, several accessed Cyt B sequences were analyzed (see paragraph below). Multiple alignments were performed with the CLUSTAL W program, version 1.8.[28] The Kimura two parameters distances were calculated using MEGA program, version 1.02.[29,30] The program Modeltest 3.7[31] was used to determine the appropriate molecular evolution model. The general time-reversible model with gamma distribution and invariant sites (GTR + G + I)[30] was used for Cyt B sequences. The phylogenetic reconstruction using Bayesian inference was performed with the MrBayes 3.1.2 program.[32–34] The analysis was run for 10 million generations, sampling trees every 100 generations. The Cyt B data were treated as three separate partitions based on codon positions. 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. The Cyt B sequences used are shown in Table 1.

Antennal phenotype analysis. Antennae were excised from each specimen at the stem level, treated with 4% KOH, and neutralized with 5% acetic acid. The individual preparations were mounted on microscope slides with glycerin to 50% (diluted with phosphate buffered saline [PBS]) and observed with a light microscope at 400×. The sensilla on the ventral side of the three distal segments were drawn using a drawing chamber. Each type of sensillum was counted separately. The receptors were classified in accordance with Catalá and Schofield[35] and counted along the pedicel (P) and flagellum segments (F1 and F2). Four types of receptors were used for this analysis: Bristles (BR, a mechanoreceptor), Basiconica (BA), thick-walled trichoid (TK), and thin-walled trichoid (TH) (chemo receptors with different functions).[35]

Statistical analysis of the antennal phenotype. Fifty-one individuals with 12 variables were analyzed. Averages and standard deviations of sensilla number were calculated by type and antennal segment. The data set were used to produce multivariate analysis: Principal components (PCA), cluster analysis, and discriminant analysis (DA) using STATISTICA version 8 (Statsoft).[25,26,35] For comparative purposes, the antennal data from 7 *T. dimidiata* females from Veracruz (Mexico) were added. These data belong to the ECLAT database for antennal phenotypes in CRILAR-CONICET, Argentina. The data set of sympatric individuals was analyzed using PADWIN version 6 (http://www.mpl.ird.fr/morphometrics) to estimate functions that identify the studied groups.

RESULTS

Sequence variations. Twenty-eight individuals were sequenced for Cyt B, and all data were deposited in GenBank (Table 1).

TABLE 1
Origin and number of triatomine analyzed

Species	Locality of origin	No. of specimens antennal phenotype	No. of specimens Cyt B
T. dimidiata	Campeche, Mexico	NA	AY859417, AY859418
***Phyllosoma* complex**	Puebla, Mexico	NA	AY859410
T. bassolsae			
T. longipennis	Nayarit, Mexico	NA	AY859412, DQ198815
*T. longipennis**	Teocuitatlan de Corona, Jalisco, Mexico	8¶	2 (EU790639¶ and EU790640‖)
T. longipennis†	Autlan de Navarro, Jalisco, Mexico	4¶	5 (EU790616,‖ EU790617,‖ EU790618,‖ EU790624,** and EU790625**)
T. mazzottii	Guerrero, Mexico	NA	AY859421, DQ198817
T. mexicana	Hidalgo, Mexico	NA	DQ118976
T. pallidipennis	§	NA	AF045724
*T. pallidipennis**	Morelos, Mexico	5§	3 (AY859419,‡¶ AY859420,‡§ and DQ198814‡)
T. pallidipennis†	Talpa de Allende, Jalisco, Mexico	6§	3 (EU790629,¶ EU790631,¶ and EU790632§)
T. pallidipennis†	Autlan de Navarro, Jalisco, Mexico	8¶	5 (EU790619,¶ EU790620,¶ EU790621,** EU790622,** and EU790623**)
*T. picturata**	Compostela, Nayarit, Mexico	7¶	1 (AY859413‡)
T. picturata	Nayarit, Mexico		AY859413
T. picturata†	Talpa de Allende, Jalisco, Mexico	6‖	2 (EU790626¶ and EU790627‖)
Triatoma sp.†	Talpa de Allende, Jalisco, Mexico	9‖	8 (EU790628,§ EU790630,‖ EU790633,§ EU790634,‖ EU790635, EU790636,** EU790637,** and EU790638**)
T. phyllosoma	Oaxaca, Mexico	NA	AY859411, DQ198818
***Protracta* complex**	Oaxaca, Mexico	NA	AY830137
T. barberi			

* Species considered as type because only the species mentioned in the table have been found in the area and they present the characteristic features reported by Lent and Wygodzinsky.[16]
† Species considered as a result of crossbreeding because they presented morphological characteristics with intermediate phenotype (see Materials and Methods).
‡ Sequences taken from GenBank.
§ Domestic origin.
¶ Peridomestic origin.
‖ Silvatic origin.
** Colony from laboratory.
NA = no analyzed species.

The size of the amplified Cyt B gene fragment was 313 bp with a number of variables (44%) and informative sites (38.7%) and an average A + T nucleotide composition percentage of 64%.

The proteins were determined for each nucleotide sequence according to the specific mitochondrial invertebrate code. No deletions, no insertions, and no stop codons were present in this amino acid sequence. Analysis based on the third codon position and use of the preferential codon supported the fact that the sequences represent mitochondrial DNA (mtDNA) and not nuclear pseudogenes.

Analysis of genetic distances. The genetic distances were calculated according to Kimura 2 parameters. Species belonging to the *Phyllosoma* complex showed small distances between 0 and 0.16 (Table 2). No differences (genetic distance = 0) were observed among the following individuals: EU790619/EU790626/EU790632/EU790636/EU790637 (*T. pallidipennis*/*T. picturata*/*T. pallidipennis*/*Triatoma* sp./*T.* sp.), EU790624/EU790628/EU790633/EU790634/EU790635 (*T. longipennis*/*T.* sp./*T.* sp./*T.* sp./*T.* sp.), EU790616/EU790618/EU790627/EU790638 (*T. longipennis/T. longipennis/T. picturata/T.* sp.), and EU790617/EU790622 (*T. longipennis/T. pallidipennis*) (Table 2). The sequence DQ198817 taken from the GenBank, identified as *T. picturata* by Pfeiles and others,[36] showed a close genetic distance with *T. pallidipennis* (AY859419, AF045724, and DQ198814), suggesting a mistake in its classification or the possibility that this sequence belongs to an individual result of natural crossbreeding.

Phylogenetic analysis. For the phylogenetic analysis, the Bayesian and the Parsimony methods (data not shown) were used, both with similar topology and support. Sequences of the species denominated type (see Materials and Methods, specimens previously reported in the GenBank with genetic and morphologic analysis)[6,17] were used as controls for these analyses. These were species of the *Phyllosoma* complex present in localities in Mexico without sympatry with other species (Figure 3).

The phylogenetic trees of the sympatric species showed genetic inconsistencies. Specimens morphologically classified as *T. pallidipennis* (individuals with keys EU790619, EU790621, EU790631, and EU790632) and *T. longipennis* (EU790624) were grouped with type specimens of *T. picturata*, which are represented by the AY859413 sequences. This clade also grouped the majority of the individuals with mixed characteristics from both species as *Triatoma* sp. (individuals EU790628, EU790633, EU790634, EU790635, EU790636, and EU790637). In the clade of the type species *T. pallidipennis/T. bassolsae*, represented by the individuals AY859419, AY859420, AF045724, DQ198814, and AY859410, individuals morphologically classified *T. longipennis* (EU790616, EU790617, EU790618, EU790625, and EU790640), *T. picturata* (EU790627 and DQ198817), and *T.* sp. (EU790638) were also found. The specimen EU790630 morphologically designated as *T.* sp. was localized in the *T. phyllosoma* clade. Individual EU790629, morphologically classified as *T. pallidipennis*, was found in the *T. mazzottii* clade. Finally, individual EU790620 did not have clear concordance with any clade (Figure 3). These data indicated the possible process of crossbreeding between species of the *Phyllosoma* complex living in sympatry. The majority of the branches showed above 70% posterior probability values.

Analysis of the antennal phenotype. To study some morphological parameters of these sympatric individuals, antennal analysis was used. The four mentioned types of sensilla (BR, BA, TK, and TH) are present in the three segments of the antenna (pedicel and Flagellum 1 and 2). Table 3 shows means and standard deviations for all sensilla types in the studied populations.

Species not in sympatry. Considering that specimens used in this work were preliminarily identified by traditional (qualitative) morphologic characteristics, we decided to check the antennal phenotype of the three species not found in sympatry: *T. pallidipennis* from Morellos, *T. picturata* from Nayarit, and *T. longipennis* from Teocuitatlan de Corona. *Triatoma dimidiata* from Veracruz was added to the analysis as an outer group. First, a principal PCA was carried out. The PCA compares the individuals by similitude without previous grouping. Figure 4 shows that the first function explains 71% of the variance and the second function explains 19%, which adds up to 90% of the variance. Two major groups were clearly identified: 1) *T. dimidiata* specimens (dmVe; Figure 4) and 2) the *Phyllosoma* complex (paMo, piNa, and loTe; Figure 4). However, within this last group *T. pallidipennis* is better separated, whereas *T. longipennis* and *T. picturata* showed mixed phenotypes.

Because the numbers of specimens for each group were insufficient for a DA with the 12 antennal variables, we used the first three factors of the PCA as variables. The analysis was significant for the two first discriminant functions ($P < 0.001$, $F = 16.05$, Wilks 0.038). All *T. dimidiata* (100%), 80% of *T. pallidipennis*, 72% of *T. picturata*, and 62% of *T. longipennis* were correctly classified. The specimens for *T. picturata* and *T. longipennis* shared almost the same phenotype and did not differ significantly. These results indicate low differentiation within the populations ("species") not in sympatry and prevent new analysis using them as true species.

Populations living in sympatry. The PCA for the populations ("species") living in sympatry in Jalisco (Autlan de Navarro and Talpa de Allende) was carried out. Only *T. dimidiata* (the outer group) was well differentiated; the *Phyllosoma* complex populations living in sympatry showed distribution with no significant Mahalanobis distances (data not show).

The DA using the two first components indicated that only 33–50% of specimens from the *Phyllosoma* complex obtain a correct classification as different phenotypes. A tree cluster with Euclidean distances (complete linkage) using two first principal components illustrates very well the position of all individuals from populations living in sympatry (Figure 5). *Triatoma dimidiata* specimens congregate on a first branch and all *Phyllosoma* complex specimens appear mixed.

The DA carried out with the two principal components for all populations together (living or not in sympatry) produces similar results. The distances between the populations were not significant with the exception of the population of *T. pallidipennis* from Morelos State (data not shown).

DISCUSSION

In this study, taxonomic analysis of sympatric populations of the *Phyllosoma* complex species from Talpa de Allende, Autlan de Navarro, and Teocuitatlan de Corona in the state of Jalisco, Mexico were performed, allowing a better understanding of the taxonomic and phylogenetic relationships between these important North American triatomines.

The collected individuals in the present study are an example of the morphological complexity of the *Phyllosoma* complex and show the genetic and morphologic classification problems that several studies have reported. For example, some species

TABLE 2
Kimura 2 parameter distances

Species	Genbank accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
T. longipennis	EU790616 (1)1																											
T. longipennis	EU790617 (2)2	0.04																										
T. pallidipennis	EU790619 (3)3	0.15	0.13																									
T. pallidipennis	EU790620 (4)	0.08	0.07	0.07																								
T. pallidipennis	EU790621 (5)4	0.16	0.16	0.02	0.08																							
T. pallidipennis	EU790623 (6)	0.15	0.14	0.06	0.08	0.08																						
T. pallidipennis	EU790625 (7)	0.04	0.01	0.13	0.07	0.16	0.14																					
T. pallidipennis	EU790629 (8)	0.14	0.14	0.14	0.1	0.13	0.1	0.13																				
T. sp.	EU790630 (9)	0.13	0.14	0.16	0.1	0.15	0.12	0.13	0.07																			
T. pallidipennis	EU790631 (10)	0.15	0.14	0.01	0.08	0.03	0.07	0.14	0.15	0.16																		
T. longipennis	EU790639 (11)	0.13	0.13	0.1	0.1	0.1	0.1	0.13	0.13	0.14	0.11																	
T. longipennis	EU790640 (12)	0.06	0.09	0.14	0.09	0.16	0.14	0.08	0.14	0.12	0.15	0.12																
T. pallidipennis	DQ198814 (13)	0.05	0.08	0.14	0.1	0.16	0.15	0.07	0.13	0.12	0.15	0.13	0.01															
T. pallidipennis	AY859419 (14)	0.07	0.09	0.14	0.09	0.16	0.14	0.08	0.14	0.13	0.15	0.13	0.01	0.02														
T. pallidipennis	AF045724 (15)	0.06	0.09	0.14	0.09	0.16	0.14	0.08	0.14	0.12	0.15	0.12	0.01	0.01	0.01													
T. pallidipennis	DQ198815 (16)	0.16	0.15	0.12	0.12	0.12	0.13	0.15	0.15	0.14	0.12	0.13	0.14	0.15	0.15	0.15												
T. pallidipennis	AY859412 (17)	0.16	0.16	0.12	0.14	0.14	0.13	0.15	0.15	0.15	0.13	0.11	0.16	0.15	0.17	0.16	0.1											
T. picturata	DQ198817 (18)	0.05	0.07	0.14	0.09	0.16	0.15	0.06	0.15	0.12	0.14	0.11	0.05	0.05	0.06	0.05	0.14	0.15										
T. picturata	AY859413 (19)	0.17	0.16	0.04	0.1	0.05	0.07	0.16	0.14	0.16	0.04	0.12	0.16	0.17	0.16	0.16	0.12	0.13	0.16									
T. mazzottii	DQ198816 (20)	0.13	0.13	0.15	0.13	0.17	0.14	0.13	0.15	0.11	0.16	0.16	0.12	0.14	0.14	0.13	0.15	0.18	0.15	0.15								
T. mazzottii	AY859421 (21)	0.15	0.14	0.12	0.1	0.11	0.08	0.14	0.06	0.12	0.13	0.12	0.13	0.14	0.14	0.14	0.14	0.14	0.15	0.12	0.15							
T. mazzottii	AY859422 (22)	0.16	0.16	0.13	0.11	0.12	0.09	0.15	0.03	0.1	0.14	0.13	0.14	0.14	0.15	0.15	0.15	0.16	0.15	0.13	0.15	0.03						
T. mexicana	DQ118976 (23)	0.15	0.17	0.15	0.13	0.14	0.13	0.17	0.1	0.13	0.16	0.12	0.14	0.14	0.15	0.15	0.14	0.14	0.14	0.15	0.15	0.09	0.09					
T. bassolsae	AY859410 (24)	0.07	0.09	0.14	0.1	0.15	0.15	0.09	0.15	0.13	0.14	0.13	0.04	0.03	0.04	0.04	0.15	0.14	0.07	0.16	0.16	0.15	0.16	0.15				
T. phyllosoma	AY859411 (25)	0.12	0.14	0.15	0.09	0.15	0.12	0.13	0.09	0.02	0.16	0.14	0.12	0.12	0.13	0.12	0.15	0.15	0.11	0.16	0.11	0.13	0.12	0.13	0.12			
T. dimidiata	AY859417 (26)	0.15	0.14	0.14	0.13	0.16	0.16	0.15	0.17	0.14	0.15	0.14	0.12	0.12	0.14	0.13	0.16	0.18	0.13	0.14	0.14	0.14	0.16	0.19	0.13	0.14		
T. barberi	AY830137 (27)	0.28	0.31	0.27	0.25	0.28	0.27	0.29	0.27	0.26	0.27	0.21	0.24	0.24	0.23	0.23	0.27	0.27	0.25	0.27	0.26	0.27	0.27	0.25	0.26	0.27	0.25	

The following sequences presented the same genetic distance values and only one representative distance data are shown in the table:
* EU790616/EU790618/EU790627/EU790638.
† EU790617/EU790622.
‡ EU790619/EU790626/EU790631/EU790632/EU790636/EU790637.
§ EU790621/EU790624/EU790628/EU790633/EU790634/EU790635.

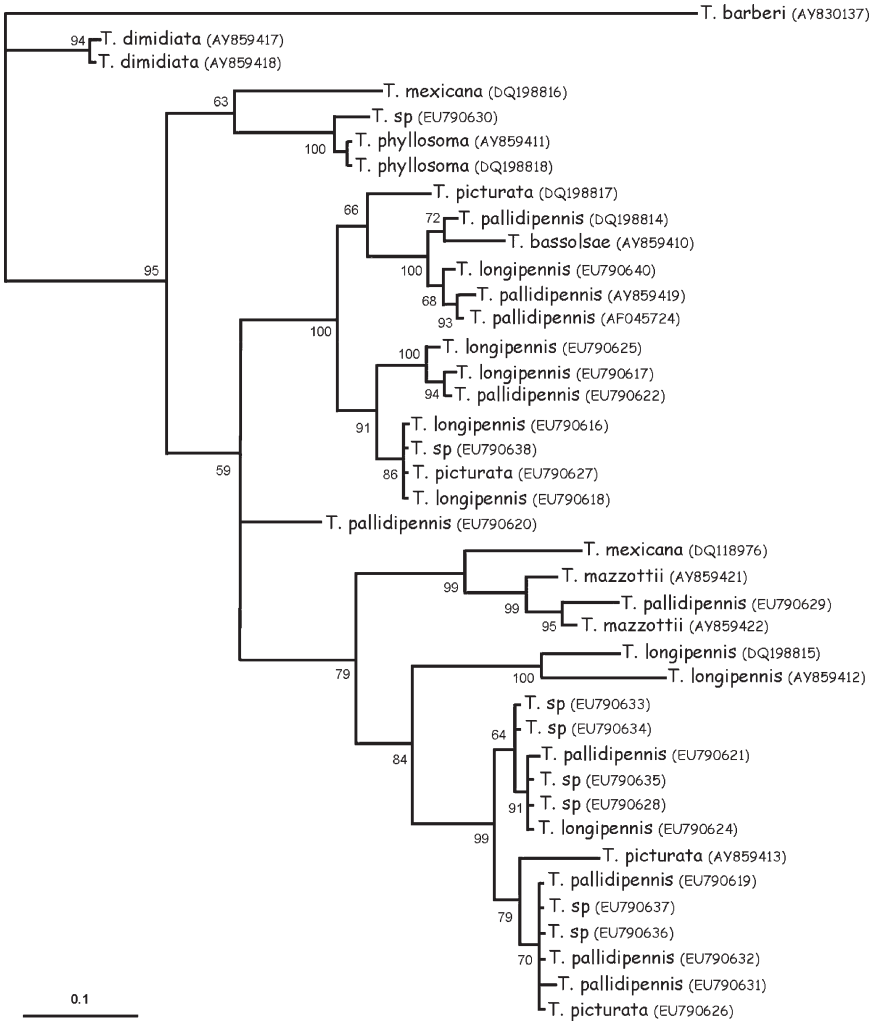

FIGURE 3. Bayesian phylogenetic trees of triatomine insects with sympatric and non-sympatric habitats using Cyt B sequences. Majority rule phylogram resulting from Bayesian analysis with GTR + G + I model of evolution. The values of the nodes indicate the percentage of the posterior probability.

such as *Triatoma brailovsky, Triatoma bolivari, T. dimidiata, Triatoma hegnery, T. mexicana, Triatoma gerstaeckeri, Triatoma sanguisuga, Triatoma ryckmani*, and *Triatoma recurva* have been tentatively included in this complex. Nevertheless, these data remain controversial, and more studies are needed to clarify their status.[13,16,17]

The results presented in this work agree with previous molecular analyses where small genetic distances with the Cyt B were found. Additionally, microsatellite data in internal transcribed spacer 2 (ITS 2) showed the same microsatellite sequence (AT_5 TTT AT_6), which indicates that the *Phyllosoma* complex species have low interspecific variation as a result of a recent divergences.[17,18]

Using Cyt B gene sequences, it was possible to genetically identify introgressions related to crossbreeding in each specimen, regardless of the fact that these species show morphologic differences, thereby showing the importance of this marker. These introgressions suggest closely related species or subspecies of recent origins that could promote adaptive evolution and speciation, which is in contrast with production of sterile descendent, that bring to an evolutionary dead-end.[37]

The phylogenetic tree constructed with the sequences of the Cyt B clearly reveals the introgression process, which means the organisms have a morphologic feature not corresponding exactly with their genetics status. Their wrong position in the tree correlates with possible mistakes in the morphological identification, which could be caused by the extensive numbers of phenotypes that resulted from natural crossbreeding. For example, in the *Phyllosoma* complex the DQ198817 sequence taken from GenBank, identified as *T. picturata*, showed genetic distance near *T. pallidipennis* (AY859419, AF045724, and DQ198814). In the case of *Triatoma brasiliensis*, 11 different phenotypes were identified in hybrid zones.[38]

The antennal phenotype analysis showed that these quantitative morphological characteristics are closely linked to adaptive genetic characteristics. Even when comparing populations not living in sympatry, the specimens show similar antennal phenotypes, which indicates close relationships. In coincidence with Catalá and others,[26] only *T. pallidipennis* from Morelos exhibits a higher morphological distance and could be identified as a separated population. All other populations, living or not in sympatry, do not show morphological distances usually found between species. These results confirm that the chromatic and qualitative characters that are traditionally used are not appropriate to identify populations from this complex.

TABLE 3
Averages and standard deviations of the number of antennal sensilla in different populations of the *Phyllosoma* complex*

Species	Locality	N	Segments											
			Pedice l				Flagellum 1				Flagellum 2			
			BR	TH	TK	BA	BR	TH	TK	BA	BR	TH	TK	BA
T. longipennis	Tecomatlan, Autlan de Navarro, Jalisco, Mexico	5	92.75 (25.8)	108 (23.05)	36.25 (25.89)	4.25 (2.217)	15.75 (2.63)	73.75 (13.28)	198.8 (59.67)	19.5 (2.646)	9.75 (3.5)	26.75 (6.70)	129.5 (53.83)	23 (12.54)
T. longipennis	Teocuitatlan de Corona, Jalisco, Mexico	8	105.4 (23.49)	175.9 (49.59)	25.25 (19.99)	2.75 (2.25)	16 (5.63)	83.13 (28.27)	247.3 (64.18)	29.63 (13.04)	11.88 (3.64)	39.25 (12.35)	200.5 (60.29)	31 (8.19)
T. pallidipennis	Tecomatlan, Autlan de Navarro, Jalisco, Mexico	8	109.3 (20.97)	135.8 (25.01)	34.13 (14.59)	3.875 (1.89)	16.25 (4.53)	68.63 (10.21)	228.1 (50.53)	32.63 (8.28)	11.88 (2.48)	36.25 (7.52)	169.5 (24.29)	27.75 (5.52)
T. pallidipennis	Cuernavaca, Morelos, Mexico	5	141 (25.97)	271.2 (46.72)	48 (20.84)	5.2 (3.49)	18.8 (5.02)	111.2 (36.09)	362.6 (62.64)	28.4 (13.01)	13.2 (2.68)	42 (13.8)	260.4 (42.38)	41.4 (14.62)
T. pallidipennis	Talpa de Allende, Jalisco, Mexico	7	125 (10.24)	163.5 (27.38)	17.33 (10.5)	3.333 (0.82)	21.33 (1.97)	82.5 (20.53)	243.7 (13.72)	24 (4.82)	15.67 (3.93)	44 (5.73)	222 (35.4)	37.33 (7.63)
T. picturata	Nayarit, Mexico	8	134.3 (17.52)	149.4 (27.99)	26.43 (8.52)	3.143 (1.35)	25.14 (2.19)	86.14 (20.76)	233.9 (21.47)	22 (7.83)	12.71 (2.29)	32.86 (8.13)	198.7 (43.72)	32.86 (10.54)
T. picturata	Talpa de Allende, Jalisco, Mexico	6	94.17 (27.63)	161.7 (24.34)	44 (15.23)	2.833 (1.72)	15.33 (4.59)	77 (10.26)	220.3 (35)	21.83 (7.63)	10.33 (1.86)	38 (6.23)	178.3 (42.46)	26 (8.67)
Triatoma sp.	Talpa de Allende, Jalisco, Mexico	9	90.44 (17.58)	158.4 (51.91)	29.33 (13.22)	3.889 (1.17)	15.78 (2.59)	80.33 (13.5)	211.9 (31.91)	24.22 (8.8)	11 (2.83)	34.11 (11.04)	184.9 (30.98)	33.44 (7.44)

*BR = bristle; TH = thin-walled trichoid; TK = thick-walled trichoid; BA = basiconic. The values in parenthesis indicate standard deviations.

FIGURE 4. Antennal phenotypes. Principal component analysis for *Phyllosoma* complex populations (species) not living in sympatry: paMo = *Triatoma pallidipennis* from Morelos (circles); loTe = *Triatoma longipennis* from Teocuitatlan de Corona (square); piNa = *Triatoma picturata* from Nayarit (rhombus); and dmVe = *Triatoma dimidiata* from Veracruz (triangles) as an outer group or reference species.

The analysis with the Cyt B marker and the antennal phenotypes has been shown to be a reliable tool for species, genera, and population classification. In this study, these markers showed that the sympatric individuals in the localities in the state of Jalisco are mixed populations and are probably products of crossbreeding between different individuals living in close proximity in this natural area, suggesting that the *Phyllosoma* complex species are morphotypes with chromatic and genetics varieties, which preserve the possibility of natural breeding and probable fertile descent.[14] Importantly, this possibility is also supported by laboratory crossbreeding experiments with the same species used in this work. A high degree of crossbreeding with fertile offspring was found between *T. longipennis* females and *T. picturata* males.[15] Our study suggests that natural crossbreeding played a crucial role in the origin and diversification of wild triatomine species as has been suggested for *T. brasiliensis*.[39]

It will be important to carefully analyze the collected individuals from the state of Jalisco and from other sympatric areas in Mexico where there are individuals of the *Phyllosoma* complex because these populations might be the result of crossbreeding, and it will be difficult to classify them with traditional morphological criteria. This study strongly suggests the need for quantitative morphologic and genetic analysis before classifying these individuals.

The consequences of the existence of individuals with mixed characteristics could be of epidemiological importance because defecation patterns, susceptibility to the *T. cruzi* infection, domiciliation, and nutritional preferences could be different to those of the parental individuals.[40] It is not known if these individuals are more capable to resist ambiental stress and better adapted to their habitat. Studies that focus on these aspects are currently being conducted in our laboratories.

In conclusion, our results strongly support the hypothesis that *T. pallidipennis, T. longipennis*, and *T. picturata* are subspecies of the *Phyllosoma* complex. The same is true for genetic studies of *T. mazzottii*, *T. phyllosoma*, and *T. bassolsae*,[17] but in this case, natural hybrid must be found and experimental stud-

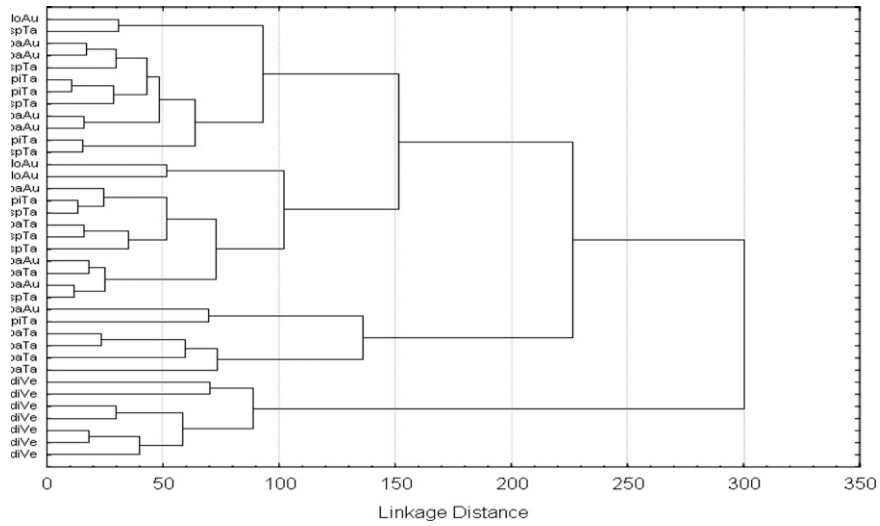

FIGURE 5. Antennal phenotypes. Tree cluster for *Phyllosoma* complex specimens living in sympatry and preliminarily classified as species. *Triatoma dimidiata* (diVe) from Veracruz, as an outgroup: paTa = *Triatoma pallidipennis* from Talpa de Allende; paAu = *Triatoma pallidipennis* from Autlan de Navarro; loAu = *Triatoma longipennis* from Autlan de Navarro; pita = *Triatoma picturata* from Talpa de Allende; and spTa = *Triatoma* sp. from Talpa de Allende.

ies that confirm the crossbreed should be made. On the basis of these results, we propose the modification of the taxonomic status of these species to subspecies and suggest that they are named *T. phyllosoma pallidipennis, T. phyllosoma longipennis, T. phyllosoma picturata, T. phyllosoma mazzottii*, *T. phyllosoma phyllosoma*, and *T. phyllosoma bassolsae.*

Received May 21, 2009. Accepted for publication October 1, 2009.

Financial support: This work was support by grant number IN 212806 from PAPIIT-DGAPA Universidad Nacional Autonoma de Mexico. Fernando Martínez-Hernandez would like to acknowledge Consejo Nacional de Ciencia y Tecnologia for the scholarship during his Ph.D. studies and the Programa de Apoyo a los Estudiantes de Posgrado from Universidad Nacional Autonoma de Mexico for the economical support to visit the Centro Regional de Investigacion Cientifica y Transferencia Tecnologica (CRILAR), Anillaco, La Rioja, Argentina.

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