MOLECULAR TAXONOMIC STUDY OF CHAGAS DISEASE VECTORS FROM THE PHYLOSOMA, LECTICULARIA, AND RUBROFASCIATA COMPLEXES

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Abstract. The Triatiominae (Hemiptera: Reduviidae) are hematophagous hemipters of importance because they transmit Trypanosoma cruzi, the causal agent of Chagas disease. The aim of this study was to define the possible relationships between species of the Phyllosoma complex (Triatoma mazzottii, Triatoma pallidipennis, and Triatoma longipennis) and species of other complexes present in Mexico that have not been previously analyzed (Triatoma lecticularia and Triatoma rubida). In addition, it was determined the inclusion of Triatoma bassolsae in the Phyllosoma complex by using 10 isoenzymatic systems (corresponding to the 14 loci). Results of isoenzymatic study show that between the species of the Phyllosoma complex including Triatoma bassolsae, the polymorphism of the analyzed enzymes ranges from 14% to 50% (P ≤ 0.95) and the species from external complexes showed polymorphism values of 43% (Triatoma lecticularia), 43% (Triatoma rubida), and 36% (Triatoma infestans). The genetic tree shows a clear difference between species of the Phyllosoma complex and the other complexes.

INTRODUCTION

Diseases of worldwide importance are often transmitted by insects, which live in close contact with humans. Among them, Chagas disease or American trypanosomiasis is one of the most important on the México, Central and South America continent. The etiologic agent of this disease is a hemoflagellate protozoan named Trypanosoma cruzi that is mainly transmitted by insects of the genera Triatoma and Rhodnius. In spite of the development of vector control programs, approximately 100 million persons are considered to be at risk and from 16 to 18 million are currently infected. In addition, 50,000 deaths are reported yearly due to the chronic complications of Chagas disease, which is therefore considered a severe public health problem in several countries in Latin America.

The classification of these insects is based primarily on morphologic characteristics. However, this type of classification offers several problems mainly concerning the species grouped in complexes. Among them, for the species of the Phyllosoma complex, the tentative inclusion of Triatoma mexicana and Triatoma dimidiata to this complex is still in discussion. Furthermore, Schofield and other authors proposed to substitute the Phyllosoma complex by the Dimidiata complex, which would contain Triatoma dimidiata, Triatoma mexicana, and Triatoma gerstaeckeri together with the other species of the Phyllosoma complex. In 1999, Alejandre-Aguilar and others described and added a new species, Triatoma bassolsae, to the Phyllosoma complex. Recently, the use of molecular markers has contributed with more data to the understanding of these classifications. Our group confirmed, with the use of isoenzymes, the morphologic classification of the species of the Phyllosoma complex published by Lent and Wygodzinsky in 1979 and the apparent taxonomic exclusion of T. dimidiata from this complex. In addition, Marcilla and others also corroborated this exclusion with the analysis of the internal ribosomal transcription spacer 2 (ITS2).

On the other hand, field work in Mexico has revealed the epidemiologic importance of these species, among them Triatoma longipennis, which is widely distributed in the states of Jalisco and Nayarit with T. cruzi infection indexes of 50 and 55%, respectively. Triatoma pallidipennis in the state of Morelos with infection index of 88%; Triatoma rubida and Triatoma recurva in the state of Sonora with infection index of 91%; and finally, T. dimidiata with infection index of 14% in Veracruz and of 9.5% in Hidalgo. These observations underline the importance of these species as important transmitters of Chagas disease in Mexico and the zones of their localization as high risk areas of transmission. The aim of the current study was to establish the relationship, by isoenzymatic markers, of one species of the Phyllosoma complex not yet analyzed (T. bassolsae), and species of other complexes, such as T. rubida and Triatoma lecticularia, which are present in Mexico.

MATERIALS AND METHODS

Triatomines. All triatomines used in the current study were adults and were obtained from the colonies established by Dr. Ricardo Alejandre (School of Biologic Sciences, National Polytechnic Institute). Information about several biological characteristics of these species had been published before.

The current study contemplated the analysis of seven Mexican species: four from the Phyllosoma complex (T. bassolsae from Puebla estate, T. longipennis from Nayarit, T. mazzottii from Guerrero, T. pallidipennis from Morelos) and two species from other complexes (T. lecticularia [Lecticularia complex] from Nuevo Leon and T. rubida [Rubrofasciata complex] from Sonora). For the phylogenetic analysis, Triatoma infestans was used as control outgroup.

Protein extraction. The thoracic muscle was extracted from the insect vector, it was frozen in a 1.6 mL tube with liquid nitrogen and macerated immediately after with a micropestle until pulverized. It was subsequently suspended in a volume of 50 to 80 μL of an enzymatic stabilizer containing 2 mM...
diethiothreitol, 2 mM E-amino caproic acid, and 2 mM EDTA. The solution was homogenized by shaking and centrifuged for 10 minutes at 25,000 × g. The supernatant containing the hydrosoluble enzymes was separated and distributed in aliquots of 10 µL, which were preserved at −70°C until used.7

Electrophoretic conditions and isoenzymatic systems. A total of 10 isoenzymatic systems were used: 4 (ME, PEP1, MDH, and PGM) have two zones of activity or loci; the remaining 6 (PEP2, GPI, 6PGDH, G6PDH, LAP, and GOT) have only one zone of activity. A total of 14 genetic loci were analyzed: aspartate amino transferase (GOT, EC 2.6.1.1), glucose 6 phosphate dehydrogenase (G6PD, EC 1.1.1.49), glucose phosphate isomerase (GPI, EC 5.31.9), leucin aminopeptidase (LAP EC 3.4.11.0.13), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), peptidase 1 and peptidase 2 (PEP, EC 3.4.11 and EC 3.4.13), 6 phosphoglucuronate dehydrogenase (6PGDH, EC 1.1.1.44), and phosphoglucomutase (PGM, EC 2.7.5.1).

Samples were separated by multilocus enzymatic electrophoresis (MLEE) standardized in cellulose acetate plates (Helena Laboratories, Beaumont, TX). Conditions for electrophoresis and enzymatic substrates have been described elsewhere.7,15–17

Analysis. ARLEQUIN Program version 2000 was used for the population genetics analysis.18 The phenogram was performed by a Nei distance matrix,19 and distances were grouped by UPGMA (unweighted pair group method with arithmetic mean). For these analyses, the program BIOSYS-1 was used.20

RESULTS

Polymorphism and heterozygosity. In the species T. bassol-sae, T. pallidipennis, T. longipennis, and T. mazzottii, ten monomorphic loci were detected: ME1, ME2, PEP1, PEP2, GOT, GPI, PGM1, PGM2, LAP, and G6PDH. Polymorphic loci for these species were for T. bassol-sae, the systems MDH1 and 6PGDH; for T. pallidipennis, the systems PEP3, MDH2, PGM2, and G6PDH, and finally, in the species T. longipennis and T. mazzottii, the systems PEP3, MDH1, MDH2, PGM2, 6PGDH, and G6PDH.

In species external to the Phyllosoma complex, the following was found: T. lec-ticularia, T. rubida, and T. infestans showed the following monomorphic loci: ME1, PEP1, PEP2, and GPI. Polymorphic loci were also different for each species: in T. lec-ticularia they were ME2, GOT, MDH1, MDH2, PGM1, and PGM2 (Figures 1a, 1c, and 1d). For T. rubida, they were PEP3, GOT, MDH2, G6PDH, 6PGDH, and PGM2, and for T. infestans, the polymorphic systems were PEP3, GOT, PGM2, LAP, and G6PDH.

Polymorphism analysis of these species revealed that T. longipennis, T. lec-ticularia, and T. rubida are the species with most genetic variabilty with a mean of 50%, 43%, and 43% of polymorphic loci, respectively (polymorphism = 0.95). This polymorphism correlates with the number of alleles per locus of these species that is around 1.5 alleles per locus. The less polymorphic species was T. bassol-sae with a mean of 14% polymorphic loci and an allelic constitution of 1.1 alleles per locus (Table 1).

Endogamic values analyzed for each locus show a large number of monomorphic alleles, that is, homozygous alleles with frequency of 1 (data not shown). For the loci that showed allelic variants, we detected 21 loci out of 28 with a clear digression from the Hardy-Weinberg equilibrium favored homozygous individuals.

Diagnostic loci. The species of the Phyllosoma complex did not show diagnostic loci, that is, no loci existed with a unique pattern that could identify them. Among the other species that do not belong to the Phyllosoma complex, T. rubida shows clear differences with the species from the Phyllosoma and other complexes, with at least six different patterns in the electrophoretic runs that differentiate it from all other species analyzed in the current work. Among the diagnostic loci for T. rubida we found ME1, GOT, GPI, MDH1, and LAP.

T. lec-ticularia also showed several diagnostic loci of which the most important were PEP1 (Figure 1b) and GPI with a very particular electrophoretic pattern. The remaining loci share the electrophoretic patterns with T. rubida.

**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean number of alleles per locus</th>
<th>Mean of polymorphic loci</th>
<th>Direct count</th>
<th>Expected HdyWbg†</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. bassol-sae</td>
<td>1.1</td>
<td>14</td>
<td>0.080</td>
<td>0.045</td>
</tr>
<tr>
<td>T. pallidipennis</td>
<td>1.3</td>
<td>29</td>
<td>0.058</td>
<td>0.118</td>
</tr>
<tr>
<td>T. longipennis</td>
<td>1.6</td>
<td>50</td>
<td>0.134</td>
<td>0.214</td>
</tr>
<tr>
<td>T. mazzottii</td>
<td>1.3</td>
<td>21</td>
<td>0.071</td>
<td>0.112</td>
</tr>
<tr>
<td>T. rubida</td>
<td>1.4</td>
<td>43</td>
<td>0.018</td>
<td>0.095</td>
</tr>
<tr>
<td>T. lec-ticularia</td>
<td>1.5</td>
<td>43</td>
<td>0.068</td>
<td>0.132</td>
</tr>
<tr>
<td>T. infestans</td>
<td>1.5</td>
<td>36</td>
<td>0.143</td>
<td>0.134</td>
</tr>
</tbody>
</table>

* An allele is considered polymorphic if the most common frequency of the allele does not exceed 0.95.
† HdyWbg, Hardy-Weinberg equilibrium.
T. infestans behaved differently from the species of the Phyllosoma complex and from T. rubida and T. lecticularia, revealing the largest number of diagnostic loci of all analyzed species. Among them were ME1, ME2, PEP2, GOT, MDH1, and LAP (Figures 1a, 1b, 1c, and 1d), which amounted to 43% of difference with the loci of other analyzed species.

**Genetic distance of Nei and phylogeny.** The genetic distance of Nei was obtained from allelic frequencies observed for each one of the species. As shown in Table 2, T. bassolsae and T. longipennis show the shortest genetic distance between them, with a value of 0.046. These two species showed a close relationship to T. pallidipennis with a distance of 0.104, while T. mazzottii displayed a distance of 0.207. The Phyllosoma complex showed a genetic distance of 0.747 with respect to T. lecticularia, of 0.809 with T. rubida, and of 1.495 with T. infestans. These relationships can be clearly observed in the phylogenetic tree constructed by UPGMA derived from Nei’s genetic distances (Figure 2). The species of the Phyllosoma complex showed close genetic distances and can therefore be grouped in a recognizable category, while species as T. lecticularia and T. rubida are clearly different to the Phyllosoma complex and to T. infestans.

**DISCUSSION**

One of the main contributions of the current work to the classification of the Phyllosoma complex species was the analysis of T. bassolsae, a species described by Alejandro-Aguilar and others in 1999, which had not been analyzed with genetic markers. It has only been described in the state of Puebla in Mexico, but its inclusion in the Phyllosoma complex demands a more detailed analysis. Another important contribution of this work was the study of the species T. lecticularia (Lecticularia complex) and T. rubida (Rubrofasciata complex). The study of these two species is necessary due to their epidemiologic importance in the north of Mexico, where they are the most important vectors of Chagas disease.

The taxonomic study showed similar values in polymorphism and allelic frequencies as those reported in the literature. In particular, the polymorphism values for the field-collected species T. longipennis and T. pallidipennis previously reported by our group were corroborated in the current study that analyzed individuals from colonies; that is, polymorphism values for T. longipennis of 50% (polymorphism = 0.95) and for T. pallidipennis of 29% (polymorphism = 0.95) were detected that are identical to the previous report. On the other hand, T. mazzottii was less polymorphic with 21% of polymorphism. Finally, T. bassolsae was the species of Phyllosoma complex with only 14% of polymorphism.

The species of the other complexes that had not been analyzed previously by isoenzymatic systems showed similar polymorphism values to T. longipennis and T. pallidipennis of the Phyllosoma complex; for example, for T. lecticularia and T. rubida polymorphism was 43%, while for T. infestans polymorphism was 36%. These results confirm that some species of the Phyllosoma complex, as well as T. lecticularia and T. rubida have enzyme polymorphism higher than T. infestans.

The scarce genetic variation in alleles per locus and monomorphic alleles among the populations analyzed in the current work could be explained by some of the characteristics of triatomines, among them, their habits of dispersion. On the other hand, breeding between sympatric species has not been proved, and this could be an important limiting factor for genetic variability in field species due to the endogamic processes, even though some of these species have been reported to share the same ecological niche.

The diagnostic loci found for T. lecticularia and T. rubida could be important for the classification of the nymphs collected in field studies and that currently cannot be identified by morphologic characters.

Interestingly, the genetic tree obtained in the current analysis reflects high similarity among species of the Phyllosoma complex with a value lower than 0.207, similarity which reveals a defined group that groups the four species of the Phyllosoma complex analyzed in this work. The short genetic distances shown by these species suggest recent evolutionary relationships and probably reflect the divergence from a common ancestor. Finally, the species T. bassolsae showed a genetic distance of 0.046 to T. longipennis and 0.104 with T. pallidipennis, which confirms that this species belongs to the Phyllosoma complex and that the three species are closely related.

The current molecular taxonomic study reveals genetic data of epidemiologically important species; such is the case of T. rubida, one of the most important vectors in the north of Mexico, which is distributed in seven states: North and South Baja California, Chihuahua, Nayarit, Nuevo Leon, Sinaloa, and Sonora. This species has been described by some authors to be constituted by five subspecies: Triatoma rubida cochimensis, Triatoma rubida jaegeri, Triatoma rubida rubida, Triatoma rubida sonoriana, and Triatoma rubida uhleri, although other authors consider it a single species with chromatic variants. This discrepancy demands a deeper phylogenetic analysis to corroborate or modify the morphologic classifications, and this first analysis is one step to address this problem. In the other hand, the species T. lecticularia has been reported with 33% of T. cruzi infection in the state of Nuevo Leon, which together with its presence in human dwellings are considered as important risk factors in

<table>
<thead>
<tr>
<th>Species</th>
<th>T. longipennis</th>
<th>T. lecticularia</th>
<th>T. infestans</th>
<th>T. bassolsae</th>
<th>T. mazzottii</th>
<th>T. rubida</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. pallidipennis</td>
<td>0.104</td>
<td>0.747</td>
<td>1.495</td>
<td>0.104</td>
<td>0.207</td>
<td>0.809</td>
</tr>
<tr>
<td>T. longipennis</td>
<td>0.747</td>
<td>1.495</td>
<td>0.046</td>
<td>0.747</td>
<td>0.809</td>
<td>0.809</td>
</tr>
<tr>
<td>T. lecticularia</td>
<td>1.495</td>
<td>0.747</td>
<td>0.747</td>
<td>1.495</td>
<td>0.809</td>
<td>1.495</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.809</td>
<td>0.809</td>
</tr>
</tbody>
</table>
In addition, distribution of these two latter species could be more extended than reported until now, as they have also been found in the southern United States.26,27 These and other vectors of Chagas disease in Mexico need to be the subject of more genetic studies to understand the complexity of genetic relationship between the nearly 28 species of vector reported until now in North America.

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