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GENETIC VARIABILITY OF *Triatoma flavida* AND *Triatoma bruneri* (HEMIPTERA: REDUVIIDAE) BY RAPD-PCR TECHNIQUE

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SUMMARY

The Triatominae (Hemiptera:Reduviidae) contains the principal and potential Chagas disease vectors present in Mexico, Central America and South America. *Triatoma flavida* and *T. bruneri* are Cuban species. These species are closely related according to morphology and were considered synonyms until 1981, when they were separated on the grounds of external characters of the body and the morphology of male genitalia. The present study seeks to analyze genetic polymorphism of *T. flavida* and *T. bruneri* populations using RAPD techniques, and to assess the genetic relationship between these species. Ten random primers were used to evaluate the genetic variability among species using RAPD-PCR. The genetic flow among them was calculated. The dendrogram based on calculated Jaccard distances showed two clearly distinguishable clusters which coincided with the studied species. Within each species, moderate genetic differentiation (Fst 0.05-0.15) and migration rates (N > 1) were found among populations, that reveal gene flow and genetic homogeneity. Between species, the Fst value showed a high genetic differentiation and the migration rate was insufficient to maintain genetic homogeneity, and confirmed the absence of gene flow between them. Our results confirm the genetic variability among *T. flavida* and *T. bruneri* species.

KEYWORDS: Triatominae; *T. flavida; T. bruneri;* RAPD; Genetic variability.

INTRODUCTION

The subfamily Triatominae (Hemiptera, Reduviidae) is currently divided in five tribes, 15 genera and 140 species[22]. Most species occur in sylvatic ecotopes, associated with small nest-building mammals or birds, but several have made the transition to domestic habitats, where they feed on humans and domestic animals[4]. In Cuba there are four species of Triatominae recorded: *Triatoma flavida* (Neiva, 1911), *Triatoma bruneri* (Usinger, 1944), *Bolbodera scabrosa* (Valdés, 1910) and *Triatoma rubrofasciata* (De Geer, 1773).

T. flavida and *T. bruneri* are autochthonous species. *T. flavida* is a sylvatic species found in the western region of Cuba that presumably is attracted to houses by electric light. This species is the most abundant in Cuba and exhibits characteristics that increase its risk as a potential vector[15]. *T. bruneri* has been found principally in the east region of Cuba, although LENT & JURBERG (1981)[16] described its presence in the western region (Pinar del Rio). There are few reports of its habitat, biology and ecology, although domestic colonies have been encountered[10].

Both species are closely related according to morphology, and between 1946 and 1981 they were considered synonymous. After 1981, they were separated on the grounds of external characters of the body and the morphology of male genitalia and considered different species[16]. HYPŠA *et al.* (2002)[13] published a phylogenetic study of 57 species of Triatominae and proposed the elevation of the ¨*T. flavida* complex¨ to the genus *Nesotriatoma* (including *N. flavida, N. bruneri* and *N. obscura*) according to the position of the clade *T. flavida* and *T. bruneri*, although this idea is not generally accepted[22].

The analysis of genetic variation in Triatominae using molecular methods constitutes an essential element for populational analysis and also for taxonomic, ecology, biosystematic, epidemiologic, and evolutionary studies. The RAPD is a useful technique[9]; RAPD is a dominant marker and therefore the analysis makes certain underlying assumptions, however the technique has been used in studies of Triatominae such as: *T. infestans*[19], *T. dimidiate*[21], *T. brasiliensis*[3], *T. venosa*[24], *T. vitticeps*[18], *Rhodnius prolixus*[14,17], *R. colombiensis*[14] and *R. robustus*[5] allowing discrimination of species, genetic variation, and estimates of gene flow between sylvatic, domiciliary and peri-domiciliary populations of these vectors.

The present study seeks to analyze genetic polymorphism of *T. flavida* and *T. bruneri* populations using the RAPD techniques and assess the genetic relationship between these species.

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MATERIAL AND METHODS

Triatominae: In this study we used 31 *T. flavida* adults collected in sylvatic habitats (caves) in different localities of Peninsula de Guanahacabibes, Pinar del Río province, and 29 *T. bruneri* adults captured inside houses in different localities of Holguín province (Table 1) (Fig. 1). All insects were classified as *T. flavida* and *T. bruneri* respectively in accordance with USINGER (1944)[23] and LENT & JURBERG (1981)[16]. As out-group we used one *T. rubrofasciata* specimen from the collection from the Vector Control Department of the Institute of Tropical Medicine ¨Pedro Kouri¨ (IPK). This specimen was collected in the province of Santiago de Cuba.

DNA extraction: Each adult bug was examined individually. A leg was selected as source of genomic DNA using the potassium acetate modified method[6]. The leg was ground to a fine powder with tissue grinders in a 1.5 mL microcentrifuge tube that contained liquid nitrogen. After grinding, the liquid nitrogen was allowed to evaporate and the powder resuspended in 150 µL of lysis buffer (20 mM tris-HCl, pH 8.25, 25 mM EDTA, 25 mM NaCl, 1% SDS). The suspension was incubated with 100 µg/mL of proteinase K (Boehringer Mannheim, Germany) for one hour at 56 °C. Nucleic acids were extracted with 100 µL of potassium acetate 3 M, ice incubation for one hour and centrifugation at 8000 xg for 10 min at 4 °C. The DNA was precipitated by adding two volumes of absolute ethanol containing 0.3 M sodium acetate and placed at - 20 °C for 30 minutes. The precipitated DNA was centrifuged at 10000 xg for 20 min. and the pellet washed in 70% ethanol. After air drying, the DNA was dissolved in 50 µL tris-EDTA buffer (TE) (1 mM tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Any remaining RNA was eliminated with RNaseH (Boehringer Mannheim, Germany) and the suspension was incubated for one hour at 37 °C. After extraction with equal volume of chloroform-isoamyl alcohol (24: 1), the aqueous phase was conserved at - 20 °C. The DNA concentration

Table 1
Data of *T. flavida* and *T. bruneri* populations studied

Specie	Number of specimens (Origin)	Sex	Collection site	Municipality, Province.
T. flavida	34 (S)	F	Caimanera	Guanahacabibes, Pinar del Río
	C (S)	M	Caimanera	Guanahacabibes, Pinar del Río
	35 (S)	M	Caimanera	Guanahacabibes, Pinar del Río
	36 (S)	F	Caimanera	Guanahacabibes, Pinar del Río
	37 (S)	M	Caimanera	Guanahacabibes, Pinar del Río
	38 (S)	M	Caimanera	Guanahacabibes, Pinar del Río
	39 (S)	F	Caimanera	Guanahacabibes, Pinar del Río
	40 (S)	F	Caimanera	Guanahacabibes, Pinar del Río
	108 (S)	F	Casito	Guanahacabibes, Pinar del Río
	127 (S)	F	Casito	Guanahacabibes, Pinar del Río
	109 (S)	M	Casito	Guanahacabibes, Pinar del Río
	128 (S)	M	Casito	Guanahacabibes, Pinar del Río
	48 (S)	M	Suerte amparo	Guanahacabibes, Pinar del Río
	110 (S)	M	Suerte amparo	Guanahacabibes, Pinar del Río
	111 (S)	F	Suerte amparo	Guanahacabibes, Pinar del Río
	49 (S)	F	Motor del Veral	Guanahacabibes, Pinar del Río
	50 (S)	F	Motor del Veral	Guanahacabibes, Pinar del Río
	107 (S)	M	Helicoptero	Guanahacabibes, Pinar del Río
	115 (S)	M	Helicoptero	Guanahacabibes, Pinar del Río
	112 (S)	F	La sorda	Guanahacabibes, Pinar del Río
	113 (S)	F	La sorda	Guanahacabibes, Pinar del Río
	106 (S)	F	Frances	Guanahacabibes, Pinar del Río
	114 (S)	M	Frances	Guanahacabibes, Pinar del Río
	117 (S)	M	Frances	Guanahacabibes, Pinar del Río
	118 (S)	F	Frances	Guanahacabibes, Pinar del Río
	43(S)	F	La Barca	Guanahacabibes, Pinar del Río
	44 (S)	M	La Barca	Guanahacabibes, Pinar del Río
	45 (S)	F	La Barca	Guanahacabibes, Pinar del Río
	46 (S)	M	La Barca	Guanahacabibes, Pinar del Río
	33 (S)	F	Iguana	Guanahacabibes, Pinar del Río
	116 (S)	M	Iguana	Guanahacabibes, Pinar del Río

Table 1
Data of *T. flavida* and *T. bruneri* populations studied (cont.)

Specie	Number of specimens (Origin)	Sex	Collection site	Municipality, Province.
T. bruneri	202 (D)	F	Coto	Urbano Noris, Holguín
	203 (D)	F	Coto	Urbano Noris, Holguín
	204 (D)	M	Coto	Urbano Noris, Holguín
	205 (D)	F	Coto	Urbano Noris, Holguín
	206 (D)	M	Coto	Urbano Noris, Holguín
	207 (D)	F	Coto	Urbano Noris, Holguín
	208 (D)	F	Coto	Urbano Noris, Holguín
	209 (D)	M	Coto	Urbano Noris, Holguín
	210 (D)	F	Coto	Urbano Noris, Holguín
	211 (D)	F	Coto	Urbano Noris, Holguín
	212 (D)	M	Coto	Urbano Noris, Holguín
	213 (D)	F	Coto	Urbano Noris, Holguín
	214 (D)	M	Coto	Urbano Noris, Holguín
	215 (D)	M	Coto	Urbano Noris, Holguín
	216 (D)	M	Coto	Urbano Noris, Holguín
	217 (D)	F	Coto	Urbano Noris, Holguín
	218 (D)	F	Coto	Urbano Noris, Holguín
	219 (D)	M	Coto	Urbano Noris, Holguín
	220 (D)	F	Coto	Urbano Noris, Holguín
	221 (D)	M	Coto	Urbano Noris, Holguín
	222 (D)	F	Iberia	Gibara, Holguín
	223 (D)	M	Iberia	Gibara, Holguín
	224 (D)	M	Iberia	Gibara, Holguín
	225 (D)	M	Iberia	Gibara, Holguín
	226 (D)	M	Guanina	Mayari, Holguín
	227 (D)	M	Guanina	Mayari, Holguín
	228 (D)	M	Dos Hermanas	Cacocum, Holguín
	229 (D)	M	Dos Hermanas	Cacocum, Holguín
	230 (D)	M	Dos Hermanas	Cacocum, Holguín

S: sylvatic; D: domestic

was estimated spectrophotometrically by reading absorbance at 260 nm and the purity of the sample was examined by electrophoresis with a 0.8% agarose gel in TBE buffer (TBE 0.5x) (0.045 M tris-borate, 0,001 M EDTA) containing ethidium bromide (0.5 mg/mL) with visualization using a UV transilluminator (Macrovue 2011, LKB).

RAPD-PCR: RAPD protocol previously optimized[8], was used for the amplification of random DNA markers to reveal genetic diversity among *T. flavida* and *T. bruneri* populations using ten oligonucleotides primers (OPA-1 to 10). The amplification was performed in a final volume of 25 µL containing 2.5 µL of 10 x PCR reaction buffer (Promega, USA) and 200 µM of each dNTPs (Promega, USA) with 5 pmol of primer, 2.5 mM of $MgCl_2$, 25 ng of template DNA and 2.0 U of Taq DNA polymerase (Promega, USA). Negative controls for each assay contained all components above, except template DNA of *T. flavida* and *T. bruneri*, with the objective of discarding any contamination. In a thermocycler (Perkin Elmer, USA) the reactions were amplified as follows: initial denaturation at 94 °C for five min, followed by 45 repetitions of one min at 94 °C, one min at 36 °C and two min at 72 °C. In the final cycle, the extension step was of 15 min. Analysis was done by electrophoresis in 1.2% agarose gels in TBE buffer containing ethidium bromide (0.5 mg/mL) with visualization using a UV transiluminator. The presence or absence of each band was scored visually.

Genetic polymorphism: For the analysis, it is assumed that (a) the *T. flavida* and *T. bruneri* insects are in Hardy-Weinberg equilibrium and therefore no selection processes favouring any particular genotype, (b) RAPD markers segregate in a Mendelian fashion with constant evolution or substitution rate and (c) recessive (band absent) alleles are identical in state among and between individuals. Individual bands were scored as present or absent (1 or 0, respectively) for each specimen and the Jaccard similarity coefficient (Sj) was used, as follows: $Sj = a/(a + b + c)$ where ¨a¨ represents the number of shared bands, ¨b¨ represents the number of bands present in specimen 1 and absent in specimen 2 and ¨c¨ represents the number of bands present in specimen 2 and absent in specimen 1.

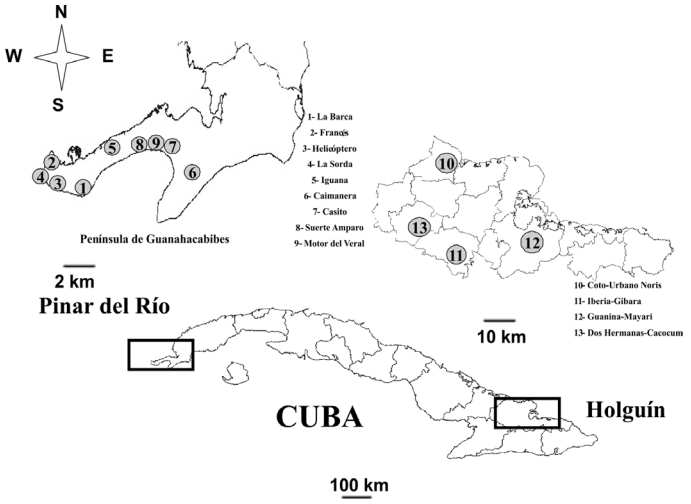

Fig. 1 - Geographical location of the capture sites of Triatominae studied.

Phylogenetic relationships were determined by the group-average clustering strategy or the unweighted pair group method with arithmetic averages (UPGMA) using FreeTree, version 0.9.1.59.[20]. Dendograms were constructed based on data from ten primers and bootstrap analysis with 2000 replicates.

Genetic flow: WRIGHT´s (1951)[26] and WEIR & COCKERHAM's (1984)[25] fixation index (Fst) and the effective migration rate (Nm) were estimated by RAPDFST[2]. The analysis was made with the assumption that the mutation rates are lower than the insect migration rates.

RESULTS

From the 115 reproducible RAPD bands generated, 85 bands showed polymorphism among species and 30 were monomorphic.

Comparison of the RAPD profiles showed the presence of species-specific bands. Amplification with OPA-2 primer (Fig. 2A) revealed a genetic marker of 630 bp in all individuals of *T. bruneri* that was absent from *T. flavida*. Also a 700 bp genetic marker was observed in all *T. flavida* individuals, that was absent in *T. bruneri*. The RAPD banding patterns obtained with OPA-4 primers (Fig. 2B) showed a genetic marker of 500 bp in all *T. flavida* and 750 bp genetic marker in all *T. bruneri* individuals.

The dendrogram based on Jaccard distances (Fig. 3) showed two clearly distinguishable clusters: (1) *T. flavida*; (2) *T. bruneri* suggested by high bootstrap value (100%). The tree also shows a correlation with geographic origin within each species. Two main groups can be recognized within *T. flavida* group with high bootstrap value (89 and 80%, respectively), they matched with the populations captured in areas from west and east of Guanahacabibes. *T. bruneri* individuals are grouped in three major clusters corresponding to the individuals of Coto, Iberia-Dos Hermanas and Guanina (Fig. 1).

The heterogeneity obtained in the banding patterns suggests high genetic differentiation between *T. flavida* and *T. bruneri*. The individuals of *T. flavida* group with a genetic distance of 0.1217 (Similarity

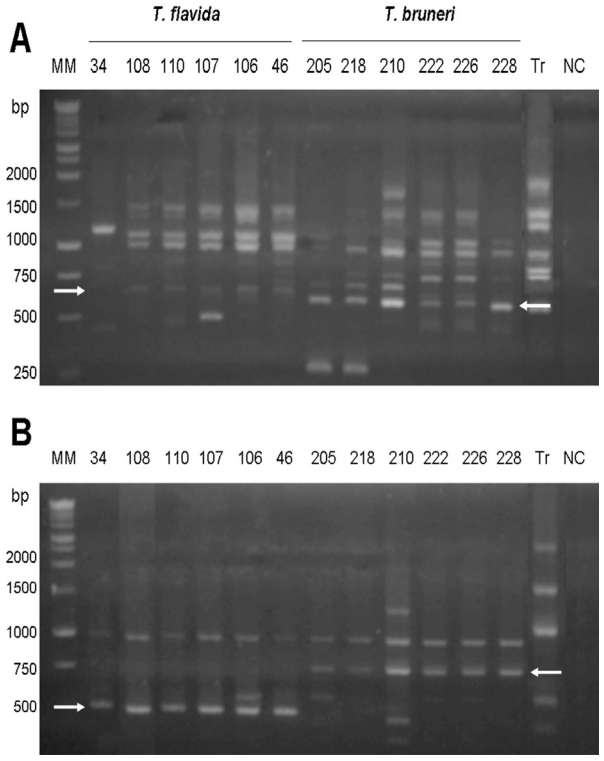

Fig. 2 - RAPD banding patterns obtained from 12 Triatominae using OPA-2 (A) and OPA-4 (B) primers. Lane MM. Molecular Weight Marker 1 kbp (Promega, USA); Lanes 34, 108, 110, 107, 106, 46: *T. flavida* bugs; Lanes 205, 218, 210, 222, 226, 228: *T. bruneri* bugs; Lane Tr: *T. rubrofasciata*; NC: Negative control without DNA. Arrows represent the specie specific RAPD genetic markers.

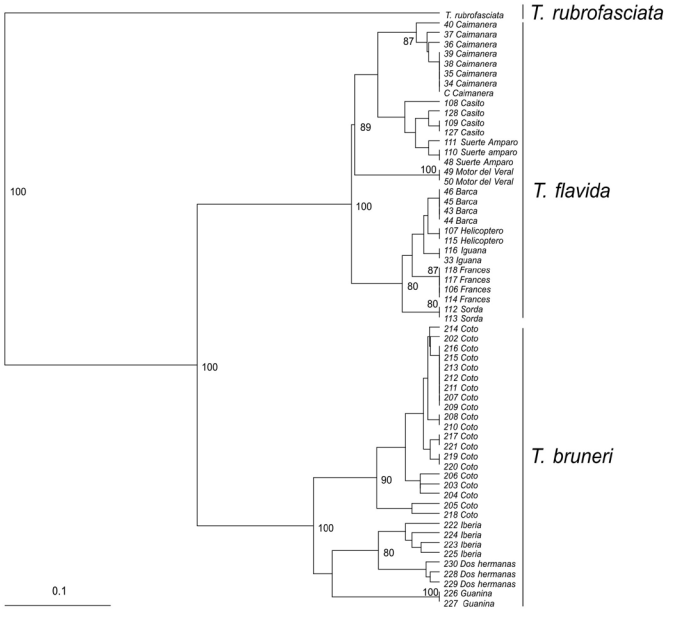

Fig. 3 - Dendogram generated for specimens populations of *T. flavida* and *T. bruneri* using Jaccard's dissimilarity index and the UPGMA algorithm. *T.rubrofasciata*: out-group. Bootstrap support of the branches was inferred from 2000 replicates, and is given in percentages at the internodes when exceeding 80%.

Table 2
Fixation index and effective migration rate values among *T. flavida* and *T. bruneri* species

Methodologies		Among *T. flavida* populations	Among *T. bruneri* populations	Among *T. flavida* and *T.bruneri*
Wright	Fst	0.055	0.090	0.36
	Nm	8.4	7.6	0.7
Weir and Cockerham	Fst	0.052	0.087	0.35
	Nm	8.6	7.2	0.6

Coefficient 87.83%) while *T. bruneri* group with a genetic distance of 0.1607 (Similarity Coefficient 83.92%). *T. bruneri* presents higher genetic variability when compared to *T. flavida*. However, within each species the Fst value showed a moderate genetic differentiation (Fst 0.05-0.15), according to Wright's classification (WRIGHT, 1978)[27] and migration rate (Nm > 1) among populations that reveal gene flow and genetic homogeneity (Table 2).

The heterogeneity in the RAPD banding pattern suggests a high genetic differentiation between *T. flavida* and *T. bruneri.* The species separate with a genetic distance of 0.4614 (Similarity Coefficient 53.86%). These results were corroborated by the fixation index and the effective migration rate. The Fst value showed a high genetic differentiation (Fst > 0.25) and the migration rate (Nm < 1) was insufficient to maintain genetic homogeneity between both species (Table 2).

DISCUSSION

Triatominae appear to be a polyphyletic group, with generalist species displaying higher genetic and phenetic variation and specialist species showing relatively reduced population variability, probably associated with a series of genetic bottlenecks (founder effects) followed by drift and modest selection[11].

RAPD markers are amplification products of DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate. A major drawback of RAPD markers in population genetic studies of out breeding organisms is that they are dominant. Thus gene frequency estimates for such loci are necessarily less accurate than those obtained with codominant markers such as allozymes and RFLPs. Some authors suggested that two to 10 times more individuals need to be sampled for dominant markers to achieve the same degree of statistical power as codominant markers such as allozymes and RFLPs[1]. The assumption of homology between bands of apparently the same molecular weight from the same primer is potentially another problem for RAPD surveys. Homology between comigrating bands in different individuals is a good assumption when individuals are from the same population. This may not be true when individuals belong to different species or widely divergent populations. Because the chance of comigrating bands being homologous becomes less as populations diverge, it was suggested that RAPD analysis gives more accurate estimates between closely related populations and less accurate estimates for distantly related populations. RAPD data has been used for phylogenetic studies and generally supported existing taxonomies based on morphology, isozymes and RFLPs[1].

For the first time we analyze the genetic variability among sylvatic *T. flavida* and domestic *T. bruneri* populations collected in the western and eastern regions of Cuba, respectively, using RAPD. We chose the OPA 1-10 primers because these primers were previously used in studies of genetic polymorphism between 16 *T. flavida* specimens from nine populations. The polymorphisms obtained with these primers were reproducible among samples of the same specimens[6-7].

In our work the heterogeneity observed in the RAPD banding patterns and the genetic distance obtained among the species together with the estimates of Fst and Nm allows concluding that *T. flavida* and *T. bruneri* have a high genetic variability although there is evidence of spatial structuring of their populations. These results are in concordance with LENT & JURBERG (1981)[16] who proposed separation of the two species based on characters of the external body and morphology of genitalia.

In the present study, we found low genetic variability (Genetic distance 0.1217) among sylvatic *T. flavida* individuals. Fst value showed a moderate genetic differentiation (Fst 0.05-0.15) and migration rate (Nm > 1) among populations, that reveal gene flow and genetic homogeneity. This result is similar with our previous study carried out with a lower number of *T. flavida* individuals (16 bugs) from nine populations collected in the same place (Peninsula de Guanahacabibes, Pinar del Río, western region of Cuba). In this study we reported low genetic variability (Genetic distance 0.107), small genetic differentiation (Fst 0.030) and migration rate (N > 1) that reveal gene flow and genetic homogeneity between sylvatic individuals of this specie[7]. *T. bruneri* individuals are more variable than sylvatic *T. flavida.* Also among populations this specie showed gene flow and genetic homogeneity. The higher genetic variability of the domiciliated *T. bruneri* may be due to a recent process of domestication.

Although the relevance of *T. flavida* and *T. bruneri* in the transmission of Chagas disease is currently unknown, and no natural infection with trypanosomes has been reported, *T. flavida* has been shown capable of transmitting the disease under laboratory conditions[12]. These two species should be considered in future epidemiological surveillance programmes.

RESUMEN

Variabilidad genética de *Triatoma flavida* y *Triatoma bruneri* (Hemiptera: Reduviidae) mediante la técnica de RAPD-PCR

La subfamilia Triatominae (Hemiptera: Reduviidae) agrupa a los vectores principales y potenciales de la Enfermedad de Chagas, presente en México, Centroamérica y Sudamérica, *Triatoma flavida* y *T. bruneri* son especies autóctonas cubanas. Estas especies están muy relacionadas desde el punto de vista morfológico y por ello fueron consideradas sinonimas hasta el 1981, cuando fueron separadas teniendo en cuenta los caracteres

externos del cuerpo y la morfología de la genitalia del macho. El presente trabajo pretende confirmar el polimorfismo genético entre las poblaciones selváticas de *T. flavida* y domiciliadas de *T. bruneri* utilizando la técnica de RAPD-PCR. Un total de 10 cebadores al azar fueron usados para evaluar la variabilidad genética entre las especies usando la técnica de RAPD-PCR, calculándose el flujo genético entre las especies. El dendrograma obtenido, basado en la distancia genética de Jaccard, mostró dos grupos que coinciden con las especies estudiadas. Dentro de cada especie estudiada se encontró una moderada diferenciación genética (Fst 0.05-015) y tasas de migración (N > 1) que revelan flujo genético y homogeneidad genética. Entre las especies estudiadas los valores de Fst muestran una alta diferenciación genética y tasas de migración insuficientes para mantener homogeneidad genética y confirman la ausencia de flujo genético entre ellas. Estos resultados confirman la variabilidad genética entre ambas especies.

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AUTHOR CONSTRIBUTION

JR, OF, YH, MC and RG collected and classified the Triatominaes; JF, AFC, JR performed the laboratory work; JF, JR, OF, YH, MC, RG and AFC analyzed and interpreted the results; JF, JR and OF drafted the manuscript; JF, YH, OF, YH, MC, AFC critically revised the manuscript.

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