

GENETIC VARIABILITY OF *TRITOMA RUBROVARIA* (REDUVIIDAE: TRIATOMINAE) FROM BRAZIL, ARGENTINA AND URUGUAY AS REVEALED BY TWO DIFFERENT MOLECULAR MARKERS

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Summary:

Randomly amplified polymorphic DNA (RAPD) and nuclear ribosomal DNA sequence analyses were used to assess the genetic population structure of the South American triatomine species *Triatoma rubrovaria* throughout its geographical distribution. To investigate the genetic variability at both intraspecific and intrapopulation levels the RAPD profiles and the nucleotide sequences of the rDNA intergenic spacers, ITS-1 and ITS-2, were analysed and compared. The phenetic analysis based on RAPD profiles show three distinct clusters diverging by similarity coefficients ranging from 0.62 to 0.96. The ITS-1 and ITS-2 sequence variability detected may be considered very high, suggesting reproductive isolation between populations. A total of seven composite haplotypes (CH) were found, among which three are specific for Brazil, other three for Uruguay, and the last one common for the three countries studied. The population studied in Argentina does not represent an independent CH. Sequence analyses proved that the five populations studied are easily differentiable and that there is heterogeneity within each one. True mutations and indels are the responsible of sequence differences between haplotypes and populations, suggesting that divergence processes may presently go on within this species. The large intraspecific variability detected may underlie the known plasticity of *T. rubrovaria*, making it a potential intradomiciliary invader and consequently an appropriate vector for Chagas disease transmission. Therefore, this triatomine species must be continuously monitored throughout.

KEY WORDS : *Triatoma rubrovaria*, RAPD, ribosomal DNA, population genetics, South America.

Résumé : VARIABILITÉ GÉNÉTIQUE DES POPULATIONS DE *TRITOMA RUBROVARIA* (REDUVIIDAE : TRIATOMINAE) DU BRÉSIL, D'ARGENTINE ET D'URUGUAY, ÉTUDIÉE PAR DEUX MARQUEURS MOLÉCULAIRES DIFFÉRENTS

L'amplification aléatoire d'ADN polymorphe (RAPD) et l'analyse des séquences de l'ADN ribosomal nucléaire ont été utilisées pour évaluer la structure génétique de populations de *Triatoma rubrovaria*, une espèce de triatomines de l'Amérique du Sud, selon leur distribution géographique. La variabilité génétique a été investiguée aux niveaux intraspécifique et intrapopulationnel, en utilisant l'analyse et la comparaison des RAPD et des séquences nucléotidiques complètes des espaces intergéniques de l'ADN ribosomal, ITS-1 et ITS-2. L'analyse phénétique basée sur des profils RAPD montre trois groupes distincts qui divergent par des coefficients de similarité allant de 0.62 à 0.96. La variabilité des séquences de l'ITS-1 et de l'ITS-2 observée est considérée comme très élevée, suggérant un isolement reproductif entre les populations. Sept haplotypes composés (CH) ont été trouvés, parmi lesquels trois sont spécifiques du Brésil, trois de l'Uruguay, le dernier étant commun aux trois pays étudiés. La population de l'Argentine ne présente pas de CH indépendant. Les analyses des séquences prouvent que les cinq populations étudiées sont facilement différenciables, bien qu'il existe une hétérogénéité au sein de chacune d'elles. Des vraies mutations et des indels sont les responsables des différences d'ordre entre les haplotypes et les populations, suggérant que les processus de divergence puissent actuellement continuer au sein de cette espèce. La grande variabilité intraspécifique observée souligne la plasticité connue de *T. rubrovaria*, en faisant un envahisseur potentiel du domicile et par conséquent un vecteur approprié pour la transmission de la maladie de Chagas. Cette espèce de triatomines doit donc être continuellement contrôlée.

MOTS CLÉS : *Triatoma rubrovaria*, RAPD, ADN ribosomal, génétique des populations, Amérique du Sud.

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INTRODUCTION

Triatoma rubrovaria (Blanchard in Blanchard et Brullé 1843), a member of the “infestans” subgroup, is a potential vector of Chagas disease. Its geographic distribution includes Southern Brazil, mainly the States of Rio Grande do Sul and Paraná, part of North-eastern Argentina and Uruguay (Dujardin *et al.*, 2000). This species is considered a generalistic insect that feeds on a variety of vertebrate and invertebrate hosts (Salvatella *et al.*, 1995). Biological aspects such as feeding, defaecation behaviour, resistance to starvation and life cycle make *T. rubrovaria* a potential vector for *Trypanosoma cruzi* transmission (Bar

et al., 2003; Almeida *et al.*, 2005). In fact, such species has already been found naturally infected with *Trypanosoma cruzi* in the three countries (Salvatella *et al.*, 1995). Previous studies have demonstrated that in the State of Rio Grande do Sul, in Brazil, two genetically distinct populations of *T. rubrovaria* are present (Almeida *et al.*, 2002; Pacheco *et al.*, 2003).

Due to the epidemiological importance of *T. rubrovaria* as a potential vector of Chagas disease, the present work aims to genetically characterize the population structure of specimens from Brazil, Argentina and Uruguay in the way to evaluate its spreading capacity and human influence on its population structure. RAPD analyses and sequencing of the complete intergenic region of the nuclear rDNA, including the spacers ITS-1 and ITS-2, are used to compare *T. rubrovaria* populations from the three South American countries.

MATERIALS AND METHODS

TRIATOMA RUBROVARIUM SPECIMENS

A total of 12 adult specimens were captured in the Municipalities of Santana do Livramento (L07, L09, L10, L11, L12, L19) and Santiago (S01, S03, S13, S14, S25, S26), State of Rio Grande do Sul, RS, Brazil. From Argentina a total of nine adult specimens (Co01, Co02, Co03, Co04, Co05, Co06, Co07, Co08, Co09) were captured in an area of natural rock piles distant 37 km from the Municipality of Mercedes, Corrientes (Bar *et al.*, 2003; Damborsky *et al.*, 2005). From Uruguay a total of four adult specimens from the Department of Salto (Sal13, Sal15) and Río Negro (RN16, RN17)



Fig. 1. – Map showing the localities of *Triatoma rubrovaria* specimens captured in the three South American countries.

were analysed (Fig. 1). All insects were taxonomically identified as *T. rubrovaria* using the keys of Lent and Wygodzinsky (1979).

RAPD AND NUMERICAL (PHENETIC) ANALYSES

23 out of the 25 specimens of *T. rubrovaria* (two from Brazil were lost during DNA extraction procedures) were studied. DNA was extracted according to the protocol previously described (Pacheco *et al.*, 2003). As negative control and outgroup, DNA from *Triatoma sordida* was included in each gel.

Phenetic analyses were carried out using the Simple-matching coefficient of similarity to determine the proportion of mismatched bands between pairs of *T. rubrovaria*. The similarity matrix was transformed into a phenogram using the UPGMA algorithm and the NTSys software programme (Exeter software, Setanker, NY).

rDNA SEQUENCE ANALYSES

For DNA extraction, one or two legs fixed in ethanol 70 % from each specimen were used and processed individually, as previously described (Marcilla *et al.*, 2001) and following the methods outlined before (Bargues *et al.*, 2006). Sequencing was performed on both strands by the dideoxy chain-termination method, and with the Taq dye-terminator chemistry kit for ABI 3730 and ABI 3700 capillary system (Perkin Elmer, Foster City, CA, USA), using PCR primers. Sequences were aligned using CLUSTAL-W version 1.8 and assembly was made with the Staden Package version 1.5. The haplotype terminology used in the present paper follows the nomenclature for composite haplotyping (CH) recently proposed by Bargues *et al.* (2006). Phylogenies were inferred from rDNA ITS-1, 5.8S, ITS-2 region using maximum-likelihood (ML) estimates with PAUP v.4.0b10 (Swofford, 2002). ML parameters were optimized using the hierarchical Likelihood Ratio Test (hLRTs) implemented in Modeltest 3.7 (Posada & Crandall, 1998). Starting branch lengths were obtained using least-squares method with ML distances. To provide an assessment of the reliability of the nodes of the trees, a quartet puzzling analysis was employed with 1000 puzzling steps and 1000 Heuristic Bootstrap replicates.

RESULTS

RAPD ANALYSES

RAPD analyses revealed genotypic heterogeneity at intra and inter-populational levels in *T. rubrovaria* specimens from the three South American countries. Genetic diversity was observed in the number and size of the fragments amplified after PCR

using six different primers. The presence of conserved bands was detected in all populations. Among the six primers tested none was found to be sufficiently specific as to discriminate a particular population. Each specimen showed a different RAPD profile (Table I). Profiles generated by the primers 4, 5 and 6 produced a total of 90 characters limited to bands ranging from 300 to 1,600 base pairs (bp), of which 27 bands were shared among the populations (Table I). For the phenetic analyses, the three-primer-consensus profiles were converted into a matrix of binary data, with 0 indica-

ting the absence of a particular character (band) and 1 its presence. The Simple-matching Similarity indices were calculated using the UPGMA algorithm by comparing each pair of specimens.

The phenogram obtained placed the 23 specimens into three great clusters diverging by similarity coefficients ranging from 0.62 to 0.96 (Fig. 2; Table I). The first cluster only comprises specimens from Santiago, RS, Brazil (S01, S03, S14, S25 and S26), with two specimens (S03 and S14) appearing very closely related because of a 96 % character sharing. The second phe-

Insect code	Geographical origin	Shared bands	Phenetic cluster	Composite haplotype
S01	Santiago, Brazil	1.2.5.6.8.9.10.12.13.14.16.17.18.19.29.21.22.23.25.27	I	CH3C
S03	Santiago, Brazil	1.2.4.5.6.8.9.10.11.12.13.16.17.18.19.20.21.22.23.25.27	I	CH3C
S14	Santiago, Brazil	1.2.4.5.6.8.9.10.11.12.13.15.16.17.18.19.20.21.22.23.25.27	I	CH3C
S25	Santiago, Brazil	1.2.4.5.6.8.9.10.13.15.16.17.18.19.20.21.22.23.25.26	I	CH3C
S26	Santiago, Brazil	1.2.4.5.8.9.10.11.13.14.15.16.17.18.19.20.21.23.25.26	I	CH3D
L07	Livramento, Brazil	1.2.3.4.5.6.8.10.12.13.14.15.16.17.18.19.20.21.24.26	II	CH2A
L12	Livramento, Brazil	1.2.4.6.8.10.12.13.14.15.16.17.18.19.20.21.24.26	II	CH1A
L10	Livramento, Brazil	1.2.5.6.8.10.12.13.14.15.16.17.18.19.21.24.26	II	CH1A
RN17	Rio Negro, Uruguay	2.5.6.8.10.12.13.14.15.16.17.18.19.20.24.26	II	CH2B
L11	Livramento, Brazil	1.2.4.6.8.9.10.12.13.15.16.17.18.19.20.21.24	II	CH1A
L19	Livramento, Brazil	1.2.3.4.5.6.8.9.10.11.12.13.15.16.17.18.19.20.21.24.26	II	CH1A
Sal13	Salto, Uruguay	2.3.5.6.8.9.10.12.13.15.16.17.18.19.20.22.25	II	CH1A
Sal15	Salto, Uruguay	2.4.5.6.8.9.12.13.14.15.16.17.18.19.20.25	II	CH1B
RN16	Rio Negro, Uruguay	2.5.6.7.10.12.13.14.15.16.17.18.19.20.25.26	II	CH4B
Co01	Corrientes, Argentina	1.2.4.5.6.7.10.13.14.15.16.18.19.20.22.24	III	CH1A
Co02	Corrientes, Argentina	1.2.4.6.7.10.11.13.14.15.16.17.18.19.20.21.22.25	III	CH1A
Co03	Corrientes, Argentina	1.2.4.7.10.11.13.14.15.16.17.18.19.20.21.22.25	III	CH1A
Co05	Corrientes, Argentina	1.2.4.6.7.10.11.13.14.15.17.18.19.20.21.24.25	III	CH1A
Co06	Corrientes, Argentina	1.2.3.4.7.10.11.13.14.15.16.17.18.19.20.21.24.26	III	CH1A
Co09	Corrientes, Argentina	1.2.4.5.7.9.10.11.13.14.15.17.19.20.21.22.24.26	III	CH1A
Co08	Corrientes, Argentina	1.2.4.5.7.10.11.13.14.15.17.18.19.20.21.22.24	III	CH1A
Co04	Corrientes, Argentina	1.2.4.5.6.7.10.11.13.14.15.17.18.19.20.21.22.25.26	III	CH1A
Co07	Corrientes, Argentina	2.3.4.7.8.10.11.12.15.17.18.19.20.21.22.24.26	III	CH1A

Table I. – Electromorphs (shared bands) observed after amplification with the three RAPD primers, the phenetic clusters and respective haplotypes obtained among the *Triatoma rubrovaria* populations analysed.

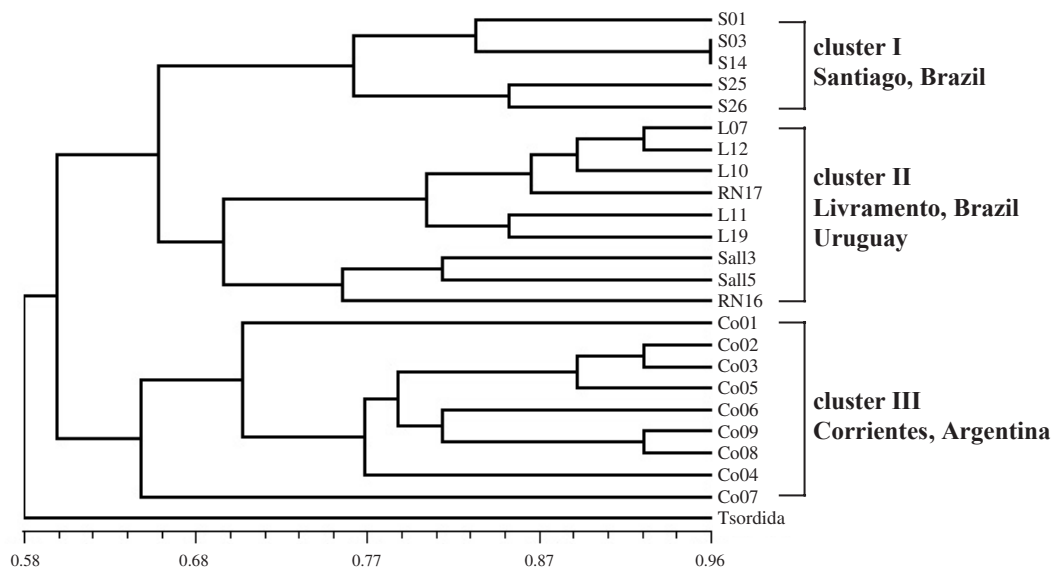


Fig. 2. – RAPD-consensus phenetic dendrogram of the triatomine specimens using the Simple Matching Coefficient of similarity.

netic group was formed by specimens of Santana do Livramento, RS, Brazil, a locality close to the Uruguay border, and by specimens from Uruguay. Within this cluster, a sub-group composed by three specimens from Uruguay (Sal13, Sal15 and RN16) appears. Interestingly, a Rio Negro specimen (RN17) clusters together with specimens from Livramento, RS, Brazil (L07, L12 and L10). The third great cluster appears composed by the nine specimens from Corrientes, Argentina, including two well-diverging specimens (Co01 and Co07) displaying similarity coefficients of 0.72 and 0.65, respectively (Fig. 2).

rDNA SEQUENCE ANALYSES

A total of seven haplotypes for the complete intergenic region analysed were detected in the populations studied. Tru-CH1A is present in Santana do Livramento (five individuals: L09, L10, L11, L12, L19), Corrientes (nine individuals: Co01 to Co09) and Salto (one individual: Sal13). Tru-CH1B, Tru-CH2A and Tru-CH2B are present in one individual from each Salto (Sal15), Santana do Livramento (L07) and Río Negro (RN17), respectively. Tru-CH3C is present in five individuals from Santiago (S01, S03, S13, S14, S25) and Tru-CH3D and Tru-CH4B are present in only one individual from each Santiago (S26) and Río Negro (RN16), respectively (Table I). When haplotype presence/absence is analysed by countries, a total of four haplotypes are present in Brazil, (Tru-CH1A, Tru-CH2A, Tru-CH3C and Tru-CH3D), four haplotypes in Uruguay (Tru-CH1A, Tru-CH1B, Tru-CH2B and Tru-CH4B), and only one in Argentina (Tru-CH1A). The only haplotype simultaneously present in the three countries analysed is Tru-CH1A. The other six previously mentioned haplotypes could be considered as specific, three for Brazil and another different three for Uruguay (Table II).

The seven haplotype sequences have been deposited in the EMBL, GenBank and DDBJ databases, where they are available under the following accession numbers: Tru-CH1A = AJ557258, Tru-CH2A = AJ557259, Tru-CH3C = AJ557260, Tru-CH3D = AJ557261, Tru-CH1B = AM259052, Tru-CH2B = AM259053, and Tru-CH4B = AM259054.

The complete alignment of the seven haplotype sequences was 1,377 bp-long, showing a genetic divergence of 1.31 %. Nucleotide length and AT content of both spacer sequences and 5.8S gene of all populations studied are noted in Table II. Concerning the ITS-1, four haplotypes were detected. These haplotypes are Tru-A, B, C and D and are distributed as follows: Tru-A is the most abundant and present in Brazil (Santana do Livramento), Argentina (Corrientes) and Uruguay (Salto); Tru-B is exclusive for Uruguay (Salto and Río Negro), and Tru-C and Tru-D are both exclusive for Brazil (Santiago). In the 730-bp-long alignment inclu-

ding the four different ITS-1 haplotypes, nine nucleotide differences (1.23 %) appeared, of which three (0.41 %) were mutations, including two transitions (ts) and one transversion (tv), plus six (0.82 %) insertions/deletions (indels). Sequence repeats were detected and included a minisatellite of 31 bp tandemly repeated two times between positions 292 and 356 of the haplotype total sequence alignment. At the level of ITS-2, four different haplotypes were detected too. These haplotypes are Tru-1, 2, 3 and 4 and are distributed as follows: Tru-1 is present in Brazil (Santana do Livramento), Argentina (Corrientes) and Uruguay (Salto); Tru-2 is present in Brazil (Santana do Livramento) and Uruguay (Río Negro), and Tru-4 is exclusive for Uruguay (Río Negro). A total of nine nucleotide differences were detected in the 492-bp-long alignment, including two ts (0.41 %) and seven indels (1.42 %), representing a 1.83 % sequence divergence. The length of the ITS-2 also differs between populations, being only four and one base shorter in specimens from Río Negro (487 bp) and Santiago (490 bp), respectively, than in specimens from Santana do Livramento, Salto and Río Negro (491 bp). In the ITS-2 sequence, the presence of the interrupted microsatellite (AT)_{5,6} TTTT (AT)₁ AA (AT)₇ was detected in all individuals and populations studied.

Phylogenetic analyses were performed including the seven *T. rubrovaria* CH and *T. sordida* (AJ576063) as outgroup. The ML model best fitting the data set was HKY85 + I using the ts/tv ratio of 2.0314. The resulting phylogeny (- Ln = 2089.0427) confirm the separation of haplotypes Tru-CH3C and Tru-CH3D from Santiago, Brazil from the rest of the haplotypes with a 96 and 67 of puzzle and bootstrap values respectively. A relatively low support values appear in the two clades comprising: a) the two haplotypes of *T. rubrovaria* from Río Negro (Uruguay) and; b) haplotypes from Livramento (Brazil), Salto (Uruguay) and Corrientes (Argentina) (Fig. 3).

DISCUSSION

In the present study, the consensus phenogram based on RAPD profiles reveals an unequivocal discrimination between two geographically distinct *T. rubrovaria* populations (Santiago in Brazil and Corrientes in Argentina). In addition, both populations appear to be geographically structured, which may suggest a founder effect. All suggests that the geographic distance between populations is an important factor determining its genetic structure, as already detected in *Triatoma infestans* (Dujardin *et al.*, 1998; Bargues *et al.*, 2006). On the other hand, the second phenetic cluster of the phenogram includes specimens geographically less distant despite proceeding from different

<i>T. rubrovaria</i> haplotypes n = 25	ITS-1											ITS-2																
	Localities	bp length	%	Nucleotide position								bp length	%	Nucleotide position														
				25	27	28	29	190	194	278	344			594	939	940	962	963	1039	1066	1327	1328	1377					
Tru-CH1A (n = 15)	Santana do Livramento Corrientes Salto	729	66.25	↔	A	T	A	T	A	A	-	G	T	491	78.00	A	T	A	T	A	T	A	T	A	T	-	G	T
Tru-CH1B (n = 1)	Salto	728	66.35	A	T	A	T	A	A	A	-	A	-	491	78.00	A	T	A	T	A	T	A	T	A	T	-	G	T
Tru-CH2A (n = 1)	Santana do Livramento	729	66.25	A	T	A	T	A	A	A	-	G	T	491	78.20	A	T	A	T	A	T	A	T	A	T	-	A	T
Tru-CH2B (n = 1)	Rio Negro	728	66.35	A	T	A	T	A	A	A	-	A	-	491	78.20	A	T	A	T	A	T	A	T	A	T	-	A	T
Tru-CH3C (n = 5)	Santiago	725	65.79	G	-	-	-	C	-	G	A	-	G	490	77.96	A	T	A	T	A	T	A	T	A	T	-	A	G
Tru-CH3D (n = 1)	Santiago	725	65.93	A	-	-	-	C	-	G	A	-	G	490	77.96	A	T	A	T	A	T	A	T	A	T	-	A	G
Tru-CH4B (n = 1)	Rio Negro	728	66.35	A	T	A	T	A	A	A	-	A	-	487	77.82	-	-	-	-	-	-	G	T	-	-	A	T	

↔ = autapomorphic positions; * = parsimony informative positions; - = insertion/deletion.

Table II. - Interhaplotype sequence differences found in the rDNA ITS-1, 5.8S and ITS-2 region of the *T. rubrovaria* populations analysed. bp = nucleotide length in base pairs; AT = composition in AT percentage; n = number of specimens analysed; CH = composite haplotype; 1,2,3,4 = different ITS-2 haplotypes; A,B,C,D = different ITS-1 haplotypes.

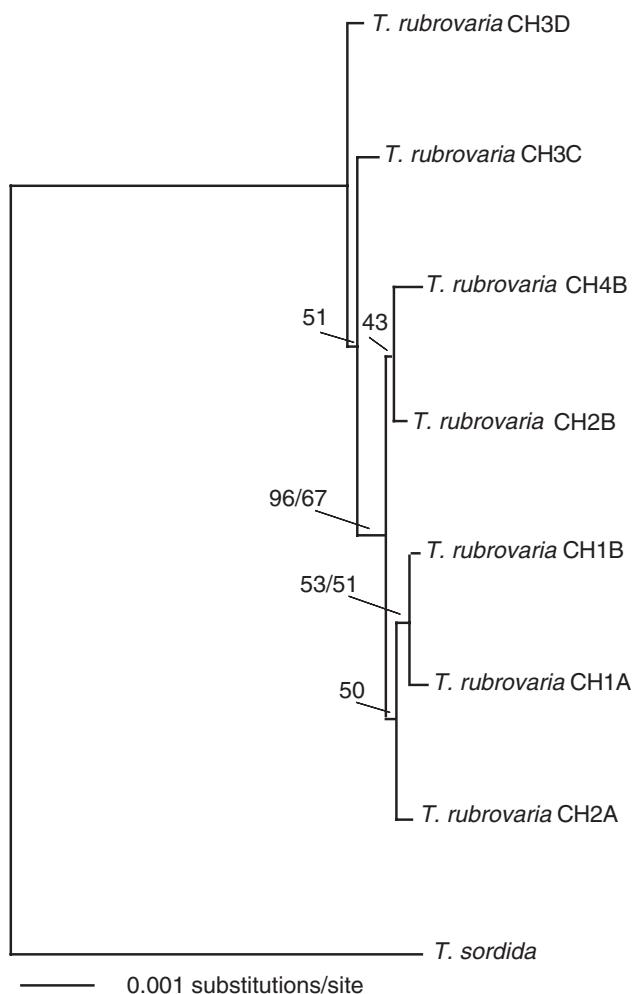


Fig. 3. – Phylogenetic tree of *T. rubrovaria* haplotypes, based on complete sequences of rDNA ITS-1, 5.8S and ITS-2, derived from the maximum likelihood (ML HKY85+I) model using *Triatoma sordida* as outgroup. Numbers represent the percentage of 1,000 puzzling/bootstrap replicates. Scale bar indicates the number of substitutions per sequence position.

countries. No genetic-geographic association was found suggesting a dispersal capability and reshuffling of the haplotypes.

Concerning ITSs, and taking into account the capacity of ITS-1 and ITS-2 sequences to differentiate species, subspecies, hybrids and populations in Triatominae (Bargues *et al.*, 2002, 2006; Marcilla *et al.*, 2001, 2002; Pacheco *et al.*, 2003), several conclusions can be reached: *a*) all individuals studied belong to the same species *T. rubrovaria*; *b*) the individuals analysed from the Brazilian localities of Santana do Livramento and Santiago belong to two clearly differentiable populations; *c*) the populations from Salto and Río Negro in Uruguay differ between them in three mutations and five indels, showing a genetic intra-population variety; *d*) only one individual from Salto (Sal13) shows the same haplotype (Tru-CH1A) as the other five individuals from Santana do Livramento in Brazil and nine

individuals from Corrientes, Argentina; *e*) three haplotypes (Tru-CH1B, Tru-CH2B, Tru-CH4B) are exclusive for Uruguay populations and other three are exclusives for Brazil populations (Tru-CH2A, Tru-CH3C, Tru-CH3D); *f*) there are only one haplotype (Tru-CH1A) simultaneously present in Brazil, Argentina and Uruguay, it appears to be the most frequent one (detected in 15 of 25 specimens analysed); *g*) in Corrientes, Argentina, all individuals analysed present the same haplotype (Tru-CH1A), without intra-population variety.

Of the total of 18 nucleotide differences detected in the 1377-bp-long alignment of the seven different sequences of the ITS-1, 5.8S and ITS-2 rDNA region, the majority (13) are indels and only five are true mutations, providing intra-population differentiation. This suggests evolving divergence processes presently going on within the species in question (Pacheco *et al.*, 2003). Phylogenetic analyses confirm the separation of haplotype Tru-CH3C and Tru-CH3D, mainly present in Santiago, Brazil, from the rest of the haplotypes.

Four haplotypes have been detected for each of both spacers. Intraspecific nucleotide differences appears to be identical in ITS-1 and ITS-2 concerning both substitutions and indels, although the nucleotide divergence is higher in ITS-2 (1.83 %) than in ITS-1 (1.23 %) because of their different length. These results are in agreement with the molecular clock dating proposed for triatomines, according to which ITS-1 evolves 1.12–2.60 times faster than ITS-2 (Bargues *et al.*, 2000, 2006). Without considering indels because of their weight less than that of mutations, the number of ts and tv in ITS-1 and ITS-2 enables population differentiation. In *T. rubrovaria* from Brazil, Argentina and Uruguay, neither minisatellites nor microsatellites in both spacers discriminate the different populations.

According to the results obtained, the rDNA intergenic region appears to be a good marker for the analysis of the heterogeneity of *T. rubrovaria* populations. RAPD analyses and sequence and phylogenetic analyses of the rDNA intergenic spacers show to be valuable tools for genetic studies of triatomines. This integrated approach provides evidence of genetic heterogeneity between *T. rubrovaria* populations from Livramento, Brazil and Salto and Río Negro, Uruguay. Furthermore, populations from Corrientes, Argentina and Santiago, Brazil appear to be isolated probably because of distances (Dujardin *et al.*, 1998).

In Southern Brazil, *T. rubrovaria* has begun to invade dwellings and peri-domiciles due to anthropic factors, related to farming activities and the replacement of the original fauna and flora. The diversity of mammals may also have contributed to the emergence of larger populations of *T. rubrovaria* in such modified environments (Almeida *et al.*, 2000, 2002). In rural areas of Argentina, the passive dispersion of *T. rubrovaria* to urban centres with stones used for house building has been

reported (Bar *et al.*, 2003). The large intraspecific variability detected may underlie the known plasticity of *T. rubrovaria*, making it a potential intradomiciliary invader and consequently an appropriate vector for Chagas disease transmission. Therefore, this triatomine species must be continuously monitored throughout (Bar *et al.*, 2003; Almeida *et al.*, 2005).

ACKNOWLEDGEMENTS

This work has been performed within the Technical Cooperation Agreement between Fiocruz (Brazil) and Universidad de Valencia (Spain). Financial support was obtained from the following Projects: PAPES 3 (Fiocruz); "Chagas Disease Intervention Activities" (CDIA-ICA4CT-2003-10049), European Commission, Brussels; and RICET No. C03/04, FIS, Spanish Ministry of Health, Madrid, Spain. D.R. Klisiowicz was on leave from the Departamento de Patología Basica, Universidade Federal do Paraná, Centro Politécnico Curitiba, PR, Brazil. F. Panzera benefited from funding by the Conselleria de Cultura i Educació of the Valencia Government, Spain and the University of Valencia for two working stays at the Parasitology Department of Valencia. We thank Roberto Salvatella (Faculty of Medicine and PAHO, Montevideo, Uruguay) for providing specimens from Uruguay. Technical support for the automatic sequencing provided by the DNA Sequencing Service of the University of Valencia.

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Reçu le 24 janvier 2007
 Accepté le 22 mai 2007

Formulaire à retourner à réception de l'accord de publication.
Form to be returned when receiving the publication agreement.

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