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RAPD analyses and rDNA intergenic-spacer sequences discriminate Brazilian populations of *Triatoma rubrovaria* (Reduviidae: Triatominae)

R. S. PACHECO*, C. E. ALMEIDA†, J. COSTA†, D. R. KLISIOWICZ‡, S. MAS-COMA‡ and M. D. BARGUES‡

**Departamento de Bioquímica e Biología Molecular, Instituto Oswaldo Cruz–FIOCRUZ, Avenida Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brazil*
†*Departamento de Entomologia, Instituto Oswaldo Cruz–FIOCRUZ, Avenida Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brazil*
‡*Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Avenida Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*

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Triatoma rubrovaria, a member of the 'infestans' subgroup, is a potential vector of *Trypanosoma cruzi* in southern Brazil. Surveillance data indicate a growing domiciliary and peridomiciliary invasion by *Tri. rubrovaria* in the rural areas of Rio Grande do Sul (RS). In fact, following effective control of *Tri. infestans*, *Tri. rubrovaria*, which seems to have pre-adaptative characteristics for anthropic ecotopes, has become the most frequent species of triatomine bug to be collected in these areas. To explore the intraspecific variability and domiciliation of *Tri. rubrovaria*, the ribosomal DNA (rDNA) of two RS populations of *Tri. rubrovaria* that were geographically separated by only 220 km was investigated. The RAPD profiles and nucleotide sequences of the intergenic region of the rDNA, including the internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) and the 5.8S gene, were analysed. In the RAPD study, the use of three decameric primers revealed polymorphisms reflecting both genetic differences between the two populations and heterogeneity within each. A phenetic dendrogram of the *Tri. rubrovaria* specimens, based on the three-primer consensus and a simple-matching coefficient of similarity, showed two clusters, clearly differentiating the bugs from the two localities studied. The rDNA sequencing revealed four different nucleotide sequences, with two different genotypes in each locality. The level of intraspecific variability detected within ITS-1 and ITS-2 of the *Tri. rubrovaria*, which was remarkably high considering the physical closeness of the two populations sampled, may indicate that the two collection sites are separated by geographical barriers that ensure the reproductive isolation of each population. The ITS sequences, like the RAPD results, clearly distinguished the two populations while showing that there is heterogeneity within each of them. The present study appears to be the first to reveal ITS length differences between populations of the same triatomine species without any associated difference in the number of microsatellite repeats. These results are in agreement with those of earlier studies on iso-enzymes, chromatic patterns, the ecological effects of environmental modification by humans, and bloodmeal sources.

Trypanosoma cruzi, the causative agent of Chagas disease, is mainly transmitted by reduviid bugs of the subfamily Triatominae and consequently the main strategies for its control are directed against these insect vectors.

Triatoma rubrovaria (Blanchard in Blanchard et Brullé, 1843) is a triatomine species which gives the name to a subcomplex within the infestans complex of the infestans subgroup of the rubrofasciata group of the genus *Triatoma* — the most diversified of all triatomine genera (Dujardin *et al.*, 2000). Although widespread in Uruguay and parts of north–eastern Argentina, in

Reprint requests to: M. D. Bargues.
E-mail: m.d.bargues@uv.es; fax: +34 96 3544769.

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Brazil this species only seems to occur in the southern states of Paraná and Rio Grande do Sul (RS). Lent (1942) considered *Tri. rubrovaria* to be a sylvatic species that is rarely found in human dwellings but Salvatella *et al.* (1994) demonstrated that it does feed on humans. Its high competence as a vector of *Try. cruzi* was experimentally verified by Silva and Silva (1993). *Triatoma rubrovaria*, now considered as a generalist species that feeds on a wide variety of vertebrate and invertebrate hosts (Salvatella *et al.*, 1994, 1995), must now therefore be regarded as a potential vector capable of infecting humans with *Try. cruzi*. In fact, surveillance data gathered by the Brazilian National Health Foundation during the national Chagas Disease Control Program indicate a growing domiciliary and peridomiciliary invasion by *Tri. rubrovaria* in rural areas of RS, and, since the control of *Tri. infestans*, the species has become the most frequent triatomine species captured in these areas (Costa, 1999; Almeida *et al.*, 2000). Although there is no consistent evidence of intradomiciliary colonization by *Tri. rubrovaria*, this species seems to have characteristics that pre-adapt it to life in anthropic ecotopes and it should therefore be kept under constant epidemiological surveillance (Almeida *et al.*, 2000).

There seem to be at least two distinct populations of *Tri. rubrovaria* in RS that differ in terms of chromatic and iso-enzymatic patterns (Almeida *et al.*, 2002*b*). The recent results of precipitin tests of bloodmeals and associated ecological observations indicate that adults of this vector species have begun to invade houses in RS (Almeida *et al.*, 2002*a*). The aim of the present study was to shed further light on the heterogeneity of the *Tri. rubrovaria* in RS and on their apparent domicilation, using RAPD analyses and DNA sequencing. DNA-sequence comparisons offer information on the evolutionary events in triatomine lineages, on the routes by which triatomine populations have spread, and on the capacity of different species to invade and became established in new areas (Bargues *et al.*, 2002). RAPD analysis has been used in studies on the identification of species and population differentiation and for genetic fingerprinting (Williams *et al.*, 1991; Garcia *et al.*, 1998; Borges *et al.*, 2000). More recently, molecular approaches using nuclear ribosomal DNA (rDNA) and sequence analyses of the internal transcribed spacers (ITS) have proved useful for inferring phylogenetic relationships in the Triatominae and for classification of species, subspecies, hybrids and populations (Bargues, 2002; Bargues *et al.*, 2000, 2002; Marcilla *et al.*, 2000, 2001, 2002).

In the present study, analyses of *Tri. rubrovaria* total DNA (by RAPD analysis) and rDNA [by the sequencing of the intergenic region, including the sequences of ITS 1 and 2 (ITS-1 and ITS-2) and the 5.8S gene] were used to increase the information available on the species' intraspecific variability and domiciliation.

ANIMALS AND METHODS

Study Areas

Twelve adult *Tri. rubrovaria* were captured in two municipalities of RS that lie 220 km apart: Santana do Livramento (30°53′27″S, 53°31′58″W; specimens L07, L09, L10, L11, L12 and L19) and Santiago (29°11′30″S, 54°52′02″W; specimens S01, S03, S13, S14, S25 and S26; Fig. 1). Savanna-like or steppe-like, subtropical, mixed prairies predominate in both areas. The study sites were selected according to the geographical distribution of *Tri. rubrovaria* in RS, notifications of domiciliary invasions by this species, and the chromatic and iso-enzymatic variations that had already been detected (Almeida *et al.*, 2000, 2002*b*). The bugs were collected in areas of pasture for sheep and cattle, about 50 m from houses, from between overlapping rocks on the ground or in the dry-stone walls used as boundary markers. The adult insects were identified as *Tri. rubrovaria* using the keys of Lent and Wygodzinsky (1979).

FIG. 1. Map of the Brazilian state of Rio Grande do Sul, showing the two localities in which the *Triatoma rubrovaria* specimens were collected.

RAPD Analyses

Ten of the 12 specimens were investigated by RAPD analysis (the other two specimens, L09 and S13, were unfortunately lost during DNA extraction). Based on the protocol described by Garcia *et al.* (1998) and modified by Lopes *et al.* (2001), DNA was extracted from a single leg of each specimen, using a commercial kit (DNA Genomic Prep; Amersham Pharmacia Biotech) followed by additional phenol–chloroform purifications. DNA concentrations and purity were determined by agarose-gel electrophoresis against known concentrations of a standard DNA. Six, 10-base primers from the Ready-To-Go RAPD analysis kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.) were selected, to give different degrees of polymorphism and well defined amplifications when the kit manufacturer's instructions were followed. For the PCR, a programmable thermal cycler (GeneAmp PCR System 9600; Applied Biosystems, Foster City, CA) was used to give 5 min at 94°C, followed by 44 cycles, each of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, and then a final extension for 5 min

at 72°C. A negative control (all reaction components except DNA) and an outgroup (DNA from *Tri. sordida*) were included in each gel. The PCR products were subjected to electrophoresis in 2% agarose gels in 1 × Tris–borate–EDTA buffer, stained with ethidium bromide and recorded on a gel documentation system (UVP, Upland, CA) with ultra-violet trans-illumination. Well defined bands ranging in size from 300 to 1600 bp were selected for the phenetic analysis and compared using the simple-matching coefficient of similarity, to determine the proportion of mismatched bands between pairs of *Tri. rubrovaria*. The similarity matrix was transformed into a dendrogram using the UPGMA algorithm (Sneath and Sokal, 1973) and version 2.0 of the NTSYS software programme (Exeter Software, Setauket, NY).

rDNA Sequence Analyses

The rDNA of all 12 specimens was partially sequenced. A single leg from each bug, fixed in 70% ethanol, was used as the rDNA source (Bargues and Mas-Coma, 1997; Marcilla *et al.*, 2001, 2002). Total DNA was isolated by standard phenol–chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). The pellet was dried and resuspended in 30 µl sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). This suspension was stored at −20°C until use.

The rDNA intergenic fragment corresponding to the ITS-1, 5.8S and ITS-2 regions of each individual bug was amplified by PCR, using 4–6 µl genomic DNA for each 50-µl reaction mix. The primers used were based on the 18S and 28S rRNA genes, as previously described (Bargues and Mas-Coma, 1997; Bargues *et al.*, 2000; Marcilla *et al.*, 2001, 2002). The PCR were run in a Peltier thermal cycler (MJ Research, Watertown, MA) set to give 30 s at 94°C, followed by 30 cycles, each of 30 s at 94°C, 30 s at 50–55°C, and 1 min at 72°C, followed by 7 min at 72°C. A 10-µl sample of each reaction mixture was then subjected to electrophoresis in a 1% agarose gel, followed by ethidium-bromide staining.

Primers and nucleotides were removed from the products of each PCR, using a commercial purification kit (Ultra Clean® PCR Clean-up DNA Purification System; MoBio, Solana Beach, CA) according to the manufacturer's protocol, and resuspended in 50 µl TE buffer. The final DNA concentration was determined by measuring absorbance at 260 and 280 nm.

Sequencing was performed, on both strands, by the dideoxy chain-termination method, using the Taq dye-terminator chemistry kit for the ABI 3700 capillary system (Perkin Elmer, Foster City, CA) and the PCR primers. Version 1.8 of the CLUSTAL W software package (Thompson *et al.*, 1994) was used for sequence alignment.

RESULTS

RAPD Analyses

Of the six decameric primers used, three — primers 4 (5′-AAG AGC CCGT-3′), 5 (5′-AAC GCG CAAC-3′) and 6 (5′-CCC GTC AGCA-3′) — revealed polymorphisms reflecting genetic differences between both populations. No two specimens gave identical RAPD profiles (Fig. 2). Although heterogeneity was observed, both in the number and sizes of the fragments amplified from different specimens, some bands detected in specimens from both populations appeared to be conserved. Amplifications with primer 4, for example, showed that specimens from both populations share a major band of around 750 bp [Fig. 2(a)]. The profiles generated by primer 5 were more polymorphic, demonstrating its usefulness in differentiating at intrapopulational level. The specimens from Santana do Livramento generally had more complex profiles than those from Santiago, with extra bands of 500–600 bp [Fig. 2(b)]. Amplification with primer 6 revealed a predominant band of 1100 bp in all of the *Tri. rubrovaria* specimens

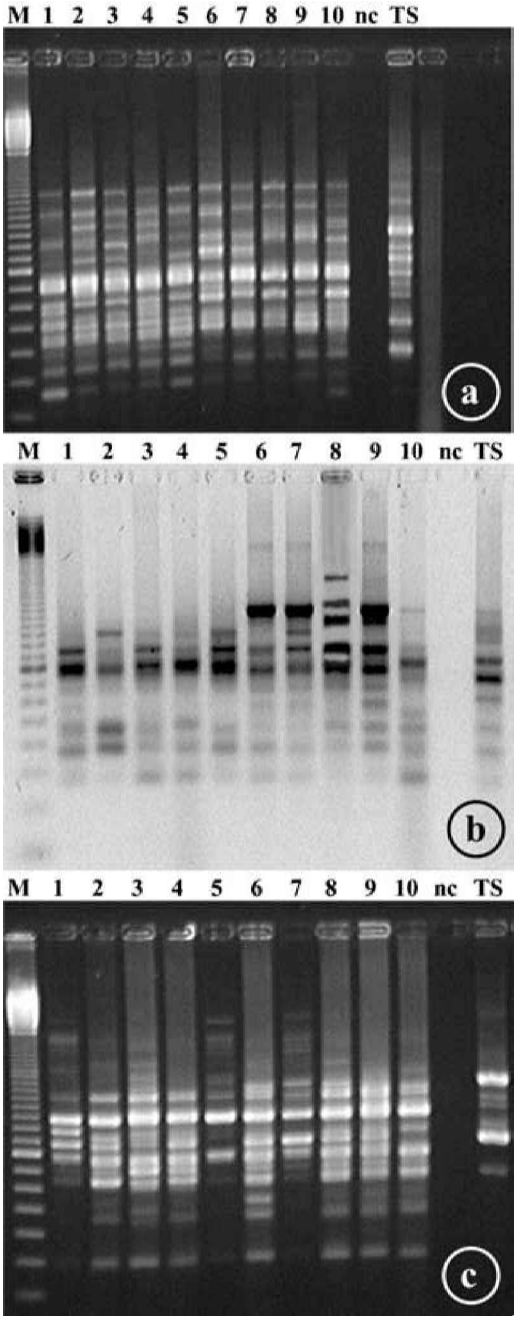

FIG. 2. The results of agarose-gel electrophoresis, showing the RAPD profiles produced when primer 4 (a), 5 (b) or 6 (c) was used. The lanes contained *Triatoma rubrovaria* samples from specimens S01 (1), S03 (2), S14 (3), S25 (4), S26 (5), L07 (6), L10 (7), L11 (8), L12 (9) or L19 (10), a negative control (nc), a sample from *Triatoma sordida* (TS), or a 100-bp DNA ladder (M).

that was absent from the *Tri. sordida* used as an outgroup. Such a primer could be a useful tool for intra- and inter-specific discrimination in studies involving both species [Fig. 2(c)].

For the numerical analyses, 65 characters (bands) resulting from the amplifications with primers 4, 5 and 6 were screened. The results for each RAPD profile were converted into a matrix of binary data, with 0 indicating the absence of a particular band and 1 its presence, and then used to calculate simple-matching similarity indices and so compare each pair of specimens. The results of the phenetic analyses placed the 10 specimens in two distinct phenetic groups, one of individuals from Santiago (first group) and one of the specimens from Santana do Livramento (second group), which diverged by a coefficient of similarity of 0.75. Interestingly, there was no overlap between the two groups, both populations being clearly separated. Although intra-populational heterogeneity can be observed, the specimens from each population were at least 75% homologous in terms of the characteristics investigated (Fig. 3).

rDNA Sequence Analyses

Four different nucleotide sequences were detected: genotype '1' (GT1) and GT2, represented, respectively, by five (L09, L10, L11, L12, and L19) and one specimen (L07) from Santana do Livramento, and GT3 and GT4, represented, respectively by five (S01, S03, S13, S14, S25) and one specimen (S26) from the Santiago population. All four sequences are new and have consequently been deposited in the EMBL, GenBank and DDBJ databases, where they are available under the accession numbers of AJ557258 (GT1), AJ557259 (GT2), AJ557260 (GT3) and AJ557261 (GT4).

The length of the fragment holding ITS-1, 5.8S and ITS-2 varied only from 1370 bp (in all specimens from the population of Santiago) to 1375 bp (in all specimens from the population of Santana do Livramento).

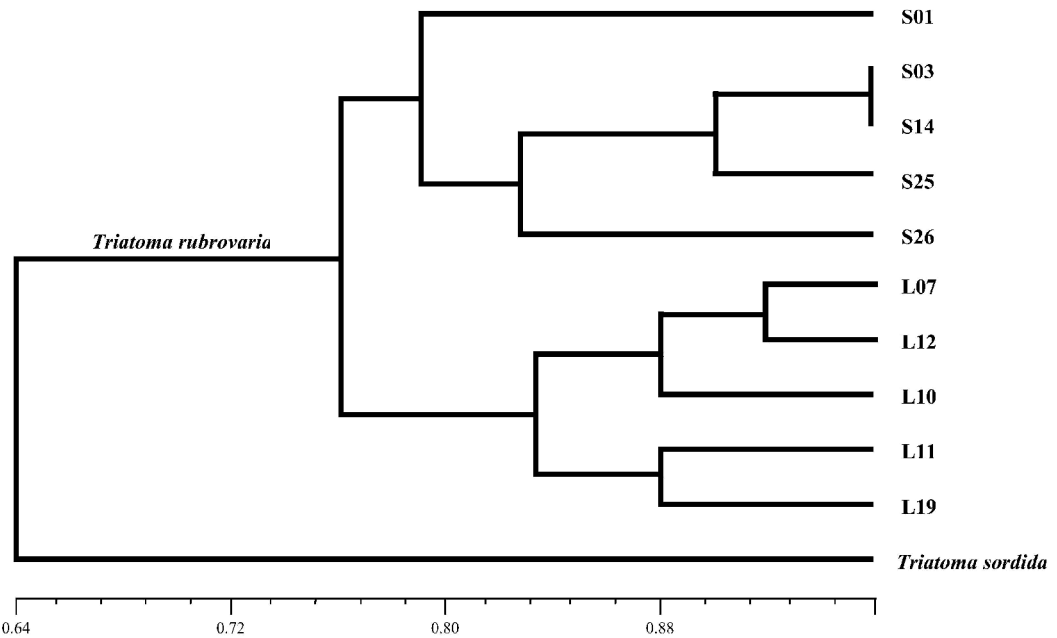

FIG. 3. The genetic polymorphism of the two *Triatoma rubrovaria* populations studied by RAPD, showing the three-primer-consensus phenetic dendrogram of the triatomine specimens, obtained using simple-matching coefficients of similarity.

The complete alignment of the four genotype sequences was 1377 bp long, showing a genetic divergence of 0.94%. The base composition was A + T-biased, with a mean of 67.7% of all bases A + T. The nucleotide lengths and AT contents of the 5.8S gene and both spacer sequences are summarized, for each of the four genotypes, in the Table.

Within ITS-1, according to the sequence comparison for the 730-bp-long alignment, nine nucleotide differences were detected: two transitions, one transversion and six insertions/deletions. Together these differences represent a sequence divergence of 1.23% between the two populations studied. This spacer was shorter in the Santiago specimens (725 bp) than in those from Santana do Livramento (729 bp). The 155-bp sequences of the 5.8S gene were identical in all of the *Tri. rubrovaria* investigated. Within the 492-bp-long alignment of ITS-2, only four nucleotide differences (one transition and three insertions/deletions) were detected, representing a sequence divergence of 0.81%. ITS-2 was just one base shorter in the specimens of *Tri. rubrovaria* from Santiago (490 bp) than in those from Santana do Livramento (491 bp). In the ITS-2 sequence, the presence of the interrupted microsatellite $(AT)_6TTTTT(AT)_1AA(AT)_7$ was detected in all of the *Tri. rubrovaria* studied, always with the same number of repeats. The Table includes a list of the nucleotide differences detected and the respective sequence positions.

DISCUSSION

RAPD Analyses

The DNA polymorphisms generated by RAPD provide a molecular basis on which to re-assess taxonomic relationships within the Triatominae (Garcia *et al.*, 1998). RAPD analysis can also be regarded as a complementary tool in eco-genetic studies,

TABLE. *Lengths and A + T compositions of the ITS-1, 5.8S and ITS-2 sequences in the ribosomal DNA of the Brazilian* Triatoma rubrovaria*, and the differences observed in the sequences*

		ITS-1											5.8S		ITS-2					
				Nucleotide at position:													Nucleotide at position:			
Genotype	Specimens	Length (bp)	A + T (%)	25	27	28	29	190	194	278	344	594	Length (bp)	A + T (%)	Length (bp)	A + T (%)	1065	1328	1329	1377
1	L09, L10, L11, L12 and L19	729	66.25	A	T	A	T	A	A	–	G	–	155	42.58	491	78.20	T	–	G	T
2	L07	729	66.25	A	T	A	T	A	A	–	G	–	155	42.58	491	78.00	T	–	A	T
3	S01, S03, S13, S14 and S25	725	65.79	G	–	–	–	C	–	G	A	T	155	42.58	490	77.96	–	A	G	–
4	S26	725	65.93	A	–	–	–	C	–	G	A	T	155	42.58	490	77.96	–	A	G	–

enabling species that are morphologically similar and closely related to be distinguished. RAPD polymorphisms were reported in *Tri. infestans* from three different localities, although none of the polymorphisms was confined to one source (Garcia *et al.*, 1998), and also indicated intra- and inter-specific differences within the *Tri. sordida* complex, *Tri. guasayana* being the most divergent species (Lopes *et al.*, 2001).

In the present study, three decameric primers were found to be useful because their use produced reproducible and discrete profiles that revealed intra- and inter-specific divergence. Although a considerable degree of heterogeneity was found among the profiles, the *Tri. rubrovaria* genotypes could be clearly grouped into two clusters. In the first cluster, composed of specimens from Santiago, S01 and S26 were found to be the most divergent pair of specimens but two other specimens in this group, S03 and S14, were found to be genetically similar, displaying 96% similarity. The second phenetic cluster, of specimens from Livramento, actually divided into two sub-groups (one of L07, L12 and L10, and the other of L11, L19) linked by a similarity coefficient of 0.85. Overall, the results of the RAPD-based phenetic analysis allowed the conclusion that the *Tri. rubrovaria* investigated fell into two distinct clusters that followed the geographical separation of the two populations sampled.

rDNA Sequence Analyses

It is clear that the sequences of ITS-1 and ITS-2 of the rDNA can be useful in distinguishing species, subspecies, hybrids and populations of Triatominae (Bargues, 2002; Bargues *et al.*, 2000, 2002; Marcilla *et al.*, 2000, 2001, 2002). In the present study, two initial conclusions can be reached from the sequencing results obtained and the sequence comparisons performed: (1) all the specimens investigated (apart from the outgroup) belonged to the same species: *Tri. rubrovaria*; and (2) the specimens analysed from the Brazilian localities of Santana do Livramento and Santiago belong to two clearly different populations.

Most (nine) of the 13 nucleotide differences detected (in the 1377-bp-long alignment of the ITS-1 + 5.8S + ITS-2 intergenic rDNA region in GT1, GT2, GT3 and GT4) were insertions/deletions and only four were true mutations. It is already known that, in other organisms, within a given species, different populations may show marked differences in terms of insertions/deletions but very few mutations, perhaps indicating how most divergence currently occurs (Bargues *et al.*, 2001).

The level of intraspecific variability detected appears to lie within the range observed in other triatomine species. The 0.81% sequence divergence detected in ITS-2 in the present study is actually less than the values reported, for the same spacer, in *Panstrongylus rufotuberculatus* (0.85%), *Tri. infestans* (0.87%), *P. geniculatus* (0.98%), or, excluding a Yucatan population, in *Tri. dimidiata* (2.10%; Marcilla *et al.*, 2000, 2001). Despite being markedly longer, the ITS-1 of *Tri. rubrovaria*, with 1.23% sequence divergence, appears to have evolved only slightly faster than the ITS-2, which fits with the patterns known in other organisms (Mas-Coma, 1999). Interestingly, the level of intraspecific variability detected within the present specimens of *Tri. rubrovaria* is very high given that all were collected within 220 km of each other (whereas the collection areas for the samples previously giving such high sequence divergences were much more widely scattered and covered several countries).

In the present study, comparison of the sequences of the intergenic rDNA fragment investigated shows that there are two groups of sequences, according to both the nucleotide composition and the ITS-1 and ITS-2 lengths (see Table). One group includes the genotypes GT1 and GT2 and corresponds to the specimens from Santana do Livramento, and the other group includes GT3 and GT4 from the other locality of Santiago.

Consequently, the ITS sequences clearly prove that there are two easily differentiable *Tri. rubrovaria* populations.

This appears to be the first time that ITS-length differences have been detected within the same triatomine species without those differences being associated with different numbers of microsatellite repeats. With a very few exceptions, ITS-2 sequences have the same length even in different species of the same genus, and when length differences are present they are usually related to variation in the number of repeats of certain microsatellites (Almeyda-Artigas *et al.*, 2000; Bargues *et al.*, 2001). Although different ITS-2 lengths have already been detected within a given triatomine species, they have always previously been linked to the number of repeats of the interrupted microsatellite $(AT)_n$ (Marcilla *et al.*, 2001, 2002). Curiously, although such a microsatellite was found in the *Tri. rubrovaria* ITS-2, it had the same number of repeats in all of the specimens investigated.

The present results indicate that the ITS-1 and ITS-2 sequences, though obtained from only 12 specimens, were sufficient to show that there is heterogeneity among each of the two *Tri. rubrovaria* populations sampled. The mutation at position 25 in the ITS-1 of the Santiago specimens, the one at position 1329 of the ITS-2 of the Santana do Livramento specimens, and the relatively high number of insertions/deletions in both ITS-1 and ITS-2 together indicate that *Tri. rubrovaria* is evolving differently in different zones of Rio Grande do Sul. According to an ITS-2 molecular clock derived from the base-substitution rate in Triatominae, which is estimated to be around 0.4%–1.0%/million years (Bargues *et al.*, 2000; Bargues, 2002), the two populations sampled here began to diverge a very long time ago, despite the two populations now being within 220 km of each other. The intrapopulational heterogeneity revealed by the ITS-1 and ITS-2 sequencing does not, however, appropriately fit in the phenetic tree produced from the simple-matching similarity indices for the RAPD profiles (Fig. 3). Thus, the divergent individuals L07 (=GT2) within the Santana do Livramento population and S26 (=GT4) in the Santiago population, according to the mutations in ITS-2 and ITS-1, respectively, do not appear as the most divergent individuals in their respective phenetic populational clusters.

Comparison with Results Furnished by Other Markers

The present results, of both the RAPD and rDNA sequence analyses, show that the *Tri. rubrovaria* populations of Santana do Livramento and Santiago are clearly different and that there is genetic heterogeneity among each one. They therefore support the results of earlier studies on the iso-enzymes and chromatic patterns of these populations, which indicated that the Santiago population is reproductively isolated from all other *Tri. rubrovaria* populations in RS (Almeida *et al.*, 2002*b*). The shorter lengths of the ITS-1 and ITS-2 of the Santiago specimens support the hypothesis that the anthropic *Tri. rubrovaria* in RS dispersed from the Santiago area, as shorter ITS are typical of older populations (Mas-Coma, 1999). The two genotypically distinct populations sampled in the present study may differ in terms of vectorial capacity and susceptibility to *Try. cruzi* infection and in insecticide resistance (Almeida *et al.*, 2002*b*).

It has been suggested that *Tri. rubrovaria* has begun to invade domestic and peridomestic areas because of environmental modification by humans — related to farming activities, the introduction of livestock and domestic animals and the replacement of the original flora and fauna (Almeida *et al.*, 2000). Increases in the diversity and abundance of mammals and birds may have helped support larger populations of *Tri. rubrovaria* in anthropic environments (Almeida *et al.*, 2002*a*). This, in turn, may have increased the numbers of *Tri. rubrovaria*

feeding on humans. When precipitin tests were used to determine the animals on which *Tri. rubrovaria* had been feeding, none of the bugs collected in both natural or artificial environments in Santana do Livramento but 6.25% of those collected in a rural setting in Santiago were found positive for human blood (Almeida *et al.*, 2002*a*).

For the moment, it remains unclear whether the large genetic differences revealed by iso-enzyme and RAPD analyses and rDNA sequencing are related to accelerated divergence as a consequence of recent environment change, in a way similar to that detected in *Tri. infestans* (Mas-Coma, 2002). The genetic differences may pre-date the relatively recent environment modifications introduced by humans, a possibility supported by the level of heterogeneity observed in the ITS-2 sequences of the *Tri. rubrovaria* (Bargues *et al.*, 2000; Bargues, 2002).

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