

# Genetic diversity of *Trypanosoma cruzi* natural populations in Paraná state, southern Brazil

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**ABSTRACT.** The genetic characteristics of twelve *Trypanosoma cruzi* strains isolated from chronic chagasic patients, triatomines and sylvatic reservoirs from Paraná state, Southern Brazil, were studied using the randomly amplified polymorphic DNA (RAPD) and simple sequence repeat-anchored polymerase chain reaction amplification (SSR-PCR) techniques. Sylvio and Esmeraldo stocks were used as reference for *T. cruzi* I and II, respectively. The DNA was amplified using three different primers for RAPD and (CA)<sub>8</sub>RY primer for SSR-PCR. Gel analyses were scored by eye for a numerical taxonomy analysis based on the proportion of shared bands and by employing computer programs. The phenograms showed the existence of two distinct genetic groups: one containing the strains from chronic chagasic patients grouped with Esmeraldo and the other with the strains from triatomines and sylvatic reservoirs grouped with Sylvio. The existence of two phylogenetic groups of *T. cruzi* in Paraná state, Southern Brazil, is described for the first time.

**Key words:** *Trypanosoma cruzi*, genetic groups, northern Paraná state, RAPD, SSR-PCR.

**RESUMO.** Diversidade genética de populações naturais do *Trypanosoma cruzi* no Paraná, Sul do Brasil. As características genéticas de doze cepas de *Trypanosoma cruzi*, isoladas de pacientes chagásicos crônicos, triatomíneos e reservatórios silvestres do estado do Paraná, sul do Brasil, foram estudadas, utilizando as técnicas de RAPD (DNA polimórfico amplificado randomicamente) e SSR-PCR (Reação em Cadeia da Polimerase ancorada em seqüências repetidas pequenas). As cepas Sylvio e Esmeraldo foram utilizadas como referências para *T. cruzi* I e II, respectivamente. O DNA foi amplificado, utilizando três diferentes iniciadores para o RAPD e o iniciador (CA)<sub>8</sub>RY para o SSR-PCR. A análise do gel foi feita visual e computacionalmente, baseada na presença e na ausência de bandas. Os fenogramas mostraram a existência de dois grupos genéticos distintos: um contendo as cepas isoladas de pacientes chagásicos crônicos, que gruparam com Esmeraldo, e o outro contendo as cepas isoladas de triatomíneos e reservatórios silvestres, que gruparam com Sylvio. A existência de dois grupos filogenéticos do *T. cruzi* no Estado do Paraná é descrita pela primeira vez.

**Palavras-chave:** *Trypanosoma cruzi*, grupos genéticos, Norte do Paraná, RAPD, SSR-PCR.

## Introduction

*Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is widely distributed in the American continent, occurring from the South of the United States to the South of Argentina (Dias, 1985). It circulates among humans, vectors, and sylvatic and domestic reservoirs. This parasite infects about one hundred different species of little sylvatic mammals and triatomine vectors already adapted to artificial habitats, among them *Triatoma infestans*, *T. braziliensis*, *T. dimidiata*, *T. sordida*, *Panstrongylus megistus* and *Rhodnius prolixus* (Forattini, 1980).

The interaction of the parasite with these natural

reservoirs and triatomine bugs is known as the sylvatic transmission cycle of the parasite. The colonization of non-natural habitats by triatomine vectors allowed *T. cruzi* to infect humans and domestic mammals, resulting in a domestic transmission cycle. It is considered that the sylvatic cycle is the ancestor of the domestic one, and that the rise of the last is attributed to ecological, social and economic conditions, which allowed contact between poor rural people and the sylvatic cycle (Dias, 1985). A recent article reports results of molecular diagnosis that prove that humans were a link in the sylvatic cycle of the disease (Aufderheide *et al.*, 2004).

In Latin America, around 15 million people are infected by the parasite (WHO, 2002) and in Brazil, 3.5 million (Dias, 1997). Paraná state has been considered the fourth endemic state in Brazil, with an estimated 166,511 infected people (Silveira and Resende, 1994). In this state, the prevalence of chagasic infection is 4% (Camargo et al., 1984). In the North and Northwest of this state, *T. cruzi* strains were isolated from chronic chagasic patients (Gomes et al., 1992), from sylvatic reservoirs and from triatomines captured in the peridomestic environment (Toledo et al., 1997; Guilherme et al., 2001). Strains isolated from chronic chagasic patients showed a homogeneous biological behavior in mice (Araújo et al., 1999), a drug susceptibility gradient that varied from 0 to 100% (Toledo et al., 1997) and a well-correlated genetic group (Gomes et al., 1998). Also, *T. cruzi* strains isolated from triatomines captured in the same region displayed low virulence in mice (Cardoso et al., 2000).

*T. cruzi* is classified into two major groups, *T. cruzi* I and *T. cruzi* II (Anonymous, 1999), using different methodologies. One of them is RAPD - Random Amplified Polymorphic DNA (Welsh and McClelland, 1990) which, together with SSR-PCR - Simple Sequence Repeat-Anchored Polymerase Chain Reaction amplification are ideal for searching for the genetic markers and are useful in the study of the genetic variability of *T. cruzi*. (Tibayrenc et al., 1993; Steindel et al., 1993; Oliveira et al., 1997).

In this paper, the genetic characteristics of *T. cruzi* strains isolated from humans, triatomines and sylvatic reservoirs from the North and Northwestern Paraná state and the phylogenetic relationships among them are presented using RAPD and SSR-PCR techniques since molecular characterization of *T. cruzi* populations from this region will allow an understanding of the epidemiological correlation among parasite populations and between parasites and their hosts.

## Material and methods

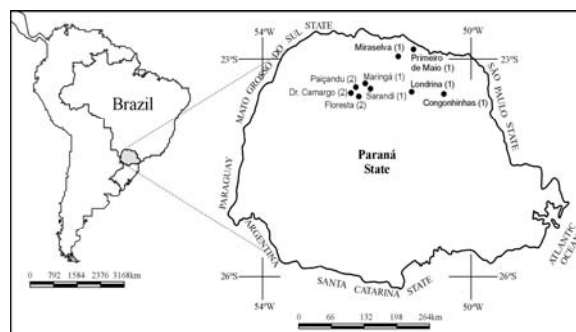
### Parasite strains

Twelve autochthonous *T. cruzi* strains, from different locations of North and Northwestern Paraná state, Brazil (Figure 1), were evaluated. Four were isolated from chronic chagasic patients, four from triatomines (*Triatoma sordida* and *Panstrongylus megistus*) and four from sylvatic reservoirs (*Didelphis* sp.) captured in the peridomestic environment.

Table 1 shows the parasite strains, hosts, isolation methods and their geographic origins.

For comparative analyses between these strains

and the groups *T. cruzi* I and II, two reference stocks, Esmeraldo strain (*T. cruzi* II) and Sylvio clone (*T. cruzi* I), were used. The strain 150, isolated from a chronic chagasic patient, was also used as reference for presenting a peculiar molecular, biochemical and biological behavior compared to the other 31 studied strains from patients from distinct endemic areas with Chagas' disease (Gomes et al., 2003).



**Figure 1.** Map of Paraná state showing the location of municipalities where *Trypanosoma cruzi* strains were isolated. The numbers in parentheses represent the quantity of analyzed *T. cruzi* strains from each city.

**Table 1.** *Trypanosoma cruzi* strains isolated from chronic chagasic patients, sylvatic reservoirs and triatomines from North and Northwestern Paraná state, southern Brazil.

Strains	Hosts	Isolation method	Geographic origin
G1	<i>Didelphis</i> sp.	Hemoculture	Sarandi
G2	<i>Didelphis</i> sp.	Hemoculture	Doutor Camargo
G3	<i>Didelphis</i> sp.	Hemoculture	Maringá
G249	<i>Didelphis</i> sp.	Hemoculture	Floresta
F3	<i>Triatoma sordida</i>	Xenoculture	Paçandu
A21A	<i>Triatoma sordida</i>	Inoculation in mice	Paçandu
N914A	<i>Triatoma sordida</i>	Xenoculture	Floresta
N120B	<i>Panstrongylus megistus</i>	Inoculation in mice	Doutor Camargo
2052	Human	Hemoculture	Miraselva
328	Human	Hemoculture	Congonhinhas
379	Human	Hemoculture	Londrina
399	Human	Hemoculture	Primeiro de Maio

### Growth of parasites

*T. cruzi* strains were kept through serial passages each 2 or 3 days in LIT medium at 28°C. To obtain the parasite mass, cumulative addition of LIT medium to the cultures was carried out until it reached  $1 \times 10^9$  cells mL<sup>-1</sup>. The cells were washed by centrifugation in KRT (Krebs-Ringer-Tris) buffer and the cellular mass was stored at -20°C before use.

### *T. cruzi* genomic DNA extraction and quantification

The DNA extraction was carried out as described by Macedo et al. (1992). The cellular mass was resuspended in 80 mM NaCl / 45 mM EDTA, pH 8.0 / 1% SDS in a concentration of  $1 \times 10^9$  cells.

Proteinase K (Sigma Company Ltda) was added to  $0.1 \text{ mg mL}^{-1}$  and the product was incubated at  $37^{\circ}\text{C}$  overnight. Sequential extraction was performed with phenol and phenol-chloroform following precipitation with absolute ethanol. The DNA was resuspended in Low-TE buffer (Tris-HCl, pH 8.0 / 1 mM EDTA, pH 8.0) in a proportion of  $100 \mu\text{L } 10^{-9}$  cells. DNA solutions were quantified by visual comparison using accepted DNA concentration standards with 1% agarose gel electrophoresis followed by ethidium bromide staining. After the DNA quantification, it was diluted to  $1 \text{ ng } \mu\text{L}^{-1}$ .

#### RAPD analysis

PCR amplification was carried out in a thermocycler, MJ Research PTC-150, with a final volume of  $10 \mu\text{L}$ . Each reaction tube contained 1.0 units of Taq-DNA Polymerase (Cenbiot, Rio Grande do Sul, Brazil),  $125 \mu\text{M}$  of each dNTP, 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, Tris-HCl, pH 8.5, together with 6.4 pmols of the primer and 1 ng of template DNA. The primers M13F-40 "forward" ( $5'\text{-GTTTTCCTCCAGTCACGAC3}'$ ), L15996 ( $5'\text{-CTCCACCATTAGCACCCAAAGC3}'$ ) and  $\lambda\text{GT11-F}$  ( $5'\text{-GACTCCTGGAGCCCG3}'$ ) were selected. The reaction mixture was overlaid with  $20 \mu\text{L}$  of mineral oil. The RAPD program consisted of an initial denaturation at  $95^{\circ}\text{C}$  for 5 minutes, two cycles for annealing at  $30^{\circ}\text{C}$  for 2 min,  $72^{\circ}\text{C}$  for 1 min for extension, and 30 sec at  $95^{\circ}\text{C}$  for denaturation, followed by 33 additional cycles in which the annealing temperature was increased to  $40^{\circ}\text{C}$ . The final extension was 5 min at  $72^{\circ}\text{C}$ . After amplification,  $5 \mu\text{L}$  of the products were electrophoresed through a 4% non-denaturing polyacrylamide gel, visualized by silver staining as described by Santos *et al.* (1993) and photographed.

#### SSR-PCR

PCR amplification was carried out as described by Oliveira *et al.* (1997) with a few modifications. Each reaction was carried out in  $10 \mu\text{L}$  containing 10 mM Tris-HCl, pH 8.5, 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl,  $125 \mu\text{M}$  of each dNTP, 2% formamide (v/v), 22.5 pmols of the primer (CA)<sub>8</sub>RY (R=purine, Y=pyrimidine), 1.0 units of Taq-DNA polymerase (Cenbiot, Rio Grande do Sul, Brazil) and 2 ng of parasite total DNA. The mixtures were overlaid with  $20 \mu\text{L}$  of mineral oil to avoid evaporation and submitted to 26 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $52^{\circ}\text{C}$  for 45 sec and extension at  $72^{\circ}\text{C}$  for 1 min. The final extension was carried out at  $72^{\circ}\text{C}$  for 7 min. Five  $\mu\text{L}$  of each PCR reaction

were submitted to electrophoresis in 4% polyacrylamide gel, visualized by silver staining (Santos *et al.*, 1993) and photographed for subsequent analysis.

#### Data analysis

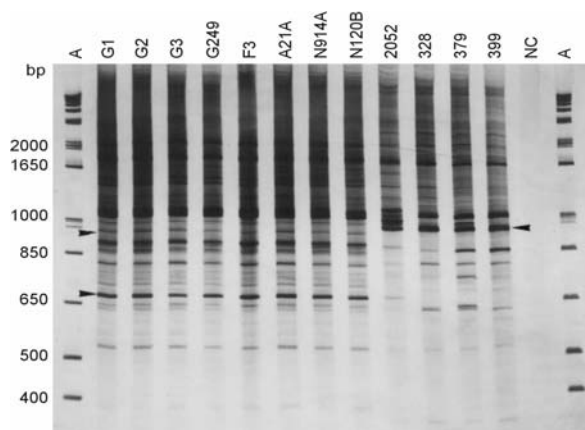
The multiple band profiles on polyacrylamide gel of *T. cruzi* strains obtained by RAPD and SSR-PCR were visually scored and analyzed for polymorphism based on the presence and absence of bands. Distance matrixes between strains, taken two by two, for RAPD data and for SSR-PCR data were obtained by the arithmetic complement of Jaccard's similarity coefficient, using the FreeTree program (Pavlicek *et al.*, 1999). Clustering was analyzed with the algorithm UPGMA (Unweighted Pair Group Methods of Arithmetic Means), according to Sneath and Sokal (1973), using the FreeTree program. Bootstrap analyses were based on 10,000 resampling. Better presentation of topology of the tree was obtained using MEGA 2.1 software (Kumar *et al.*, 2001).

#### Results

##### RAPD data

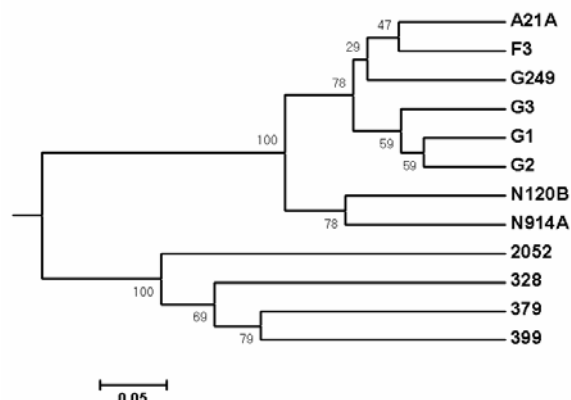
The amplified band patterns for each strain of *T. cruzi* showed a characteristic profile composed of multiple bands that varied from 200 to 2000 base pairs (bp). No identical RAPD profiles among the studied strains were observed. The average of the shared bands among the different pairs for all the primers was  $14.65 \pm 4.62$ , with a minimum of 10 and a maximum of 29 analyzed bands, depending on the primer and the sample. The mean proportion of shared bands among all the studied strains was 63.8%, taking into account all the three primers.

A representative result of the amplified band patterns with the primer M13F-40 is shown in Figure 2. Very similar band patterns were exhibited in gel for *T. cruzi* strains isolated from vectors and reservoirs, indicating low genetic diversity among them. This fact can be confirmed through the great sharing of bands that was observed, for this primer, among the strains isolated from vectors and reservoirs (83%) when compared to the proportion of shared bands among the ones isolated from vectors and those isolated from humans (48%), and among the ones isolated from reservoirs and those isolated from humans (50%). On the other hand, the band patterns observed among the strains isolated from vectors and reservoirs were very different from those observed in the strains isolated from humans.



**Figure 2.** RAPD profiles of *Trypanosoma cruzi* strains using the primer M13F-40 “forward”. On the left and on the right the molecular weight size markers (A) of some DNA fragments of 1 Kb Plus DNA ladder scale (GIBCO BRL) are indicated. G1, G2, G3 and G249 represent the strains isolated from sylvatic reservoirs; F3, A21A, N914A and N120B from triatomines; 2052, 328, 379 and 399 from humans. NC represents the negative control. Left side arrows indicate bands with about 670 bp and 900 bp. Right side arrow indicates band with about 950 bp.

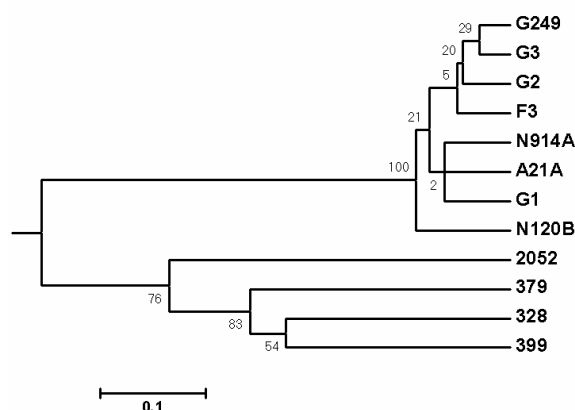
In Figure 2, bands with about 670 bp and 900 bp (Arrows, left side) were observed only in parasite strains obtained from vectors and reservoirs, while one band with about 950 bp (Arrow, right side) was present only in strains isolated from humans. Data obtained with 147 RAPD markers from three primers were put in a distance matrix of the complement of Jaccard's similarity coefficient and used to build a phenogram by UPGMA algorithm (Figure 3). It showed the existence of two different genetic groups: one containing strains isolated from vectors and reservoirs and the other containing strains isolated from humans.



**Figure 3.** Phenogram of twelve *Trypanosoma cruzi* strains, constructed by the UPGMA method based on the complement of Jaccard's similarity coefficient, from RAPD markers obtained from three different arbitrary primers (L15996, M13F-40 “forward” and  $\lambda$ gt11-F). Bootstrap values were based on 10,000 resampling.

#### SSR-PCR data

Twelve *T. cruzi* strains were amplified with the primer (CA)<sub>8</sub>RY and polymorphic banding patterns ranging from 200 to 2000 bp were obtained. An average of  $8.2 \pm 5.7$  bands were detected with 53% of shared bands between any two strains. As in RAPD, the SSR-PCR profiles showed 40 markers with a great sharing of bands among the strains isolated from vectors and reservoirs compared to strains isolated from humans. Besides this, it was possible to detect the existence of two different genetic groups by observing the phenetic tree (Figure 4).



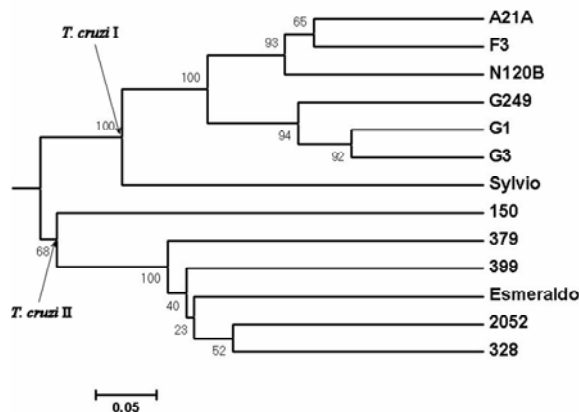
**Figure 4.** Phenogram of twelve *Trypanosoma cruzi* strains, constructed by the UPGMA method based on the complement of Jaccard's similarity coefficient from SSR-PCR markers. The number on each node indicates bootstrap probability based on 10,000 resampling.

#### Phylogenetic relationship between *T. cruzi* strains from Paraná and *T. cruzi* I and II reference stocks

RAPD analysis of *T. cruzi* strains from Paraná, using three different primers, as previously mentioned, together with *T. cruzi* I and II reference stocks (Sylvio and Esmeraldo, respectively), pointed out that the strains isolated from vectors and reservoirs had 57% of shared bands with Sylvio, against 46% with Esmeraldo. In SSR-PCR analysis, this percentage was 51% with Sylvio versus 25% with Esmeraldo. The phenogram showed that these strains grouped with the *T. cruzi* I reference stock.

On the other hand, by RAPD, strains isolated from humans showed 62% of shared bands with Esmeraldo and 38% with Sylvio, while by SSR-PCR, the shared percentages were 59 and 19%, respectively. The phenetic tree was constructed, for both techniques, demonstrating that these strains grouped with the *T. cruzi* II reference stock. The phenogram obtained with RAPD data, taking into account the 213 RAPD markers obtained from three different arbitrary primers, is illustrated in Figure 5.

This figure shows that the 150 reference stock, a human isolate, grouped with Esmeraldo despite presenting a bigger genetic distance with regard to the other studied strains that were also isolated from chronic chagasic patients.



**Figure 5.** UPGMA phenogram of *Trypanosoma cruzi* strains, obtained from the complement of Jaccard's similarity coefficient from RAPD markers obtained with three different arbitrary primers (L15996, M13F-40 "forward" and  $\lambda$ gt11-F). The tree shows the relationships between the ten *T. cruzi* strains from North and Northwestern Paraná state, Brazil, and the reference stocks, Sylvio, Esmeraldo and 150. Bootstrap values were based on 10,000 resampling.

The bootstrap analysis confirmed the consistency of the two genetic groups with the strains isolated from North and Northwestern Paraná state, when a UPGMA tree derived from the complement of Jaccard's similarity coefficient was constructed.

## Discussion

Chagas' disease is endemic in Paraná state, being known since the early 1920s with the description of several human cases in the Northeastern area (Camargo *et al.*, 1984; Souza Araújo, 1919).

For the first time, *T. cruzi* strains isolated from Paraná state have been analyzed by both RAPD and SSR-PCR techniques. The analysis of twelve strains displayed two distinct genetic groups of the parasite, one containing strains from reservoirs and vectors, indicating its preferential circulation in a peridomestic and/or sylvatic environment, and another with strains from humans, demonstrating that it is more related to the domestic transmission cycle of the parasite. These two genetic groups proved to be relevant, even in this limited sample, where the strains studied were isolated from different sources and several localities, being representative of the North and Northwestern region of Paraná state. Moreover, the bootstrap analysis done with the complement of Jaccard's

similarity coefficient, confirmed the consistency of these two groups.

In Rio Grande do Sul, another state in Southern Brazil, Fernandes *et al.* (1997), analyzing isoenzyme and RAPD profiles of *T. cruzi* strains isolated from reservoirs, triatomines and humans, two independent groups of strains were also observed, related to the sylvatic transmission cycle and the domestic one. These authors observed a close correlation between the main groups defined by RAPD and by isoenzyme profiles.

The data of this study are also in agreement with results obtained by several other authors that worked with the isoenzyme technique (Miles *et al.*, 1977; Tibayrenc *et al.*, 1993) and with other genetic markers, including RAPD. This led to congruent results, dividing the *T. cruzi* taxon into two main groups (Tibayrenc, 1995; Souto *et al.*, 1996; Brenière *et al.*, 1998). However, Acosta *et al.* (2001) did not obtain the same observations when researching the isoenzyme profiles of twenty one *T. cruzi* stocks isolated from humans, domiciliary triatomines and one sylvatic animal, obtained from different areas of Paraguay. The authors classified all stocks as the same lineage, using MNcl2 as the *T. cruzi* II reference stock, which could be explained by the fact that the great majority of the stocks were isolated from the domestic transmission cycle.

In the present work, two major genetic groups were observed, and their intra-group genetic diversities were low. *T. cruzi* strains isolated from vectors and sylvatic reservoirs constituted one group and displayed very similar RAPD and SSR-PCR profiles, with a high number of shared bands among them.

Soccol *et al.* (2002) obtained similar results using multilocus isoenzyme electrophoresis and 31 isolates from sylvatic reservoirs and triatomines from two regions of Paraná state. The data found by the authors cited above, together with those of this paper, have shown that the genetic variability among these sylvatic isolates is very limited, unlike what has been observed for isolates of sylvatic environments in other countries. This fact demonstrates that there is a geographical difference in the genetic diversity of the strains. Soccol *et al.* (2002) suggest that the limited clonal variability is due to the recent common origin of the sylvatic populations of the parasite in Paraná state, which is considered a special case.

In the same way, the other group observed here, was constituted of isolated human strains, which also displayed low genetic diversity among themselves. Gomes *et al.* (1998) concluded the same using RAPD and SSR-PCR techniques and 30

strains isolated from chronic chagasic patients living in this region.

The similarity between RAPD and SSR-PCR data confirmed the existence of two genetic groups of *T. cruzi* in North and Northwestern Paraná. Based on this fact, our next step was to verify the correspondence of these very different genetic groups with the major *T. cruzi* I and II groups.

We observed that strains isolated from vectors and reservoirs of Paraná state were closer to the *T. cruzi* I reference stock and that strains isolated from humans were closer to the *T. cruzi* II reference stock. The finding, for the first time, of the *T. cruzi* II genetic group in Paraná state is important, as it complements the existing data about the presence of *T. cruzi* I in the sylvatic transmission cycle of this state (Soccol *et al.*, 2002).

It seems obvious that the differences in biological behavior are related to variations in the genomic constitution expressed by the amplification of DNA fragments. Toledo *et al.* (2002) found correlations between the biological behavior in mice and the genetic distance of *T. cruzi* clonal genotypes. Recently, we demonstrated that *T. cruzi* strains isolated from humans in Paraná state could be divided into two genetic groups. We showed that one group, constituted of the 150 strain, differed from the other one in biochemical and biological parameters (Gomes *et al.*, 2003). For this reason, the 150 strain was also used as reference for comparison with the strains studied here. We observed that the 150 strain grouped with other strains isolated from humans together with Esmeraldo, in spite of having exhibited a larger genetic distance with regard to the others. This fact confirms the data already obtained for this strain and opens up the possibility of verifying the possible correlation among genetic, biological and biochemical characteristics of the presented isolated Paraná state strains.

## Conclusion

Even though Paraná is considered the fourth endemic state in Brazil, little is known about the genetic characteristics of the populations of *T. cruzi* parasite that circulate in it. The finding of the two genetic groups of *T. cruzi* circulating in the North and Northwestern Paraná, increases the general data about this parasite and opens perspectives for the study of several biological parameters of these two groups of strains. It also opens up the possibility to better investigate the impact of these two genetic groups in the clinical evolution of human Chagas' disease, thereby improving diagnosis. Genetic markers of benznidazole resistant strains should also

be identified, since it is the only available drug for etiologic treatment of chagasic patients. Henceforth, it may become feasible to conduct a study with alternative drugs that might arise. Thus, the search around such information is according to the premise that groups, which work with basic research and attend to chagasic patients, have moral and ethical obligation of offering improvements to those patients.

## Acknowledgments

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