

Usefulness of Microsatellite Typing in Population Genetic Studies of *Trypanosoma cruzi*

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Through microsatellite analysis of 53 monoclonal populations of Trypanosoma cruzi, we found a remarkable degree of genetic polymorphism with no single multilocus genotype being observed more than once. The microsatellite profile proved to be stable during 70 generations of the CL Brener clone in culture. The microsatellite profiling presented also high diagnostic sensitivity since DNA amplifications could be achieved with less than 100 fg DNA, corresponding to half parasite total DNA content. Based on these technical attributes the microsatellite assay turns out to be an important tool for direct typing T. cruzi in biological samples. By using this approach we were able to type T. cruzi in feces of artificially infected bugs and in single cells sorted by FACS. The microsatellites have shown to be excellent markers for T. cruzi phylogenetic reconstruction. We used maximum parsimony based on the minimum number of mutational steps to build an unrooted Wagner network, which confirms previous conclusions based on the analysis of the D7 domain of the LSU rDNA gene that T. cruzi is composed by two major groups. We also obtained evidence that strains belonging to rRNA group 2 are subdivided into two genetically distant clusters, and that one of these clusters is more related to rRNA group 1/2. These results suggest different origins for these strains.

Key words: *Trypanosoma cruzi* - microsatellites - genetic typing - rDNA classification - phylogenetic inference

Chagas disease caused by the protozoan *Trypanosoma cruzi* exhibits distinct clinical manifestations, taking an asymptomatic indeterminate course or evolving as chronic cardiomyopathy and/or gastrointestinal pathology. It is believed that such clinical pleiotropism is the product of a complex interaction of environmental and genetic factors from both host and parasite (reviewed Macedo & Pena 1998). Despite the great clinical variability, studies based on rRNA and mini-exon gene sequence and RAPD markers have indicated a clear division of *T. cruzi* into two major lineages pre-

senting a high phylogenetic divergence (Souto et al. 1996, Zingales et al. 1998). The division of *T. cruzi* into two major phylogenetic lineages has been officially recognized and they were named *T. cruzi* I and *T. cruzi* II (Momen 1999). The existence of the two groups is now well established on the basis of biological, epidemiological, biochemical and molecular data (revised in Zingales et al. 1999). However, the significance of these divisions is still under debate (Brisse et al. 1998, Souto et al. 1998, Macedo & Pena 1998). In order to try to understand the significance of the two major lineages, the parasite should be studied with genetic markers presenting distinct rates of molecular evolution.

In 1998, we described the first eight polymorphic microsatellite *loci* with cytosine-adenine (CA) repeats in the *T. cruzi* genome (Oliveira et al. 1998). Preliminary studies on these microsatellite *loci* have provided valuable information about the structure of *T. cruzi* populations (Oliveira et al. 1998, 1999).

In this work we evaluate the sensitivity and stability of microsatellite profiles under laboratory conditions and investigate their application in *T. cruzi* profiling directly in biological samples as well in phylogenetic analysis.

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MATERIALS AND METHODS

Parasites - *T. cruzi* stocks were obtained from the Laboratório de Biologia do *Trypanosoma cruzi* (Departamento de Parasitologia, UFMG, Brazil) and from the Centre d'Etudes sur le Polymorphisme des Microorganismes (Montpellier, France) (Table). Epimastigote forms were grown in LIT (liver infusion tryptose) medium and DNA preparations were obtained following extractions with phenol-chloroform as previously described (Macedo et al. 1992).

Microsatellite assay - *T. cruzi* stocks were analyzed with the MCLE01, MCLE08, SCLE11, MCLF10, MCLG10 and MCL05 microsatellite loci or, alternatively, with single microsatellite locus, the SCLE10. Amplification was achieved as described earlier by Oliveira et al. (1998). After PCR, the amplified microsatellites were loaded on a 6% denaturing polyacrylamide gel and analyzed on an ALF sequencer (Pharmacia) using the Allelinks software. To determinate the allele size the samples were directly compared with band sizes from an allelic ladder prepared by amplification of an artificial mixture of DNA from 60 *T. cruzi* strains.

Microsatellite stability - The stability of microsatellites was investigated in samples of CL Brener clone obtained after 10, 20, 30, 40, 50, 60 and 70 generations of subcultivation in LIT medium. The initial inoculum of each passage was of $20\text{-}25 \times 10^6$ parasite/ml and the doubling time of the cultures was calculated as 58 ± 13 hs (Zingales et al. 1997). DNA from 3×10^8 parasites from each generation above-indicated was extracted and stored at -70°C until used. Aliquots containing 1 ng of parasite DNA were submitted to PCR for all microsatellite loci.

Vector intestinal analyses - The third-instar nymphs of *Rhodnius neglectus* or *Dipetalogaster maximus* were simultaneously infected with bloodstream trypomastigotes from four monoclonal strains of *T. cruzi* (JG strain, Coll.7G2 clone, CL Brener clone and PNM strain) in concentration 50 cells/ml of each population (Azambuja & Garcia 1997). Sixty days after the repast, the entire intestinal tract was removed by centrifugation as described by Garcia and Azambuja (1997) and collected in 0.85% NaCl solution. After treatment with 6M guanidine, 0.2M EDTA, pH 8.0 (1:1), total DNA was obtained by sequential extraction with equal volumes of phenol/chloroform and chloroform, followed by 3M sodium acetate-pH 5.2 and ethanol precipitation (Gomes et al. 1998). Further DNA purification was performed by using GeneClean II Kit (Bio 101 Inc.) to remove PCR inhibitors. Aliquots of 1/10 dilutions were typed by PCR of the SCLE10 microsatellite locus.

Single cell analyses - For single parasite sorting an artificial mixture of two monoclonal population of *T. cruzi* (Rosa and MLBM strains) was submitted to the Clone Cyt apparatus from the FACS Vantage (Becton Dickinson). An aliquot of fixed cells on 50% Master Facs Fix (Becton Dickinson) and 50% PBS was applied to the FACS apparatus and diluted in PBS until 300 events/sec. The procedure for cell cloning was performed using the program Clone Cyt according to the manufacturer. Assorted cells were collected into microtiter plates containing 5 μl of water. After adding mineral oil in each well, the microtiter plate was heated at 80°C for 20 min for cell lysis and then submitted to SCLE10 microsatellite PCR.

rRNA 24S α gene amplification - Amplification of the D7 divergent domain of the 24S α rRNA gene was achieved by PCR with D71 and D72 primers following protocols described previously (Souto et al. 1996). Within individual isolates, one of the three amplification products was observed: a 125-bp fragment (defining group 1 - isolates); a 110-bp fragment (group 2 - isolates) and both fragments (group 1/2 - isolates) (Souto et al. 1996).

Phylogenetic inference - As previously described (Oliveira et al. 1998, 1999) to make phylogenetic inferences, we assumed a stepwise mutation model for the microsatellites. As a measure of genetic distance between any two strains we used the minimum number of mutational steps necessary to transform one into the other. The microsatellite multilocus genotypes were transformed into binary characters using the FACTOR program from the PHYLIP package version 3.57c (Felsenstein 1993). These data were then used to construct unrooted Wagner parsimony trees using the MIX program also from the PHYLIP package. The significance levels of the branching in the Wagner network were achieved by bootstrapping (1,000 reiterations) using the program SEQBOOT from the PHYLIP software package.

RESULTS

Genetic variability, stability and sensitivity of *T. cruzi* microsatellite assay - In this work we have chosen 53 monoclonal populations (see Table) among 72 *T. cruzi* isolates that have been typed before by microsatellite markers (Oliveira et al. 1999). The hypervariability previously described for *T. cruzi* microsatellite loci was confirmed in this expanded analysis since no single repeated multilocus genotype was detected among the analyzed samples.

The stability of the microsatellite loci was analyzed in samples of CL Brener clone obtained from sequential subcultivation in LIT medium. No changes in the microsatellite profiles were observed

TABLE
 Characteristics of *Trypanosoma cruzi* monoclonal stocks used in this work

Stock	Host/Vector	Group ^a	Stock	Host/Vector	Group ^a
1005	<i>Triatoma infestans</i>	1	MC	Human	1
1022	<i>Triatoma infestans</i>	1	NHR	Human	1
CL Brener	<i>Triatoma infestans</i>	1	NO	Human	1
TU18 cl93	<i>Triatoma infestans</i>	1	RTL	Human	1
1014	<i>Panstrongylus megistus</i>	1	Tula cl2	Human	1
138	Human	1	WG	Human	1
167	Human	1	Y	Human	1
169/1	Human	1	IVV cl3		1
182	Human	1	A87	<i>Didelphis marsupialis</i>	2
207	Human	1	1001	<i>Didelphis marsupialis</i>	2
209	Human	1	M5631 cl5	<i>Didelphis novemcinctus</i>	2
229A3	Human	1	Gamba cl1	<i>Didelphis azarae</i>	2
237	Human	1	Cutia cl1	<i>Dasyprocta aguti</i>	2
239	Human	1	SC43cl1	<i>Triatoma infestans</i>	2
402	Human	1	222	Human	2
Bas	Human	1	231	Human	2
Be62	Human	1	Col1.7G2	Human	2
CANIIIcl1 Z3	Human	1	M6241 cl6	Human	2
DSR	Human	1	X10 cl1Z1	Human	2
Esmeraldo cl3	Human	1	S13		2
Gil	Human	1	SO3	<i>Triatoma infestans</i>	1/2
JAF	Human	1	84	Human	1/2
JG	Human	1	115	Human	1/2
JHF	Human	1	226	Human	1/2
JSM	Human	1	MN cl2	Human	1/2
LPS	Human	1	NR	Human	1/2
MAS	Human	1			

a: group of strains defined from the amplification fragment produced from the 24Sα rRNA gene (Souto et al. 1996) (see Materials and Methods).

up to 70 generations of growing (Fig. 1). This suggests that despite of the high degree of genetic variability observed among natural *T. cruzi* populations, the microsatellites present satisfactory stability under laboratory conditions that indicate their use as genetic markers for these parasites.

The sensitivity of parasite detection by the microsatellite assay was also examined. After 35 cycles of PCR amplification, the minimum amount of template DNA required for visualizing the allele products by silver staining varied from 10 to 100 fg depending on the microsatellite locus analyzed (data not shown).

Microsatellite analysis for T. cruzi profiling - We have investigated the usefulness of the microsatellite analysis for *T. cruzi* characterization directly in biological samples. For this purpose, specimens of *D. maximus* and *R. neglectus* were infected, respectively, with a mixture of three or four monoclonal populations of *T. cruzi*. Parasites were recovered from the triatomine intestinal tracts 60 days post-infection and the extracted DNA was analyzed for the SCLE10 microsatellite locus. Fig.

2 shows the *T. cruzi* allele profiles identified in *D. maximus* feces infected with a mixture of CL Brener + PNM + JG and in *R. neglectus* feces infected with a mixture of CL Brener + Col1.7G2 + PNM + JG. The data indicate that the assay allows the identification of all *T. cruzi* population added to the infection mixture. In some *R. neglectus* specimens we could not detect the presence of the PNM strain. It should be emphasized that positive microsatellite amplification was obtained even in feces samples where microscopic inspection failed to show parasites.

The sensitivity and specificity of the microsatellite assay was also investigated in single cells sorted by FACS. In this experiment two *T. cruzi* stocks (Rosa and MLBM) were mixed and submitted to FACS apparatus. The cells were collected into microwells and analyzed for microsatellite allele size. In some of the wells the SCLE10 allele corresponding to Rosa or to MLBM strains could be detected (Fig. 3). Both strain alleles were never observed in a unique well as expected for cloned parasites.

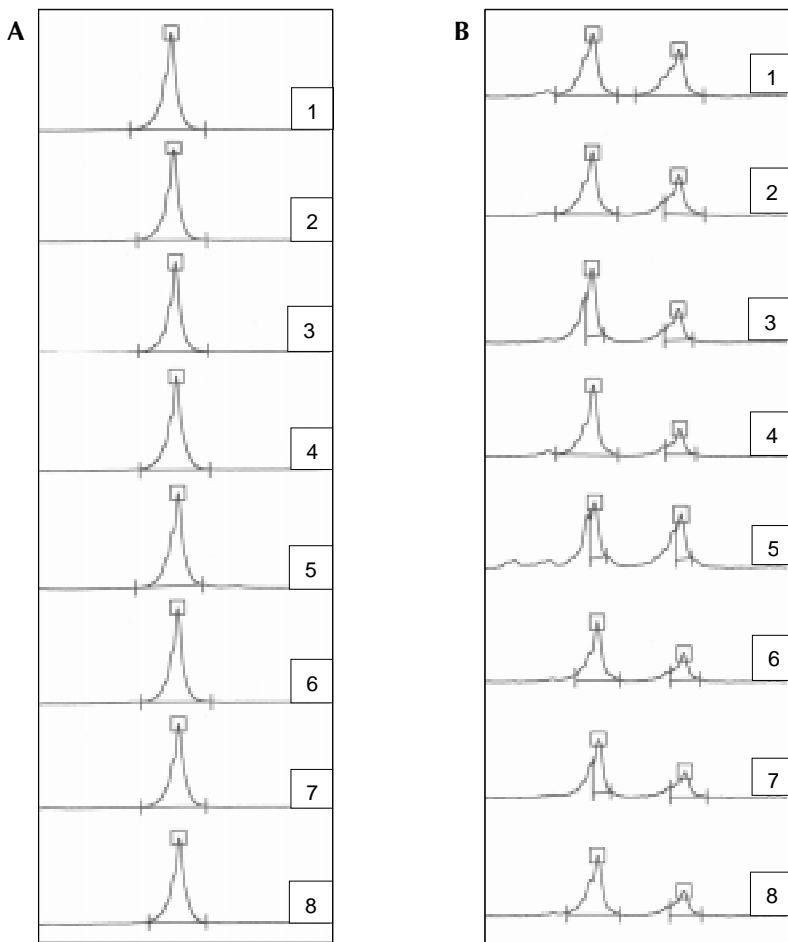


Fig. 1: microsatellite profile electrofluorogram from CL Brener clone obtained by sequential subcultivation up to 70 generations with MCLG10 (box A) and MCLE05 (box B) loci. In line 1: parental clone; lines 2 to 8: respectively 10th, 20th, 30th, 40th, 50th, 60th and 70th generations

T. cruzi phylogenetic relationship - To further evaluate the phylogenetic value of *T. cruzi* microsatellite markers, we compared microsatellite polymorphism with *T. cruzi* classification based on the divergent domain D7 of the 24S α rRNA gene, which corresponds to a more conserved genetic trait. To investigate the phylogenetic relationship among the 53 monoclonal strains we used the multilocus genotype obtained with six microsatellite loci to investigate the phylogenetic relationships among them. We used the Wagner parsimony algorithm to obtain one most parsimony unrooted network that is shown in Fig. 4. This analysis shows a clustering tendency of the samples belonging to rRNA group 1 from those stocks typed as group 2 or 1/2. The rRNA group 2 strains are arranged into two major clusters, genetically distant one from each other. Interestingly, one cluster is more related to the strains belonging to rRNA group 1/2.

DISCUSSION

The disclosure of the *T. cruzi* microsatellites has provided a new tool for the analysis of the parasite population structure. These DNA markers are extremely polymorphic and dispersed throughout the parasite nuclear genome and constitute a simple mean to determine if a *T. cruzi* strain is a monoclonal or multiclonal population (Oliveira et al. 1998, 1999).

In the present study we demonstrate that besides its technical simplicity and the high resolving power, the microsatellite analysis also presents good stability under laboratory conditions and high detection sensitivity. In fact, we demonstrated conservation of the alleles corresponding to the CL Brener microsatellite loci during 70 generations of continuous subculture of the original clone. It was also concluded that microsatellite amplifications

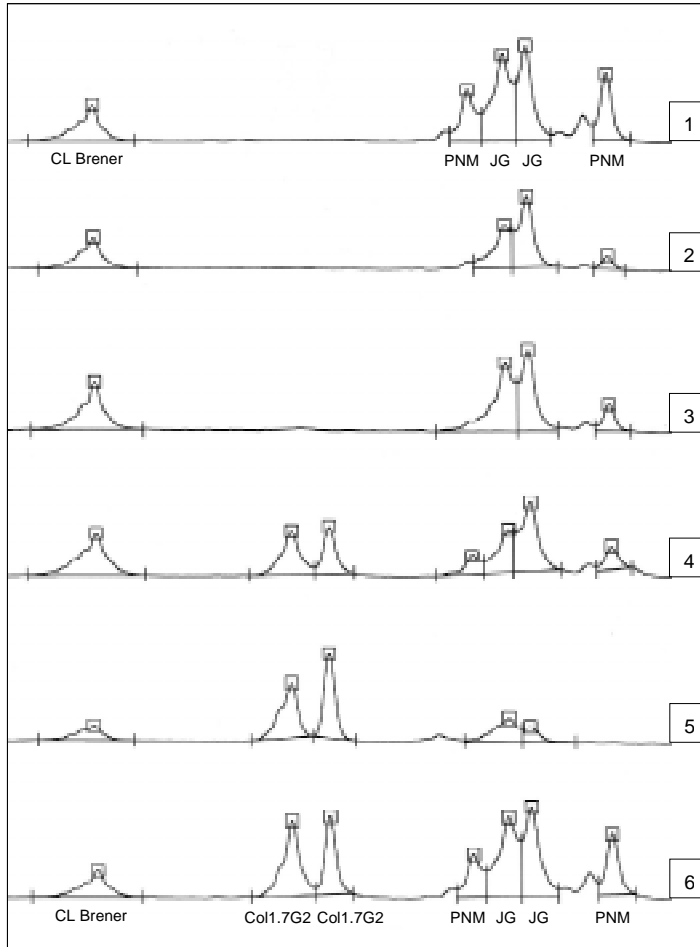


Fig. 2: microsatellite profile electrofluorogram with SCLE10 locus of parasites recovered from triatomine feces artificially infected with monoclonal *Trypanosoma cruzi* populations. In line 1 and 6: allelic profiles of all *T. cruzi* populations used to meal *Dipetalogaster maximus* and *Rhodnius neglectus* respectively; in lines 2 and 3: allelic profiles obtained from parasites recovered from feces of two *D. maximus* specimen infected with CL Brener + PNM + JG; in lines 4 and 5: allelic profiles obtained from parasites recovered from feces of two *R. neglectus* specimen infected with CL Brener + Col1.7G2 + PNM + JG.

can be obtained with less than 100 fg of parasite DNA, which corresponds to less than half of the parasite genome. These data suggest that microsatellite PCR is suitable for *T. cruzi* detection in biological samples with low parasite content.

The potentialities of the microsatellite tool were further investigated following two approaches. Firstly, we analyzed the intestinal content of triatomines infected with mixtures of *T. cruzi* populations. By using microsatellite PCR, we were able to directly profile each *T. cruzi* population in bug feces, even in samples in which microscopic fresh examination failed to identify the presence of parasites. Secondly, we could type *T. cruzi* microsatellite on single parasites isolated by cell sorting

and verify that different wells gave PCR products characteristic of either of the two previously mixed strains. These pilot experiments clearly demonstrate the possibility of sorting and analyzing *T. cruzi* individual cells opening new perspectives for the *T. cruzi* population studies. This issue is particularly relevant since occasionally some strains with interesting biological properties cannot be cloned.

We also investigated the usefulness of microsatellite as genetic markers for *T. cruzi* phylogenetic reconstruction in comparison with the 24S α rRNA gene polymorphism. The tree topology obtained from microsatellite data from 53 *T. cruzi* populations clearly indicated clustering of strains belonging to rRNA group 1, group 1/2 and

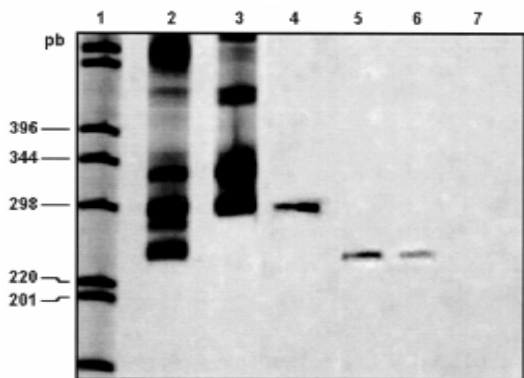


Fig. 3: microsatellite profiles on silver stained acrylamide gel with SCLE10 locus of *Trypanosoma cruzi* single cells sorted by FACS Vantage from a mix of MLBM and ROSA strains. In line 1: 1Kb ladder (Pharmacia Biotech); line 2: MLBM strain control DNA (1ng); line 3: ROSA strain control DNA (1ng); lines 4 to 6: amplification of wells containing one *T. cruzi* single cell; and line 7: amplification of a well containing no cells (negative control).

group 2 previously defined by Souto et al. (1996). We obtained evidence that the rRNA group 2 can be further split into two distant subgroups, and that one of these subgroups is more related to group 1/2. This observation suggests that the evolutionary origin of *T. cruzi* rRNA group 2 is more complex

than predicted in previous studies (Souto et al. 1996, Oliveira et al. 1998).

Different hypotheses have been proposed to explain the origin of the major lines of *T. cruzi* (Souto et al. 1996). One of these hypotheses suggests the independent evolution of rDNA genotypes 1 and 2. The origin of group 1/2 is explained by an eventual rRNA gene transfer from an individual of group 2 to organisms of group 1. Supporting this hypothesis RAPD and mini-exon data have demonstrated that strains belonging to rRNA group 1/2 have a genetic structure closely related to group 1 (Souto et al. 1996). The presence of organisms with rDNA genotype 2 more related to 1/2 could be explained by a subsequent deletion of the rRNA gene type 1 cistron from the individual of group 1/2.

There are still different opinions about the significance of the division of *T. cruzi* into the two major phylogenetic lineages, but the basis for that is better and better supported by biological, epidemiological, biochemical and molecular data.

It is predicted that microsatellite analyses of natural *T. cruzi* multiclonal strains will certainly allow the refinement of *T. cruzi* genetic population studies, which until now have been conducted by comparing different populations instead of individuals. In this direction, our future goal is to

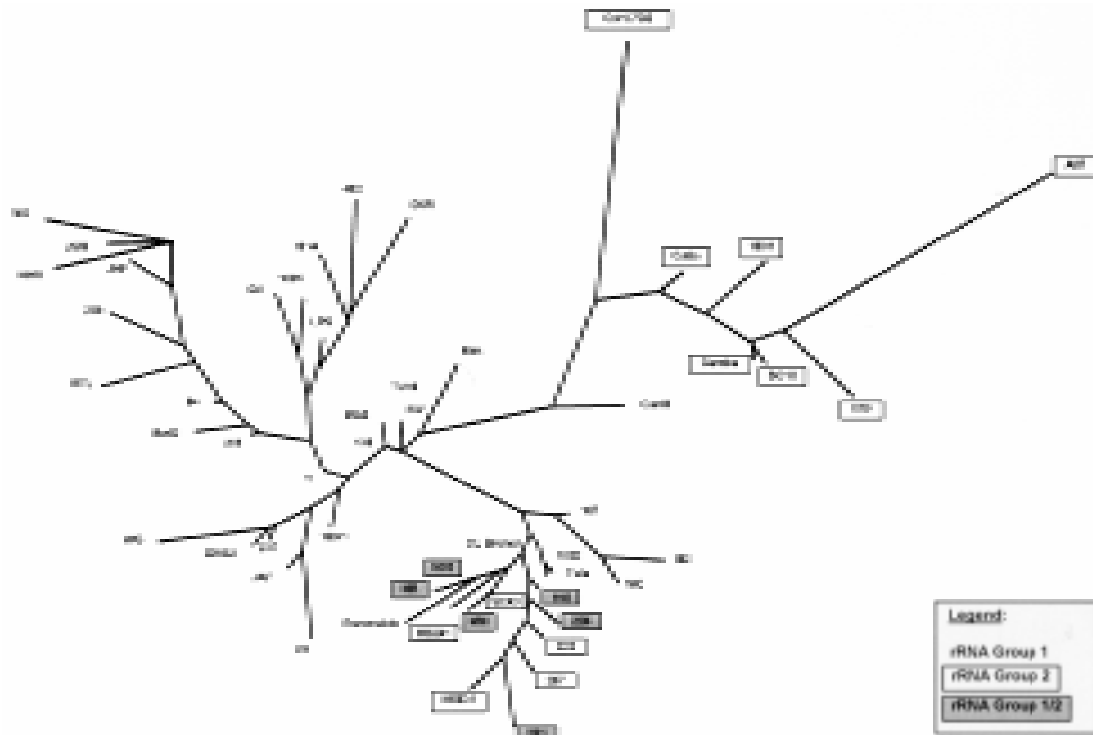


Fig. 4: unrooted Wagner network of 53 *Trypanosoma cruzi* strains and clones based on the amplification of five microsatellite loci of (CA)_n repeats. The classification of each strain from the divergent domain D7 of the 24Sα rRNA gene is also indicated. The strains belonging to rRNA group 1/2 and 2 are highlighted by a shaded box and a white box respectively. The strains belonging to rRNA group 1 are not marked.

achieve the direct genetic typing of *T. cruzi* in infected host tissues for a better understanding of the pathogenesis of Chagas disease.

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