Restriction Fragment Length Polymorphism of 195 bp Repeated Satellite DNA of *Trypanosoma cruzi* Supports the Existence of Two Phylogenetic Groups

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The restriction fragment length polymorphism of the 195 bp repeated DNA sequence of Trypanosoma cruzi *was analyzed among 23* T. cruzi *stocks giving a reliable picture of the whole phylogenetic variability of the species. The profiles observed with the enzymes* Hinf I *and* Hae III *were linked together and supported the existence of two groups. Group 1 shows a 195 bp repeated unit (*Hinf I*) and high molecular weight DNA (*Hae III*), while group 2 presents a ladder profile for each enzyme, which is a characteristic of tandemly repeated DNA. The two groups, respectively, clustered stocks pertaining to the two principal lineages evidenced by isoenzyme and RAPD markers. The congruence among these three independent genomic markers corroborates the existence of two real phylogenetic lineages in* T. cruzi. *The specific monomorphic profiles for each major phylogenetic lineage suggest the existence of ancient sexuality and cryptic biological speciation.*

Key words: *Trypanosoma cruzi* - satellite DNA - 195 bp repeat unit

Satellite DNA, as distinct from kinetoplast DNA, was observed in *Trypanosoma cruzi* (the causative agent of Chagas disease) and *T. brucei* protozoan parasites (Borst et al. 1980). *T. cruzi* satellite DNA located in minichromosomes constituted approximatively 9% of the nuclear DNA and was made of a long tandem array of 195 bp repeated (Lanar et al. 1981, Castro et al. 1981, Sloof et al. 1983). Satellite DNA diverges rapidly from one species to another and provides a useful tool to distinguish close related species of the genus trypanosome (Ole-MoiYoi 1987, Dickin & Gibson 1989, Jarman & Wells 1989, Brenière et al. 1993a,b). Moreover, *T. cruzi* satellite DNA is a target for a specific PCR-based diagnosis of Chagas disease in blood samples (Gonzales et al. 1984).

The genetic variability of *T. cruzi* is significant as evidenced by isoenzyme (Miles et al. 1978, Romanha et al. 1979, Tibayrenc et al. 1981, 1986), restriction fragment length polymorphisms (RFLP) of kinetoplast DNA (Morel et al. 1980, Tibayrenc & Ayala 1987), DNA fingerprinting (Macedo et al. 1992) and random amplification of polymor-

phic DNA (RAPD) (Tibayrenc et al. 1993, Steindel et al. 1993). *T. cruzi* is present as numerous natural clones (Tibayrenc & Ayala 1988) clustered in two principal phylogenetic lineages each highly polymorphic (Tibayrenc et al. 1993, Souto et al. 1996).

In this work satellite DNA RFLPs were compared between *T. cruzi* stocks previously characterized by RAPD (7 loci) and multilocus enzyme electrophoresis (MLEE, 22 loci), using the 195 bp repeated as probe. The observed dimorphism of 195 bp-RFLP preserved the two lineages giving a further evidence of a clear upper subdivision of *T. cruzi* taxon.

MATERIALS AND METHODS

Samples and genetic characterization - The Table summarizes places, and host origins of 24 stocks. Among them, 18 were previously characterized using 22 isoenzyme loci (Table and unpublished data) and 7 RAPD loci (Tibayrenc et al. 1993). Five additional stocks were analyzed at the same 29 loci. Genetic differences between stocks were estimated by Jaccard's distance (Jaccard 1908). A distance matrix was established from combined RAPD and MLEE data. Phylogenetic relationships were evaluated by the clustering method using the UPGMA (unweighted pair-group method with arithmetic averages) algorithm method (Sokal & Sneath 1973). The accuracy of phylogenetic divisions was tested by a bootstrap analysis; we selected the A0087 stock as an

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TABLE

Origin, of the 24 *Trypanosoma cruzi* stocks, studied

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Stocks	Host	Locality	MLEE[b]
Cutia cl1[a]	*Dasylprocta aguti*	Espírito Santo, Colatina, Brazil	1
Cuica cl1[a]	*Philander opossum*	São Paulo, Riberão Preto, Brazil	1
SO34 cl4[a]	*Triatoma infestans*	Potosi, Toropalca, Bolivia	1
Gamba cl1[a]	*Didelphis azarae*	São Paulo, Riberão Preto, Brazil	2
P11 cl3[a]	Human	Cochabamba, Bolivia	1
Esquilo cl1[a]	*Sciurus aestuans ingrami*	São Paulo, Franca, Brazil	1
SP104 cl1[a]	*Triatoma spinolai*	IVa region, Combarlalá, Chile	ND
P209 cl1[a]	Human	Chuquisaca, Sucre, Bolivia	1
13379 cl7[a]	Human	Santa-Cruz, Bolivia	1
X10 cl1 Z1[a]	Human	Pará, Belém, Brazil	1
OPS4	*Didelphis marsupialis*	Carabobo, El Yagual, Venezuela	1
CBB cl3[a]	Human	IVa region, Tulahuén, Chile	1
TU18 cl2[a]	*Triatoma infestans*	Potosi, Bolivia	ND
Esmeraldo cl3 Z2[a]	Human	Bahia, São Felipe, Brazil	1
SO3 cl5[a]	*Triatoma infestans*	Potosi, Otavi, Bolivia	3
MN cl2[a]	Human	IVa region, -, Chile	1
SC43 cl1[a]	*Triatoma infestans*	Santa-Cruz, Santa-Cruz, Bolivia	1
NR cl3[a]	Human	IIIa region, Salvador, Chile	1
TPK1	*Triatoma infestans*	La Paz, Khala Khala, Bolivia	3
Bug 2148 cl1[a]	*Triatoma infestans*	Rio Grande do Sul, Brazil	ND
Tulahuen FKIIA cl2[a]	Human	IVa region, Tulahuen, Chile	1
M6241 cl6[a]	Human	Pará, Belém, Brazil	1
Can III cl1 Z3[a]	Human	Pará, Belém, Brazil	1
A0087	*Didelphis marsupialis*	Montsinery, French Guiana	4

a: laboratory cloned stocks; *b*: previous isoenzyme characterization for 15-20 enzymatic loci, 1: Tibayrenc et al. (1993); 2: Tibayrenc & Ayala (1988); 3: Brenière et al. (1991); 4: Lewicka et al. (1995); ND: not done.

outgroup because it is genetically unrelated to other stocks (Felsenstein 1985).

Restriction fragment length polymorphism - DNAs were phenol chloroform extracted from 100 mg of parasites (Véas et al. 1991). A complete digestion of 3 µg of the DNA preparation was obtained overnight with 5UI/µg of *Eco* RI, *Hae* III and *Hinf* I restriction enzymes (Boehringer, France). Digested DNAs were electrophoresed in a 20 cm-long 0.8% agarose gel in 1X TBE at 30 V for 16 hr and transferred by capillarity onto nylon membranes Hybond N+[TM] (Amersham, Buckinghamshire, UK) (Sambrook et al. 1989).

The 195 bp probe was purified by electroelution from NR cl3 stock PCR products obtained with TCZ1 (5'CGAGCTCTTGCCCACACGGGTGCT3') and TCZ2 (5'CCTCCAAGCAGCGGATAGTT CAGG3') primers (Genset, Paris, France). These primers were designed to anneal sites at the extremities of the *T. cruzi* satellite DNA repeated unit (Sloof et al. 1983, Moser et al. 1989). The concentration was assessed by electrophoresis.

Labeling and hybridization were performed with the enhanced chemiluminescence gene detection system (ECL, Amersham, Buckinghamshire, UK). Briefly, the membranes were pre-hybridized

in the hybridization buffer (0.25 ml cm^{-2}) for 20 min at 42ºC. The purified probe (20 ng ml^{-1} of buffer) was labeled for 10 min at 37ºC. Hybridization was carried out overnight at 42ºC. The membranes were then washed twice for 20 min at 42ºC, under high stringent conditions (6 M urea, 0.1% SDS, 0.1 SSC) and twice for 10 min in a 2 x SSC at room temperature. The detection was performed in autoradiography films (Hyperfilms[TM]-MP, Amersham, Buckinghamshire, UK).

RESULTS

RFLP patterns - Twenty four *T. cruzi* DNAs stocks digested by *Eco* R1, *Hae* III and *Hinf* I restriction enzymes were hybridized with the 195 bp probe. Three different hybridization patterns were observed: (1) a hybridization limited to high molecular weight DNAs associated with few smaller bands for some stocks (Fig. 1a,b); showing the scarcity of the restriction site in satellite DNA. This pattern was observed for all the DNAs stocks digested by *Eco* RI and half the stock DNAs digested by *Hae* III; (2) one main intense band of 195 bp corresponding to the monomer of the satellite DNA (Fig. 1c).; showing the presence of the restriction site in each repeat unit. This pattern was observed

for half of the DNAs stocks digested by *Hinf* I; (3) a ladder of 195 bp polymer bands (18 detectable polymers) (Fig. 1b,c), was observed for half of the DNAs stocks digested by *Hinf* I and *Hae* III. The clear interpretation of this profile is a tandem array of nearly identical elements, many of which lack the *Hinf* I and *Hae* III sites as a result of sequence heterogeneity.

The *Eco* RI RFLP did not show a clear polymorphism due to the scarcity of the restriction site. Two distinct patterns were observed for the *Hinf* I and *Hae* III RFLPs. Moreover the stocks showing a *Hinf* I unit always presents *Hae* III high molecular weight profiles and those with a *Hinf* I ladder also presents *Hae* III ladder patterns. Thus the stocks were clustered in only two different RFLP groups.

Clustering and RFLP 195 bp - Fig. 2 presents the dendrogram constructed from RAPD and MLEE data (29 loci) and shows two principal lineage named 1 and 2. Lineage 1 and 2 occur 100 and 83.2 times out of 100 replicates, respectively (bootstrap analysis). The stocks belonging to each lineage (1 and 2) present a specific RFLP pattern, except Can III stock which was clustered in lineage 2 but has a RFLP pattern identical to the stocks of lineage 1.

DISCUSSION

Taxonomical considerations - The present phylogenetic study, plotting RAPD and MLEE data together, allows an analysis of 29 loci and clusters the stocks in two distinct lineages. The same lineages were previously evidenced separately by each marker (Tibayrenc et al. 1993). We introduced the bootstrap analysis to test the confidence limits of the two present groups. The bootstrap places high confidence limits only for lineage 2 (100%), while the monophyly of lineage 1 is more uncertain (83.2%); a group can be considered monophyletic if it occurs in at least 100 - 5/n-1 percent of the bootstrap estimated, where n is the number of groups (Felsenstein 1985).

The analysis of *T. cruzi* RFLP satellite DNA polymorphism, shows only two distinct profiles among the same set of *T. cruzi* stocks. This dimorphism preserves the two lineages with the exception of Can III stock which presents a lineage 2 RFLP profile. If we consider the polymorphism of satellite DNA, Can III stock, belonging to zymodeme 3 (Z3), is more related to lineage 2 that includes the X10 cl1 stock belonging to zymodeme 1 (Z1) (Miles et al. 1977, 1981). Pioneering work showed that Z1 and Z3 have a closer relationship (Ready & Miles 1980). Moreover, in Brazilian, French Guianan and Bolivian (unpublished data) sylvatic cycles, stocks belonging to Z1 and Z3 zymodemes are largely represented (Miles et al. 1981, Lewicka et al. 1995, Carrasco et al. 1996). The profile showed by 195 bp RFLP seems to support a common evolution of Z1 and Z3 zymodemes.

In conclusion, the satellite DNA polymorphism would truly support the presence of two lineages
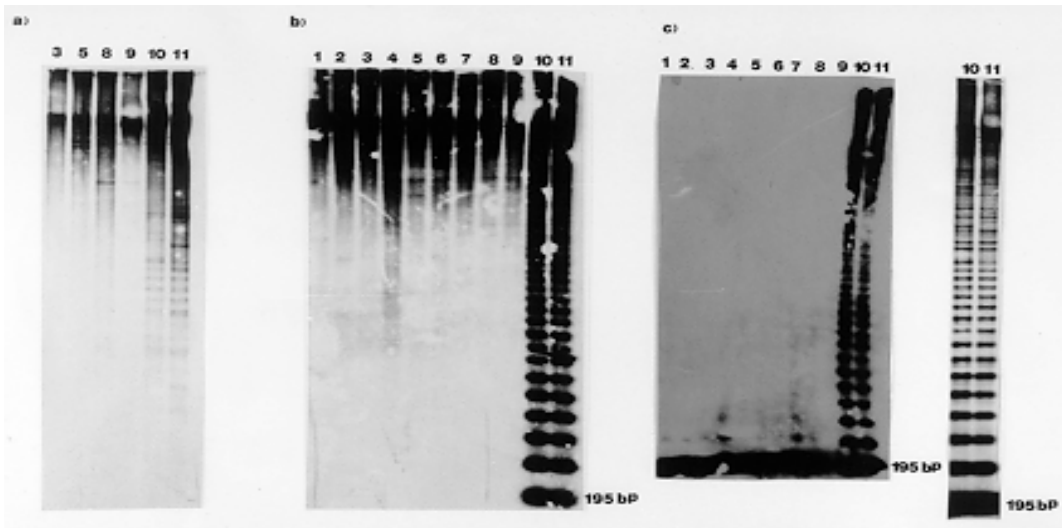

Fig. 1: autoradiographs of Southern blots showing typical banding observed when (a) *Eco* RI (b) *Hae* III and (c) *Hinf* I digests of DNA from *Trypanosoma cruzi* stocks, hybridized with the 195 bp repeated probe.
Lanes 1 to 9: P209 cl1, Sp104 cl1, Cutia cl1, Gamba cl1, 13379 cl7, P11 cl3, So34 cl4, Cuica cl1, Esquilo cl1. These stocks belong to lineage 2 evidenced by RAPD and MLEE markers and present a unit profile for *Hinf* I and high molecular weight for *Hae* III.
Lanes 10 to 11: Sc43 cl1, Bug 2148 cl1. These stocks belong to lineage 1 and present a ladder profile for *Hinf* I and *Hae* III.
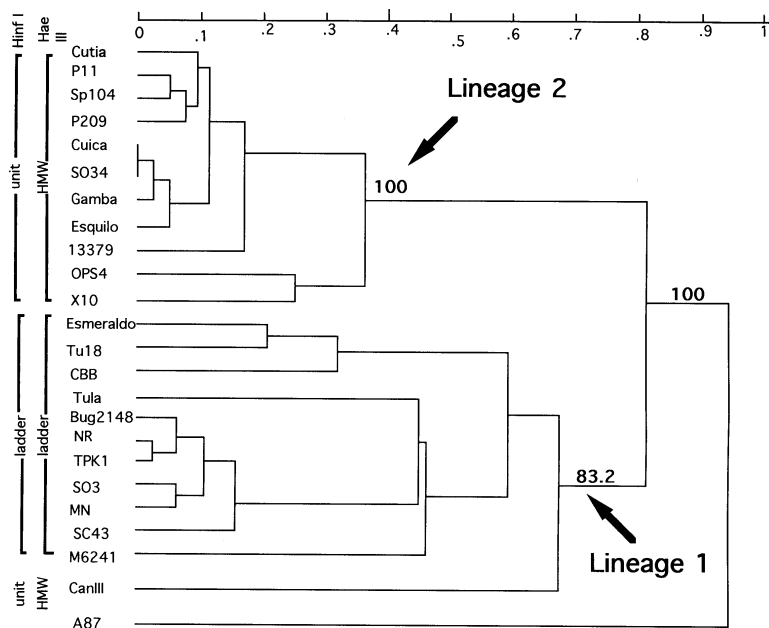
Fig. 2: dendrogram of the 24 *Trypanosoma cruzi* stocks constructed by UPGMA method from Jaccard's distances obtained by RAPD and MLEE (29 loci) method joined toghether. The values of bootstrap are indicated for the two lineages. Unit, ladder and high molecular weight (HMW) refer to the RFLP profile obtained with *Hinf* I and *Hae* III restriction enzymes.

in the natural *T. cruzi* population. This attractive result deserves to be investigated in a larger set of *T. cruzi* stocks of geographic and host diversified origins.

What is the origin of the two major phyloge-netic lineages of T. cruzi? - A classical mechanism of homogenization of tandem repeat is the recombination that occurs constantly in sexual species. According to our results, *T. cruzi* satellite DNA shows no polymorphism of its tandem repeated sequences within each of the two major phylogenetic lineages described (Tibayrenc 1995, Souto et al. 1996). This drastic satellite DNA sequence divergence between the two phylogenetic groups, together with their homogeneity within each of them, suggests events of cryptic speciation in a basically sexual species followed by a sequence homogenization in each species. Such a hypothesis of a cryptic biological speciation has been proposed (Tibayrenc et al. 1984) in order to explain the origin of the two *T. cruzi* major phylogenetic lineages. As mentioned above, a great homogeneity of sequences is expected within a biological species, if one assumes a tandem arrangement of sequences. The present populations of *T. cruzi* show a typical clonal structure. In case of long-lasting clonal evolution, we would expect a divergence of the repeated sequences between clones due to the accumulation of divergent muta-tions not only between the two major phylogenetic lineages but also within each of them. The satellite DNA data, therefore, suggest the existence of ancient sexuality and cryptic biological speciation within *T. cruzi* followed by a more recent clonal evolution in the two lineages. Nevertheless, isoenzyme and RAPD data do show considerable genetic heterogeneity and strong linkage disequilibrium within each of the major phylogenetic lineages, which supports the hypothesis that they are the result of a long-term clonal evolution with only occasional events of hybridization followed by clonal propagation of the hybrids (Tibayrenc 1995, Bogliolo et al. 1996, Brisse et al. 1998). An alternative explanation for the satellite DNA monomorphism within each of the two lineages is that in *T. cruzi,* and possibly in other kinetoplastid parasites, these sequences have a specific evolutionary rate and a slow molecular clock. In this case, the specific monomorphic profiles recorded in the present study for each major phylogenetic lineage would correspond to common-place synapomorphic characters, as it is the case for many isoenzyme, RAPD, and mini-exon characters (Tibayrenc 1995, Souto et al. 1996, Brisse et al. 1998).

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