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A phylogenetic analysis of the *Trypanosoma cruzi* genome project CL Brener reference strain by multilocus enzyme electrophoresis and multiprimer random amplified polymorphic DNA fingerprinting

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Abstract

We have assessed the phylogenetic status of the *Trypanosoma cruzi* Genome Project CL Brener reference strain by multilocus enzyme electrophoresis (MLEE) and multiprimer random amplified polymorphic DNA (RAPD) including a set of cloned stocks representative of the whole genetic diversity of *T. cruzi*. MLEE and RAPD data gave congruent phylogenetic results. The CL Brener reference strain fell into the second major phylogenetic subdivision of *T. cruzi*, and was genetically very close to the Tulahuen reference strain. No reliable RAPD character and only one MLEE character permitted us to distinguish between the CL Brener and Tulahuen reference strains. In contrast, many RAPD and MLEE characters were able to distinguish between the CL Brener reference strain and the other *T. cruzi* genotypes analyzed here, in particular the formerly described principal zymodemes I, II and III. It is suspected that both CL Brener and Tulahuen are hybrid genotypes, a fact that should be taken into account when interpreting sequence data. Moreover, our study confirms that the species *T. cruzi* is genetically very heterogeneous. We recommend future comparison of sequencing data from the CL Brener reference strain with those of at least one radically distinct *T. cruzi* genotype, belonging to the other major phylogenetic subdivision of this species.

Keywords: Chagas' disease; Genetic characterization; Hybrid genotype; Random amplified polymorphic DNA; Multilocus enzyme electrophoresis

Abbreviations: MLEE, multilocus enzyme electrophoresis; RAPD, random amplified polymorphic DNA; UPGMA, unweighted pair-group method with arithmetic averages.

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1. Introduction

The *Trypanosoma cruzi* Genome Project was launched in 1994 by the WHO Special Programme for Research and Training in Tropical Diseases with the aim of establishing a detailed physical map, and ultimately the entire sequence, of the genome of the aetiological agent of Chagas' disease. The CL Brener clone, derived in 1987 from the long-studied reference strain CL isolated in Southern Brazil [1] was selected as the reference clone of the Genome Project.

Trypanosoma cruzi, the agent of Chagas' disease, shows considerable genetic polymorphism. This was first demonstrated by the pioneering multilocus enzyme electrophoresis (MLEE) work of Miles et al. [2], and fully confirmed by the analysis of MLEE and random amplified polymorphic DNA (RAPD) data in evolutionary genetics terms, which has shown that *T. cruzi* has a clonal population structure [3,4]. The natural clones that subdivide *T. cruzi* are distributed into two major phylogenetic lineages, each considerably heterogeneous, and separated from each other by evolutionary distances that are four times greater than those that separate men from chimpanzees [5].

The CL mother strain has been previously characterized by isoenzyme studies [6]. However, it was shown to be a heterogeneous population containing a mixture of at least two genotypes, on the basis of RFLP analysis of kinetoplast DNA [7] and MLEE [8].

Considering both the extreme intraspecific phylogenetic diversity of *T. cruzi* and the high risk of experimental stock mix-up, it is important to have an accurate idea of the genetic identity of clone CL Brener. With this intention, we carried out an extensive analysis of the reference strain of the *T. cruzi* Genome Project by both MLEE (22 genetic loci) and RAPD (72 primers).

2. Materials and methods

2.1. Selection and culturing of the stocks

In order to reliably set the phylogenetic status of the clone CL Brener, ten stocks representative of the whole genetic diversity of *T. cruzi*, as shown by previous MLEE analysis [3], have been included in this study. All these stocks have been cloned in the laboratory by micromanipulation under the microscope. Table 1 gives the name of the stocks, as well as their origin, date of isolation and previous genetic identification. The stocks were bulk-cultured in disposable plastic flasks in liver infusion tryptose (LIT) culture medium containing 10% fetal calf serum (FCS) and 50 μg ml^{-1} gentamycin.

Table 1
List of the 11 *Trypanosoma cruzi* stocks under study

Stock	Zymodeme[a]	Host	Geographic origin
X10 cl1	17 (ZI[b])	Human	Belém, Brazil
Cuica cl1	20	*Opossum cuica philander*	São Paulo, Brazil
Can III cl1	27 (ZIII[b])	Human	Belém, Brazil
TU18 cl2	32	Not known	Bolivia
Esmeraldo cl3	30 (ZII[b])	Human	Bahia, Brazil
NR cl3	39	Human	Atacama, Chile
SC43 cl1	39	*Triatoma infestans*	Santa Cruz, Bolivia
M6241 cl6	35	Human	Belém, Brazil
M5631 cl5	36	*Didelphis novemcintus*	Belém, Brazil
Tulahuen cl2	43	Human	Chile
CL Brener		*Triatoma infestans*	Southern Brazil

[a] Zymodeme numbers are given in [3].
[b] [2].

2.2. Preparation of the samples

Stocks were harvested by centrifugation (2800 × *g*, 20 min, 4°C) and washed in phosphate-buffered saline (PBS) (Na_2HPO_4 10 mM, NaH_2PO_4 10 mM, NaCl 150 mM, pH 7.2). Cells were lysed in an equal volume of hypotonic enzyme stabilizer (EDTA 2 mM, dithiotreitol 2 mM, ε-aminocaproic acid 2 mM), on ice for 20 min. The lysates were again centrifuged (13000 × *g*, 10 min, 4°C). The soluble fraction was stored at −70°C until used in MLEE analysis, whereas the pellet of lysed cells was used for DNA extraction, according to the following protocol. Pellets were resuspended in 400 μl Tris–HCl 100 mM pH 8.0, NaCl 400 mM, $EDTANa_2$ 10 mM. SDS was then added to a final concentration of 1% and the tubes were incubated for 2 h at 37°C in the presence of 100 μg ml^{-1} RNase A and then overnight at 55°C with 200 μg ml^{-1} proteinase K. After two phenol extractions, two chloroform :isoamyl alcohol (24:1) extractions and ethanol precipitation, the DNA was resuspended in sterile double-distilled water. DNA concentration was estimated by spectrophotometry at 260 nm. DNA extracts were stored at −20°C until analysis.

2.3. Protocol for isoenzyme analysis

MLEE analysis was carried out on cellulose acetate plates (Helena) according to [9], with slight modifications. The 20 following enzyme systems were used: aconitase (ACON, E.C.4.2.1.3.), alanine aminotransferase (ALAT, E.C.2.6.1.2.), diaphorase (DIA, E.C.1.6.), glyceraldehyde-3-phosphate dehydrogenase (GAPD, E.C.1.2.1.12.), glutamate dehydrogenase NAD^+ (GDH-NAD^+, E.C.1.4.1.2.), glutamate dehydrogenase $NADP^+$ (GDH-$NADP^+$, E.C.1.4.1.4.), glutamate oxaloacetate transaminase (GOT, E.C.2.6.1.1.), glucose-6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49.), glucose-phosphate isomerase (GPI, E.C.5.3.1.9.), isocitrate dehydrogenase (IDH, E.C.1.1.1.42.), leucine aminopeptidase (LAP, E.C.3.4.11.1.), malate dehydrogenase NAD^+ (MDH, E.C.1.1.1.37.), malate dehydrogenase $NADP^+$ or malic enzyme (ME, E.C.1.1.1.40.), mannose-phosphate isomerase (MPI, E.C.5.3.1.8.), nucleoside hydrolase (inosine) (NHi, E.C.2.4.2.-.), peptidases 1 and 2 (substrates: L-leucyl-leucine-leucine and L-leucyl-L-alanine) (PEP1 and PEP2, respectively, E.C.3.4.11 or 13.-.), 6-phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44.), phosphoglucomutase (PGM, E.C.2.7.5.1.), and superoxide dismutase (SOD, E.C.1.15.1.1.). These 20 enzyme systems correspond to 22 different genetic loci, since diaphorase and malic enzyme exhibit the activity of two distinct loci each.

2.4. RAPD protocol

One hundred and twenty different decameric primers were screened, corresponding to the A, B, F, N, R and U kits from Operon Technologies (Alameda, CA). Amplification was carried out according to [10] with slight modifications. Briefly, the amplification reactions were carried out in a final volume of 60 μl containing 0.9 units Taq Polymerase (Boehringer, Mannheim, Germany), 100 μM each dNTP, 200 nM primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8.3 and 20 ng template DNA. Forty five cycles (denaturation: 1 min at 94°C; annealing: 1 min at 36°C; elongation: 2 min at 72°C) were followed by a final elongation step of 7 min at 72°C. Amplification was carried out in a PTC-100 thermocycler (MJ Research, Watertown, MS). RAPD products were analyzed by electrophoresis in 1.6% agarose gels in TAE buffer (Tris–acetate 40 mM, EDTA 1 mM) stained with ethidium bromide and visualized by ultraviolet light.

2.5. Data analysis

MLEE and RAPD data were computed with the Genetics software, designed in our laboratory and operated on SUN stations (S. Noël). For both MLEE and RAPD data, the Jaccard's distance [11] was used to estimate the genetic differences among the stocks. Each MLEE and RAPD gel band was coded with a number, starting with one for the fastest band in the case of MLEE and one for the slowest band in the case of RAPD. In

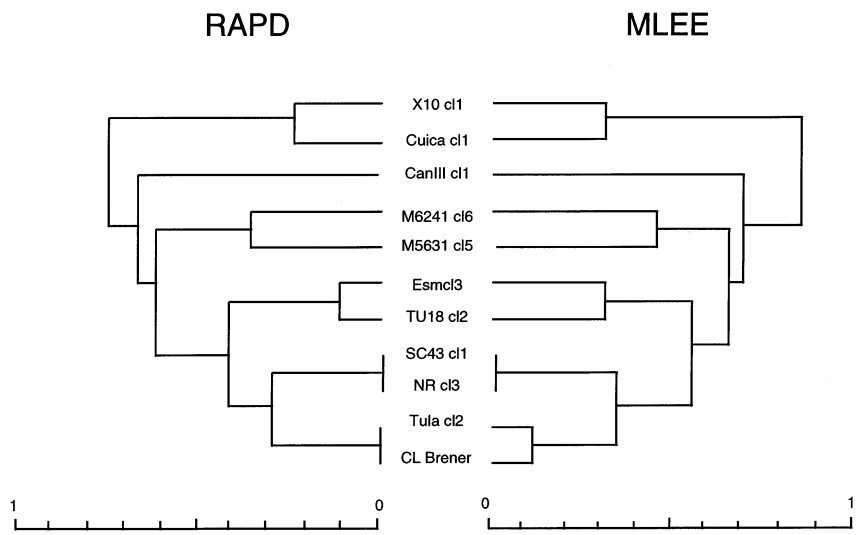

Fig. 1. Two UPGMA dendrograms [12] obtained from MLEE (right) and RAPD (left) data. The scale indicates the genetic distances [11] that separate the genotypes studied. The stocks X10 cl1 (principal zymodeme I [2]) and Cuica cl1 correspond to the first major clade described in [5], whereas the other stocks (including Esmeraldo cl3 and Can III cl1, respectively principal zymodemes II and III, [2]) correspond to the second major clade.

the case of MLEE at some loci, the numbering did not start with one, since the band numbering of a more extensive study involving 440 stocks (Barnabé, unpublished) was kept here. The distance was estimated after the following formula:

$D = 1 - [a/(a + b + c)]$.

a = Number of bands that are common to the two compared genotypes;

b = number of bands present in the first genotype and absent in the second;

c = number of bands absent in the first genotype and present in the second.

The unweighted pair-group method with arithmetic averages (UPGMA) [12] was used to cluster the genotypes together according to their Jaccard's distances.

Agreement between MLEE and RAPD genetic distances was tested by a correlation analysis based on the nonparametric test of Mantel [13]. Briefly, this test relies on a Monte Carlo simulation with 10^4 iterations, which randomly permutes the different cells of one of the distance matrices. In contrast to the classical correlation test, this randomization procedure does not make any assumptions about the number of degrees of freedom.

3. Results

3.1. MLEE analysis

As previoulsy noted [3,4], the present set of stocks exhibited considerable MLEE polymorphism. Each stock proved to have a distinct genotype with the MLEE data, with the exception of clones NR cl3 and SC43 cl1 that appeared identical. Table 2 gives the genotypes of the 11 stocks under study.

Fig. 1 shows the UPGMA dendrogram computed from MLEE data. The phylogenetic picture depicted by this dendrogram corroborates previous MLEE analysis [4].

Fig. 2 shows the five different genotypes observed for the *Gpi* locus. As previously noted [3], two different three-banded patterns were observed, which probably correspond to heterozygous genotypes for a dimeric enzyme. The *3/4* heterozygous genotype was observed in both the CL Brener and the Tulahuen cl2 reference stocks and permitted us to distinguish them from any other genotype. It is worth noting that the fastest and slowest *Gpi* alleles of the CL Brener (and the Tulahuen cl2) strain correspond to the

Table 2
Multilocus genotypes of the 11 *T. cruzi* stocks characterized with 22 enzymatic loci[a]

Stock	GPI	IDH	G6PD	GDH NAD^+	GDH $NADP^+$	LAP	MDH	ME1	ME2	6PGD	PGM	MPI	PEP1	PEP2	GOT	GAPD	DIA1	DIA2	ACON	NHI	ALAT	SOD
X10 cl1	*5/5*	*3*	*4/4*	*5/5*	*3/3*	*3/3*	*3/3*	*3/3*	*3/3*	*5/5*	*4/4*	*2/2*	*4/4*	*1/1*	*3*	*5/5*	*2*	*1/1*	*2/2*	*2/2*	*3/3*	*5-6-9*
Cuica cl1	*5/5*	*3*	*4/4*	*5/5*	*3/3*	*4/4*	*3/3*	*3/3*	*3/3*	*4/5*	*4/4*	*3/3*	*2/2*	*1/1*	*3*	*5/5*	*2*	*1/1*	*2/2*	*2/2*	*3/3*	*5-6-9*
Can III cl1	*4/4*	*5*	*5/5*	*4/4*	*5/5*	*6/6*	*3/3*	*3/3*	*2/2*	*6/6*	*7/7*	*4/4*	*6/6*	*3/3*	*2*	*6/6*	*2*	*3/3*	*3/3*	*1/1*	*3/3*	*5-6-7*
TU18 cl1	*3/3*	*5-6*	*2/2*	*4/4*	*5/5*	*4/4*	*3/3*	*3/3*	*4/4*	*2/2*	*5/10*	*3/3*	*5/5*	*2/2*	*2*	*6/6*	*3*	*3/3*	*2/2*	*1/1*	*2/2*	*5-6-7*
Esmeraldo cl3	*1/3*	*5-6*	*2/2*	*4/4*	*5/5*	*4/4*	*3/3*	*3/3*	*4/4*	*2/2*	*5/8*	*3/3*	*3/3*	*2/2*	*2*	*6/6*	*2*	*3/3*	*2/2*	*1/1*	*2/2*	*5-6-7*
NR cl3	*2/4*	*5*	*3/3*	*3/3*	*3/3*	*4/4*	*3/3*	*2/2*	*4/4*	*2/5*	*5/8*	*2/2*	*5/5*	*2/2*	*2*	*6/6*	*3*	*1/1*	*2/2*	*1/1*	*3/3*	*5-6-7*
SC43 cl1	*2/4*	*5*	*3/3*	*3/3*	*3/3*	*4/4*	*3/3*	*2/2*	*4/4*	*2/5*	*5/8*	*2/2*	*5/5*	*2/2*	*2*	*6/6*	*3*	*1/1*	*2/2*	*1/1*	*3/3*	*5-6-7*
M6241 cl6	*4/4*	*5*	*4/4*	*2/2*	*3/3*	*5/5*	*3/3*	*2/2*	*4/4*	*5/5*	*5/5*	*1/3*	*6/6*	*1/1*	*1*	*6/6*	*3*	*1/1*	*2/2*	*1/1*	*3/3*	*5-6-7*
M5631 cl5	*4/4*	*5*	*4/4*	*2/2*	*3/3*	*5/5*	*3/3*	*2/2*	*4/4*	*5/5*	*7/7*	*5/5*	*6/6*	*2/2*	*3*	*4/4*	*3*	*1/1*	*2/2*	*1/1*	*3/3*	*5-6-7*
Tulahuen cl2	*3/4*	*5*	*2/2*	*4/4*	*5/5*	*4/4*	*3/3*	*2/2*	*4/4*	*2/5*	*5/9*	*2/2*	*4/4*	*2/2*	*2*	*6/6*	*3*	*2/2*	*2/2*	*1/1*	*3/3*	*5-6-7*
CL Brener	*3/4*	*5*	*2/2*	*4/4*	*3/3*	*4/4*	*3/3*	*2/2*	*4/4*	*2/5*	*5/9*	*2/2*	*4/4*	*2/2*	*2*	*6/6*	*3*	*2/2*	*2/2*	*1/1*	*3/3*	*5-6-7*

[a] An allelic interpretation of the enzymatic profiles was made for all loci except *Idh*, *Got*, *Dia1* and *Sod*.

alleles of the TU18 cl2 stock on one hand, and either Can III cl1 or M5631 cl5 or M6241 cl6 on the other hand (Table 2 and Fig. 2).

The *Pgm* locus showed other putative heterozygous genotypes, with two bands since PGM is a monomeric enzyme. As it was the case for the *Gpi* locus, the *5/9* genotype was specific for the CL Brener and Tulahuen cl2 clones (Table 2). Differently from the *Gpi* locus, one of the alleles of the *Pgm* genotype of CL Brener and Tulahuen cl2 was specific to these strains, since it was present in none of the other genotypes studied here.

Fig. 3 shows the results obtained for the *Gdh*-NADP$^+$ locus. For this locus, the CL Brener strain was unambiguously distinct from the Tulahuen cl2 strain, whereas it had the same genotype as other stocks of the present sample. The combined analysis of both *Gpi* and *Gdh*-NADP$^+$ therefore distinguishes the clone CL Brener from any other stock in the present sample.

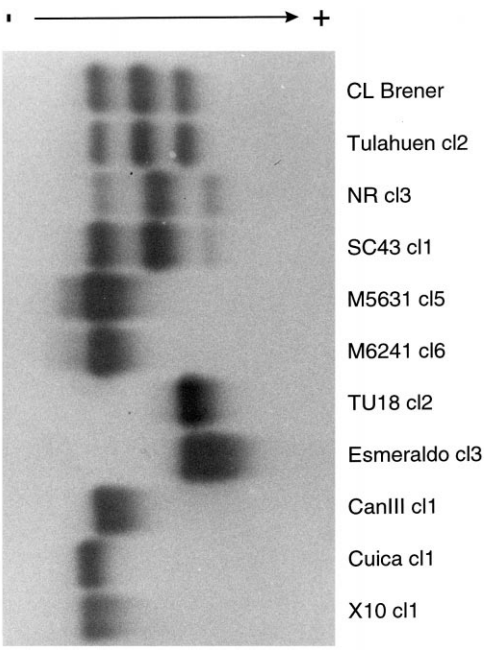

Fig. 2. Profiles obtained for the *Gpi* isoenzyme locus for the 11 *T. cruzi* stocks under study. The CL Brener and Tulahuen cl2 clones both show a three-banded heterozygous genotype *3/4* (Table 2) with alleles corresponding to those of clone TU18 cl2 on one hand, and either Can III cl1, or M6241 cl6, or M5631 cl5 clones on the other hand.

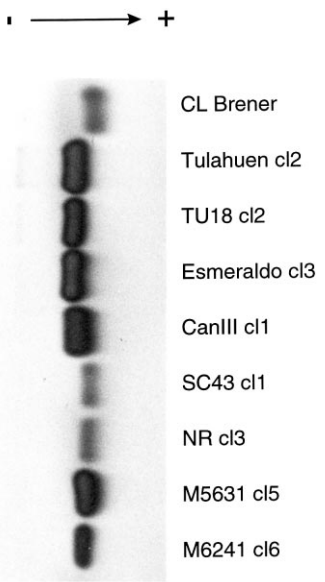

Fig. 3. Profiles obtained for the *Gdh*-NADP$^+$ isoenzyme locus for nine of the 11 stocks under study. Clone CL Brener is clearly distinct from Tulahuen cl2.

3.2. RAPD analysis

Out of 120 different primers screened, the 72 that gave the more readable profiles were selected. This yielded a total of 656 scoreable RAPD fragments.

Only 45 RAPD fragments were shared by all the stocks included in the study. As a matter of fact, RAPD diversity, like MLEE variability, proved to be considerable in this sample of *T. cruzi* stocks, which confirms previous RAPD analysis carried out with a more limited set of primers [4].

Fig. 1 shows the UPGMA dendrogram computed from RAPD data. The phylogenetic picture depicted by this dendrogram corroborates previous RAPD analysis and shows that MLEE and RAPD dendrograms are congruent, confirming previous results obtained from a more limited set of primers [4]. This agreement between MLEE and RAPD data is statistically confirmed by the Mantel test ($r = 0.97$; $P < 10^{-4}$). As for MLEE data, RAPD results show that the CL Brener and Tulahuen cl2 clones are genetically very close. Actually, they did not show any different banding

pattern. The only differences were observed with the R5 and R12 primers, which both showed variations of intensity for some RAPD fragments between CL Brener and Tulahuen cl2. Such differences are not taken into account in computing genetic distances, and are hardly usable as diagnostic characters. In contrast, it is possible to distinguish both CL Brener and Tulahuen cl2 from all other *T. cruzi* stocks surveyed here. As an example, Fig. 4 shows the RAPD profiles obtained with the U7 primer. The pattern of CL Brener and Tulahuen cl2 is clearly distinct from that of all other stocks. A great number of such diagnostic primers, specific for the CL/Tulahuen pair, was recorded, and is listed in Table 3. Some of these primers have been tested on 38 additional stocks, which fully confirmed their reliability as diagnostic characters for the CL/Tulahuen pair (Brisse, unpublished) (Table 3).

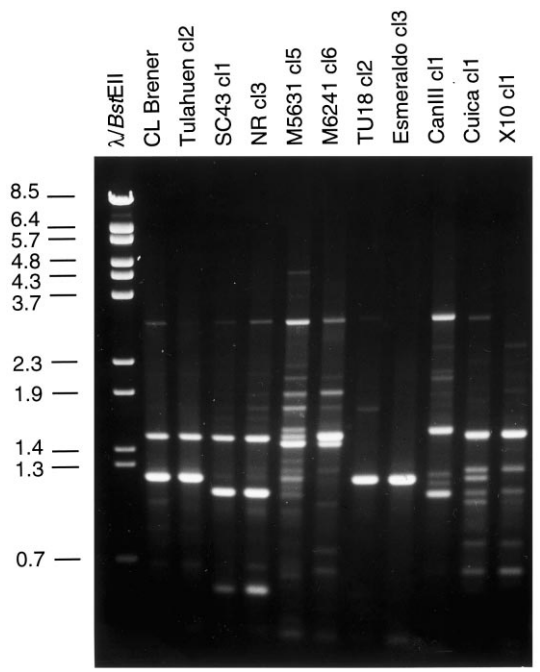

Fig. 4. RAPD profiles obtained with the U7 primer. The first lane corresponds to standard size markers from λ phage digested with *Bst*EII. The two-banded profile of CL Brener and Tulahuen cl2 is specific for this pair of clones. One fragment of this profile is shared with the profiles of other stocks, of which M6241 cl6 and M5631 cl5, and the other fragment is common to the profiles of stocks Esmeraldo cl3 and TU18 cl2.

Table 3
List of the 72 RAPD primers used in the study

Kit B	B1[a], B2, B3[a], B4, B5, B6, B7, B8[a], B10, B11, B12, B13, B15, B17, B18, B19[a,b,c]
Kit R	R1, R2, R3, R4, R5, R6, R7[a], R8[a], R9, R10, R11[a], R12, R13, R14[a], R16, R20[a]
Kit U	U2, U3, U6, U7[a,c], U8, U11[a,c], U12, U13[a], U14[a,c], U16
Kit N	N1, N8, N9, N10, N11, N12, N13[a,b], N14, N16, N17[a], N18, N19, N20
Kit A	A10, A15
Kit F	F1[a], F2[a], F3, F4, F5, F8, F9, F10, F11, F12[a], F13[a,c], F14, F15[a,c], F16, F20

[a] Primers whose overall profile is specific for both CL Brener and Tulahuen cl2.
[b] Primers which exhibit individual fragments specific for both CL Brener and Tulahuen cl2.
[c] The specificity of the characters was confirmed on 38 additional stocks.
Sequences (5′–3′) of primers B19, N13 and U7 are ACC CCC GAA G, AGC GTC ACT C and CCT GCT CAT C, respectively.

Although such diagnostic primers generated overall profiles that were specific for CL/Tulahuen, most of them included individual RAPD fragments that were shared with other *T. cruzi* stocks. This is the case for the U7 primer (Fig. 4). Only the B19 and N13 primers showed RAPD fragments specific for the CL/Tulahuen pair (Fig. 5 and Table 3).

As for the *Gpi* isoenzyme locus, many RAPD primers showed that the CL Brener and the Tulahuen cl2 clones have composite profiles that include fragments from either TU18 cl2 or M5631 cl5/M6241 cl6. This is the case for the U7 primer (Fig. 4).

4. Discussion

4.1. Parity between MLEE and RAPD results

Previous studies [4,14] have used the RAPD technique to survey *T. cruzi* genetic variability. When compared with these earlier works, the present study relies on a much broader range of primers, which permits assessment of a phylogenetic analysis based on more characters. As previ-

ously noted [4], our results showed a fair agreement between the dendrograms obtained from MLEE and RAPD data (Fig. 1), statistically verified by the Mantel test. Nevertheless, in the present case, MLEE and RAPD clusterings are absolutely identical, whereas they were only very similar in the earlier study [4]. Two reasons can explain this difference: first, the much broader set of primers used here permits construction of a more accurate phylogenetic picture. Second, we surveyed a more limited number of stocks than was done previously [4] (11 vs 24). All things being equal, this proportionately reduces the probability of incompatibilities.

The strict agreement between MLEE and RAPD data indicates that the phylogenetic picture obtained with our results is robust. Moreover, it is a telling manifestation of a strong linkage disequilibrium, or nonrandom association between genotypes occuring at different loci. Practically speaking, the very strong linkage observed here makes it possible to infer RAPD genotypes from MLEE genotypes, and vice versa. Linkage disequilibrium is a classical circumstantial evidence for a clonal population structure. Our results show that genetic exchange has been either severely restricted or absent among the present set of stocks, which is in agreement with previous data [3].

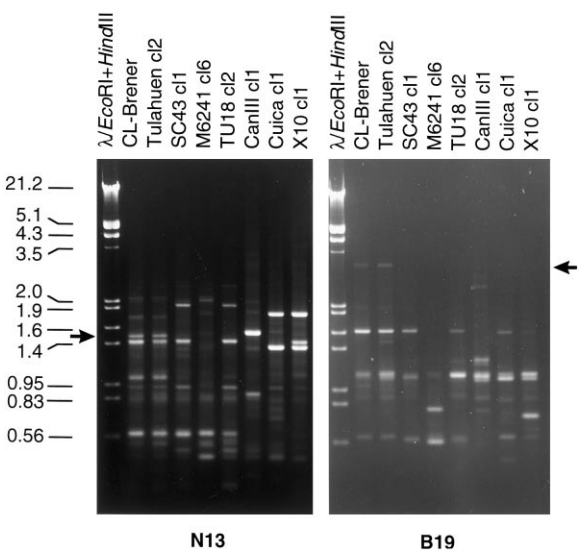

Fig. 5. RAPD profiles obtained with primers N13 (left) and B19 (right). The first lane corresponds to standard size markers from λ phage digested with *Eco*RI and *Hind*III. With each primer, CL Brener and Tulahuen cl2 show a RAPD fragment that is specific for this pair of clones (indicated by the arrow on the left for N13 and on the right for B19).

4.2. Genetic and phylogenetic characterization of the clone CL Brener

The original CL strain, isolated in 1963, was unambiguously shown to be a mixture of genotypes at least by two studies, relying on RFLP analysis of kinetoplast DNA [7] and MLEE [8]. Moreover, the clone CL Brener was produced in 1987 and, for such a long period of time, the risk of laboratory mix-up is high. It appeared therefore indispensable to get a clear idea of the genetic characteristics and phylogenetic situation of the clone CL Brener, which is now the basis for a great research effort of many laboratories.

The present study clearly shows that clone CL Brener is extremely close to the Tulahuen cl2 reference strain. Actually, out of the many MLEE and RAPD characters surveyed here, only one (the *Gdh*-NADP$^+$ isoenzyme locus) distinguishes the CL Brener clone from Tulahuen cl2 with no ambiguity. This confirms previous results [15], where no difference was found between the original CL and Tulahuen strains with 12 isoenzyme loci. This extreme genetic similarity between CL Brener and Tulahuen is worrying, since Tulahuen is also a widely used reference strain, and it will be therefore more difficult to distinguish between these two reference strains when laboratory mix-ups are suspected.

In the cladistic terminology, CL Brener and Tulahuen both fall into the same clade (monophyletic subdivision), at a very low level of phylogenetic divergence. All MLEE and RAPD characters that are specific to these two genotypes can be considered as synapomorphic characters (derived characters that are shared by all members of a given clade). This lower clade corresponds to the clonal genotypes 40–43 identified in [3]. Although comparisons between different laboratories are more tentative, according to the *Gpi* genotype, it is probable that this clade corresponds also to the ZB group of isoenzyme profiles

described by Romanha [16]. The more obvious synapomorphic character of this lower clade is the three-banded heterozygous *Gpi* genotype (*3/4*), which must not be confused with another three-banded *Gpi* genotype whose fastest allele is faster (*2/4*), and is seen in the SC43 cl1 and NR cl3 stocks isolated in Bolivia and Chile, respectively and in many other stocks [3]. The status of this *Gpi* 3-banded genotype as a synapomorphic character of the lower clade that contains both CL Brener and Tulahuen (named lower clade 43 in the rest of this text, according to the number of the clonal genotype of the Tulahuen strain noted in [3]) has been fully confirmed by a more extensive MLEE analysis dealing with 440 *T. cruzi* isolates (Barnabé and Tibayrenc, unpublished). In contrast, in the same study, the *Pgm 5/9* genotype was found to be not strictly specific of the lower clade 43.

When RAPD characters are considered, 20 primers yielded synapomorphic profiles for the lower clade 43, which was confirmed for some of them by the analysis of 38 additional *T. cruzi* stocks (Table 3).

In summary, only the *Gdh*-NADP$^+$ isoenzyme locus reliably distinguishes between the clones CL Brener and Tulahuen cl2. In contrast, many MLEE characters and RAPD profiles are available to distinguish these clones from any *T. cruzi* stock not belonging to the lower clade 43.

4.3. Phylogenetic situation of the CL Brener clone in the whole Trypanosoma cruzi species

T. cruzi appears to be subdivided into two major phylogenetic lineages (clades) [5,17], each being genetically very heterogeneous [5]. According to our results (Fig. 1), the CL Brener clone falls into the second major clade, which also includes the principal zymodemes II (stock Esmeraldo cl3) and III (stock Can III cl1) formerly described by Miles et al. [2,18], whereas the principal zymodeme I (stock X10 cl1) falls into the first major clade.

Our results, based on a very broad range of genetic markers, confirms the now well-accepted notion that the genetic diversity of this parasite is considerable [19] and is structured into highly divergent phylogenetic subdivisions that probably correspond to clonal lineages [3]. Accordingly, it would be extremely tentative to consider that the CL Brener clone is representative of the whole genetic diversity of the parasite. It is therefore advisable to complete the studies presently involved in the *T. cruzi* genome project with additional work dealing with one or more *T. cruzi* genotypes, selected to be drastically different from CL Brener. This is all the more desirable since growing evidence suggests that genetic variability on one hand, biomedical and eco-epidemiological diversity on the other hand, are linked in the agent of Chagas' disease [20–22].

4.4. Possible hybrid origin of clone CL Brener

T. cruzi shows strong evidence for a clonal population structure [3]. This does not exclude the possibility of occasional exchange of genetic information. Preliminary evidence for hybridization events has been recently published [23,24]. Our data are consistent with the hypothesis that the genotype of the CL Brener clone has such a hybrid origin, with the genotypes of the TU18 cl2 stock on one hand, the genotypes of either the M5631 cl5 or the M6241 cl6 stocks on the other hand, as putative parental genotypes. This is suggested by the fact that the two alleles corresponding to the *Gpi* heterozygous genotype of clone CL Brener corresponds to the ones of the putative parents, whereas most of the RAPD profiles of clone CL Brener present various combinations of the putative parents' profiles. This is for example the case for the U7 primer (Fig. 4). The *Gpi* genotype *4/4* of the putative parents M5631 cl5 and M6241 cl6 is also shared by the Can III cl1 stock. Nevertheless, considering all genetic characters together, in the present sample, M5631 cl5 and M6241 cl6 appear to be more plausible parents of CL Brener. The few characters that show some discrepancy with this hypothesis, such as the isoenzyme locus *Pgm*, could be explained in two ways: (i) the hybridization event occured a long

time ago, and some mutations have accumulated since then; (ii) TU18 cl2 and M5631/M6241 are not the parental genotypes, but rather, genotypes closely related to the real parental genotypes.

The hybrid nature of clone CL Brener would imply that many loci will be found in a heterozygous state when sequencing its genome. At the present state of this research, the alternative possibility of identity of characters by convergent mutations still has to be carefully weighed, and needs further exploration by in-depth molecular analysis of RAPD characters. Nevertheless, the hypothesis that CL Brener is the result of a hybridization event must be considered here and now, due to its considerable potential implications in the *T. cruzi* genome project.

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