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Infection, Genetics and Evolution 2 (2003) 173–183

Infection, Genetics and Evolution

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Evidence for genetic exchange and hybridization in *Trypanosoma cruzi* based on nucleotide sequences and molecular karyotype☆

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Received 9 January 2002; received in revised form 6 August 2002; accepted 29 August 2002

Abstract

Trypanosoma cruzi is thought to undergo predominant clonal evolution, as determined by population genetics studies. However, this model does not exclude occasional recombination, which existence is strongly suggested by several recent studies. We sequenced a portion of the maxicircle cytochrome *b* (*CYb*) gene and of the nuclear rRNA promoter region from representative strains of six *T. cruzi* genetic lineages isolated from anthroponotic environments and man (lineages IIb, IId and IIe), sylvatic environments (lineages IIa and IIc) or both (lineage I). Phylogenetic analyses based on the two genes were incongruent. Remarkably, in lineage IIe, CYb and rRNA sequences were very closely related to those of lineages IIc and IIb, respectively. One stock of lineage IId showed rRNA sequence heterogeneity, with both IIb-like and IIc-like copies. Analysis of the size variation of six distinct pairs of putative homologous chromosomes revealed a bimodal distribution of chromosomal sizes across *T. cruzi*. Notably, stocks of lineages IId and IIe had several chromosomal pairs distributed in distinct modes, with the corresponding modes individually found in lineages IIb and IIc. Together, these data indicate the origin of lineages IId and IIe by hybridization between representatives of lineages IIb and IIc. CYb and rRNA sequences clustered into three and four major lineages, respectively. Data were in agreement with the distinction of six genetic lineages, but not with their proposed grouping into two primary lineages, as lineage II was not monophyletic. Based on a CYb substitution rate of 1% per million years (Myr), the major lineages are estimated to have diverged around 10 million years ago.

Keywords: *Trypanosoma cruzi*; Molecular phylogeny; Congruence test; Chromosomal size variation; Cytochrome *b*; Evolution

1. Introduction

Population genetics analyses have shown that *Trypanosoma cruzi*, the agent of American trypanosomiasis, has a predominantly clonal population structure, with genetic exchange and recombination being restricted to such a degree that individual cell lines persist as stable clonal genotypes and propagate over broad geographic scales (Tibayrenc et al., 1986; Tibayrenc and Ayala, 1988). However, the clonal model does not state that recombination is totally absent, and is compatible with occasional genetic recombination (Tibayrenc et al., 1990). Although it would not disrupt the clonal population structure, the occurrence of rare events of genetic exchange, on an evolutionary scale, could have profound significance for the adaptive evolution of *T. cruzi* parasites to new environments, including new vectors and hosts, and possibly humans. In addition, cryptic sexuality would

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the following accession number(s): cytochrome *b* gene, AJ130927–38 and AJ439719–27; rRNA promoter sequence: AJ439878.

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PII: S1567-1348(02)00097-7

have important consequences on attempts to determine the phylogenetic relationships among *T. cruzi* genotypes.

Natural populations of *T. cruzi* exhibit considerable genetic heterogeneity (Miles et al., 1978; Tibayrenc et al., 1986). *T. cruzi* isolates have been ordered into two primary phylogenetic lineages based on multilocus enzyme electrophoresis (MLEE) (Tibayrenc et al., 1993), random amplified polymorphic DNA (RAPD) (Tibayrenc et al., 1993; Tibayrenc, 1995), and on structural and functional variation of the 24Sα LSU rRNA and mini-exon genes (Souto et al., 1996; Nunes et al., 1997). Stocks corresponding to the MLEE/RAPD lineage II were classified into five smaller subdivisions called IIa to IIe (Brisse et al., 2000a, 2001; Barnabé et al., 2000). The two main subdivisions consensually referred to as *T. cruzi* I and II by a group of experts (Anonymous, 1999; Momen, 1999) correspond to lineages I and IIb, respectively (Brisse et al., 2001). Thus, considering the major lineage I and the five subdivisions of major lineage II, it is possible to distinguish six discrete lineages in *T. cruzi*. Representative MLEE genotypes belonging to distinct lineages show distinctive biological properties in experimental studies (Laurent et al., 1997; Revollo et al., 1998) and evidence for differences of infectivity and virulence in man has been presented (Montamat et al., 1996; Brēnièr et al., 1998).

In contrast with the generation of experimental hybrids of *Trypanosoma brucei* (Jenni et al., 1986), evidence for genetic exchange in *T. cruzi* has been scant until recently. MLEE profiles taken as evidence for heterozygosity and diploidy have been previously described (Tibayrenc et al., 1981). Putative *T. cruzi* hybrid genotypes have been described in restricted localities (Bogliolo et al., 1996; Carrasco et al., 1996), and MLEE and RAPD data have previously suggested a hybrid origin for lineages IIe (Brisse et al., 1998, 2000a; Barnabé et al., 2000) and IId (Tibayrenc and Miles, 1983; Brisse et al., 2000a), which are both highly prevalent among human isolates and geographically widespread (Barnabé et al., 2000). However, apparent heterozygous states could be created, in the course of completely asexual evolution, by gene duplication or allele sequence divergence (Tibayrenc et al., 1981; Birky, 1996), and homoplasy of MLEE, RAPD or RFLP characters could result in apparently hybrid genotypes. Finally, although experimental *T. cruzi* 'hybrids' were obtained (Stothard et al., 1999), the exchanged genetic material was restricted to extra-chromosomal elements, and mating experiments involving the nuclear genome in *T. cruzi* have so far been unsuccessful. However, clear evidence for occasional, ancient genetic exchange in *T. cruzi* has recently been provided based on sequence data from two nuclear genes and one portion of the maxicircle DNA (Machado and Ayala, 2001).

In order to further investigate genetic exchange events in the evolution of *T. cruzi*, we have analyzed samples representative of the six *T. cruzi* subdivisions by two complementary approaches. First, we compared the phylogenies of the nuclear ribosomal RNA (rRNA) promoter region and of the 5′-half of the cytochrome *b* (*CYb*) gene coding region, encoded in the maxicircle DNA of the mitochondrial genome. Comparison of the phylogenies of independent genes is a powerful means to determine if genetic exchange has occurred. Under strict clonal evolution, independent sequences would have the same history and are expected to show the same pattern of descent, whereas significant incongruence between them would indicate that genetic exchange has occurred among genomes (Dykhuizen and Green, 1991; Lecointre et al., 1998). Second, we investigated the size variation between homologous chromosomes corresponding to six linkage groups (Henriksson et al., 1995). Extensive chromosomal size variation has been demonstrated in *T. cruzi*, including between putative homologous chromosomes (Gibson and Miles, 1986; Engman et al., 1987; Aymerich and Goldenberg, 1989; Henriksson et al., 1990; Wagner and So, 1990; Henriksson et al., 1993, 1995). Here, we investigated whether the distribution of chromosomal heteromorphy (Birky, 1996) across *T. cruzi* lineages reveals past events of hybridization.

2. Materials and methods

2.1. Parasites

The stocks analyzed are given in Table 1. Most stocks were laboratory-cloned, with verification under the microscope. Lineage assignment of the stocks have been previously determined by MLEE, RAPD and/or SCAR markers (Brisse et al., 2000a,b; Barnabé et al., 2000). Parasite culture and DNA preparation were as described (Brisse et al., 2000a).

2.2. Analysis of cytochrome b

Sequence of the 5736-nt fragment TRCKPMAX (GenBank U43567) from the *T. cruzi* stock Tulahuen (Ochs et al., 1996) was used to design primers targeting the 5′-half of the *CYb* gene: p18 (5′-GAC AGG ATT GAG AAG CGA GAG AG-3′) and p20 (5′-CAA ACC TAT CAC AAA AAG CAT CTG-3′). Amplifications were performed during 35 cycles (94 °C, 1 min; 50 °C, 30 s; 72 °C, 90 s) followed by a final elongation step (5 min, 72 °C). Sequencing of PCR products was carried out using Big Dye technology (Perkin-Elmer).

2.3. Analysis of the rRNA promoter region

Sequence of the rRNA promoter region was obtained after PCR amplification with primers p5′dIII1 and p3′dIII for stocks of major lineage II, or with primers p5′dIII2 and p3′dIII for stocks of lineage I (Nunes et al., 1997). Only one PCR product of the expected size was obtained in all stocks. For only one stock (IId-MN cl2), the sequence obtained from the PCR amplified products showed multiple

Table 1
Origin and previous characterisation of the *Trypanosoma cruzi* stocks analyzed

Stock	Lineage	Country	Locality	Host	Analyzed for
133–79 cl7	*T. cruzi* I	Bolivia	Santa Cruz	Human	PFGE
Cuica cl1	*T. cruzi* I	Brazil	Sao Paulo	*Philander opossum*	Cyb, rRNA, PFGE
Cutia cl1	*T. cruzi* I	Brazil	Espiritu Santo	*Dasyprocta aguti*	PFGE
Esquilo cl1	*T. cruzi* I	Brazil	Sao Paulo	*Sciurus aestuans ingrami*	PFGE
Gamba cl1	*T. cruzi* I	Brazil	Sao Paulo	*Didelphis azarae*	PFGE
LGN	*T. cruzi* I	Chile	Illapel	Human	PFGE
OPS21 cl11	*T. cruzi* I	Venezuela	Cojedes	Human	PFGE
OPS22	*T. cruzi* I	Venezuela	Cojedes	*Panstrongylus geniculatus*	PFGE
P11 cl3	*T. cruzi* I	Bolivia	Cochabamba	Human	PFGE
P209 cl1	*T. cruzi* I	Bolivia	Sucre	Human	PFGE
SO34 cl4	*T. cruzi* I	Bolivia	Potosi	*Triatoma infestans*	PFGE
SP104 cl1	*T. cruzi* I	Chile	Combarbalá	*Triatoma spinolai*	PFGE
X10 cl1	*T. cruzi* I	Brazil	Belém	Human	Cyb, rRNA, PFGE
SC13	*T. cruzi* I	Colombia	Antioquia	*Rhodnius pallescens*	Cyb, rRNA
CanIII cl1	*T. cruzi* IIa	Brazil	Belém	Human	Cyb, rRNA, PFGE
Stc33R	*T. cruzi* IIa	USA	St. Catherines Island	*Procyon lotor*	Cyb, rRNA
DogT	*T. cruzi* IIa	USA	Oklahoma	*Canis familiaris*	Cyb, rRNA
CBB cl3	*T. cruzi* IIb	Chile	Tulahuén	Human	Cyb, rRNA, PFGE
Esmeraldo cl3	*T. cruzi* IIb	Brazil	Bahia	Human	Cyb, rRNA, PFGE
TU18 cl2	*T. cruzi* IIb	Bolivia	Tupiza	*Triatoma infestans*	Cyb, rRNA, PFGE
M6241 cl6	*T. cruzi* IIc	Brazil	Belém	Human	Cyb, rRNA, PFGE
M5631 cl5	*T. cruzi* IIc	Brazil	Marajo	*Dasypus novemcinctus*	Cyb, rRNA
X109/2	*T. cruzi* IIc	Paraguay	Makthlawaiya	*Canis familiaris*	Cyb, rRNA
Bug2148 cl1	*T. cruzi* IId	Brazil	Rio Grande do Sul	*Triatoma infestans*	PFGE
Bug2149 cl10	*T. cruzi* IId	Brazil	Rio Grande do Sul	*Triatoma infestans*	PFGE
MN cl2	*T. cruzi* IId	Chile	Region IV	Human	Cyb, rRNA, PFGE
NR cl3	*T. cruzi* IId	Chile	Salvador	Human	PFGE
SC43 cl1	*T. cruzi* IId	Bolivia	Santa Cruz	*Triatoma infestans*	Cyb, rRNA, PFGE
9280 cl1	*T. cruzi* IId	Bolivia	Santa Cruz	Human	Cyb, rRNA
CL Brener	*T. cruzi* IIe	Brazil	Rio Grande do Sul	*Triatoma infestans*	Cyb, rRNA, PFGE
Tulahuen cl2	*T. cruzi* IIe	Chile	Region IV	Human	Cyb, rRNA, PFGE
Guateque	*T. cruzi* IIe	Colombia	Guateque, Bocaya	*Rhodnius prolixus*	Cyb, rRNA
X57/3	*T. cruzi* IIe	Paraguay	Makthlawaiya	*Canis familiaris*	Cyb, rRNA
M1117	*T. cruzi marinkellei*	Brazil	Para	*Phyllostomum hastatus*	Cyb

ambiguous base calls. PCR product from this stock was cloned as described (Nunes et al., 1997) and five clones were sequenced.

2.4. Phylogenetic analysis

Nucleotide sequences were manually aligned with the ED editor of the MUST package (Philippe, 1993). To reduce potential biases introduced by unequal base compositions, different substitution rates between nucleotides, and heterogeneous substitution rates between sites, CYb and rRNA promoter phylogenies were reconstructed using maximum likelihood (ML) (Felsenstein, 1981). The evolution of sequences was described by the General Time Reversible (GTR) model of nucleotide substitutions, combined to a eight-categories discrete Gamma (Γ) distribution of parameter α for the heterogeneity in substitution rates among sites (Yang, 1996). All tree reconstructions have been conducted with PAUP* (Swofford, 1998), Version 4 beta 4. Robustness of the trees was assessed by the bootstrap (Felsenstein, 1985). For each node, bootstrap percentages (BP) were computed after 200 resamplings, each followed by a heuristic ML reconstruction (bootstrap option in PAUP*, with neighbor-joining starting trees, and tree bisection–reconnection branch swapping). The *T. cruzi marinkellei* M1117 stock was used as an outgroup for mitochondrial CYb analyses, based on its divergent biochemical characteristics as compared to all other *T. cruzi* stocks (Baker et al., 1978), but for technical reasons this outgroup could not be investigated for rRNA promoter region.

The degree of incongruence between the ML CYb and rRNA promoter topologies was evaluated through crossed Kishino & Hasegawa tests (Kishino and Hasegawa, 1989), corrected according to Shimodaira & Hasegawa (S–H tests; Shimodaira and Hasegawa, 1999). The significance level of the decrease in log-likelihood introduced by the best CYb topology (and reciprocally by the best rRNA promoter topology) relative to the highest-likelihood rRNA promoter (and reciprocally CYb) topology was measured for the rRNA promoter (and reciprocally CYb) sequences. The likelihood ratio test (Felsenstein, 1988) was conducted to evaluate the potential clock-like substitution pattern of the CYb sequences. All incongruence and clock tests were conducted with PAML (Yang, 2000), Version 3.0b, assuming

an independent GTR + Γ model for each of the three codon positions in order to reflect the codon structure of the CYb.

2.5. Pulse field gel electrophoresis

Chromosomal separations were performed as previously described (Henriksson et al., 1995). Six probes were selected because they corresponded to distinct linkage groups and were not linked to the rRNA genes (Henriksson et al., 1995; Cano et al., 1995): among these were three gene fragments (1F8/F29, FFAg6 and Tc2) and three anonymous genomic markers (CA7:12, CA7:32 and P19) (Henriksson et al., 1995). Probes CA7:12 and CA7:32 are linked in lineage I stocks but not in lineage II (Henriksson et al., 1995). Sizes of the chromosomal bands were estimated as described (Henriksson et al., 1995).

2.6. Multimodality analysis

For each chromosomal probe, the size-distribution analysis was performed on a population of 50 individual chromosomes, considering the 25 stocks diploid. The number of modes in each individual population was determined as described by Silverman (Silverman, 1981) and Manly (Manly, 1996). Briefly, the minimum width (h_k) yielding k modes in a kernel density estimation was determined and its likelihood was established by means of smoothed bootstrap resampling. Determination of h_k and bootstrapping (1000 samples per value of h_k) were carried out in Extend™ (Diamond and Hoffman, 1995). The actual locations of the modes were obtained in Stata (StataCorp, 1999). A probability level $P = 0.05$ was used throughout.

3. Results

3.1. Mitochondrial cytochrome b phylogeny

A 516-nt sequence of the 5′-half of the mitochondrial *CYb* gene non-edited coding region was determined for 19 *T. cruzi* stocks and stock *T. cruzi marinkellei* M1117. An important polymorphism was found in the coding region: 516 sites were aligned without indels, and 113 and 81 appeared variable and phylogenetically informative, respectively. The ML analysis of the CYb matrix (Fig. 1, left) robustly evidenced three major clades of *T. cruzi* stocks (BP > 96):

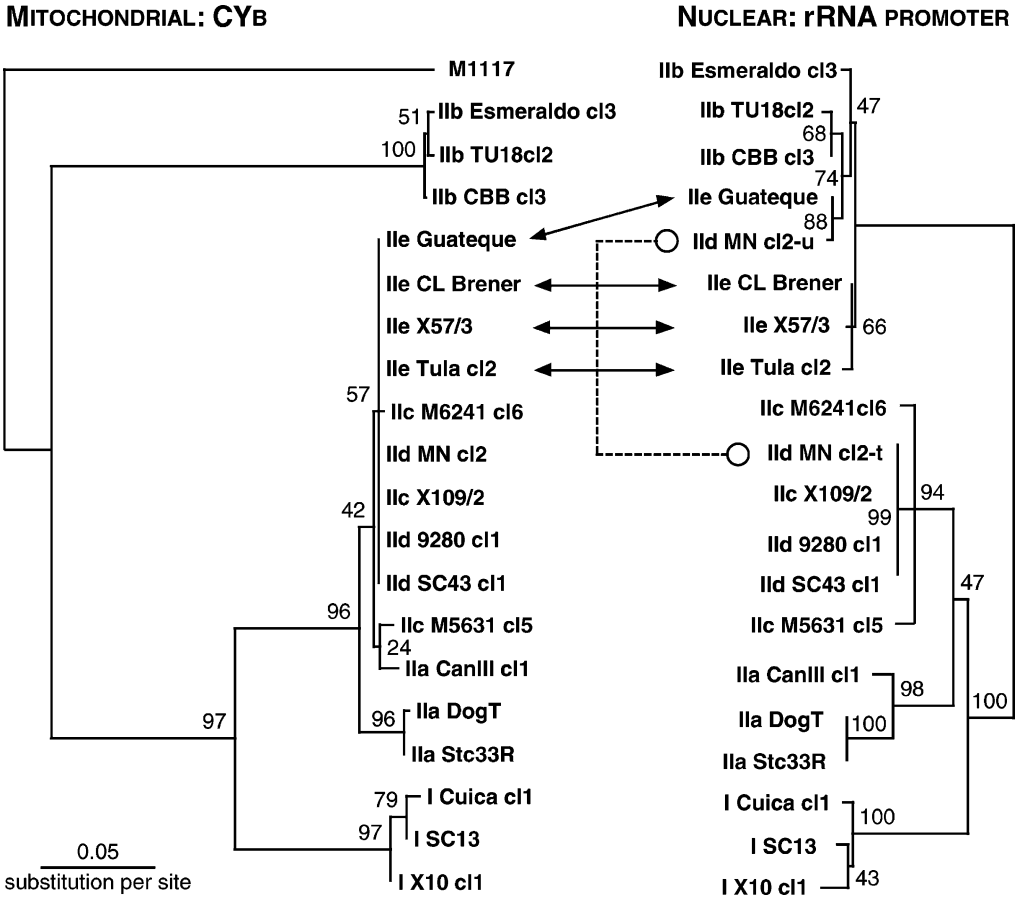

Fig. 1. Maximum likelihood phylograms of 19 *Trypanosoma cruzi* stocks reconstructed from mitochondrial CYb (left; $-\ln L = 1158.9$) and nuclear rRNA promoter region (right; $-\ln L = 1626.1$) sequences. Bootstrap percentages obtained by ML after 200 replicates are given for each node. Phylogenetic trees are drawn using midpoint rooting. The arrows connect stocks whose phylogenetic position is highly incongruent. The white circles indicate that two highly divergent rRNA promoter sequences ("t" and "u") have been isolated from stock IId-MN cl2. Note that the scale is the same for both phylogenetic trees.

lineage IIb, lineage I, and a group containing lineages IIa, IIc, IId and IIe. The strong association of the latter two clades (BP = 97) showed that *T. cruzi* lineage II was paraphyletic. Unexpectedly, the seven CYb sequences belonging to lineages IId and IIe were identical or very similar to those of stocks IIc-X109/2 and IIc-M6241 cl6.

3.2. Nuclear rRNA promoter phylogeny

The sequence of a 561–585-nt portion of the rRNA promoter region was determined for 19 *T. cruzi* stocks. In only one stock (IId-MN cl2), direct sequencing of the single PCR product revealed sequence heterogeneity. After cloning, two distinct alleles were revealed (IId-MN cl2-u and IId-MN cl2-t). Across the 20 *T. cruzi* sequences established, numerous nucleotide substitutions and small insertion/deletions were observed, in agreement with previous findings (Nunes et al., 1997). The alignment yielded 618 sites, of which 179 were variable and 144 phylogenetically informative. The ML analysis of the data produced the phylogram depicted on Fig. 1 (right). The sequence of the rRNA promoter of *T. cruzi marinkellei* was not available for the present study. Because the stocks of lineage IIb appeared to be the most divergent in the CYb phylogeny, we used them to root the rRNA promoter phylogeny.

Four major groups were robustly individualized: the lineage I (BP = 100), the lineage IIa (BP = 98), and two lineages comprising sequences from other lineages. One comprised stocks of lineages IIc and IId and one sequence of stock IId-MN cl2 (BP = 94), whereas the second included sequences from lineages IIb, IIe and the other sequence of stock IId-MN cl2. Constraining the sequences of the two alleles IId-MN cl2-t and IId-MN cl2-u to cluster together induced a significantly drop in the log-likelihood of the topology ($-\ln L = 1778.4$ versus 1626.1, $P < 0.001$ for the S–H test). It is interesting to note that stock MN cl2 belong to major MLEE genotype 39 (Tibayrenc et al., 1986), which was previously shown to be heterozygous at several loci (Tibayrenc and Miles, 1983).

3.3. Incongruence between mitochondrial and nuclear phylogenies

Incongruence between the mitochondrial and nuclear phylogenies appeared evident when considering the position of the four sequences of lineage IIe (Fig. 1). The position of stock IIa-CanIII cl1 also seemed incongruent, as the CYb sequence of stock IIa-CanIII cl1 was not associated with the North American lineage IIa stocks DogT and Stc33R. However, the latter was not significant, since constraining the CYb of stock CanIII cl1 to branch with DogT + Stc33R did not involve a significant drop in log-likelihood (1159.9 versus 1158.9; $P = 0.32$ for the S–H test).

Strong incongruence between the nuclear and mitochondrial phylogenies was demonstrated. The highest-likelihood CYb topology was significantly less likely than the one of the rRNA promoter: $\ln L = -1148.4$ versus -1010.2 after removal of the CYb of M1117 and MN cl2 sequences of both markers (S–H test: $P < 0.001$ for the rRNA promoter sequences). Conversely, the highest-likelihood rRNA promoter topology was significantly less likely than the one of the CYb ($\ln L = -1993.5$ versus -1617.9; S–H test: $P < 0.001$ for the CYb sequences). Moreover, the incongruence could be attributed to the apparent hybrid genotypes of lineages IId and IIe (see Section 4) since when sequences IId MN cl2-u and the four IIe stocks were removed, the two phylogenies were no longer incongruent under the CYb matrix ($\ln L = -1014.3$ versus -1010.1; $P = 0.11$ for the S–H test). Under the rRNA promoter matrix, the CYb topology remained less likely than the rRNA promoter topology ($\ln L = -1605.8$ versus -1555.0; $P < 0.001$). When CanIII cl1 was constrained to cluster with the two other IIa stocks, the CYb and rRNA promoter phylogenies became congruent under the rRNA promoter matrix ($\ln L = -1558.0$ versus -1555.0; $P = 0.16$).

3.4. Divergence date estimates of the CYb lineages

The clock-like pattern of CYb nucleotide substitution was controlled before estimating divergence times between CYb sequences. To this aim, a likelihood ratio test was conducted between ML trees reconstructed with and without a clock assumption. When the root was placed along the branch leading from M1117 to the ingroup, the hypothesis of a clock-like behavior of the 20-taxa topology was not rejected (likelihood ratio test statistics = 16.94; 19 d.f.; χ^2 (P-value) = 0.59). The CYb evolution was, therefore, considered to behave according to a clock-like pattern within *T. cruzi*. In particular, no higher rate of evolution was observed in the IIb lineage. Based on ML branch lengths, and assuming a 1% substitution rate per million years (Myr) and per lineage (Martin and Palumbi, 1993), the following divergence dates were estimated for the mitochondrial lineages: 10.6 ± 2.1 Myr for the split between lineage IIb and the remaining *T. cruzi* stocks, 4.4 ± 1.0 Myr for the one between lineage I and lineages IIa + IIc + IId + IIe, and 1.0 ± 0.4 Myr for the one between stocks IIa DogT + Stc33R and stocks IIa-CanIII cl1 + IIc + IId + IIe.

3.5. Chromosomal size variation

With all six chromosomal probes, one or two hybridizing bands were observed in the *T. cruzi* stocks, as illustrated in Fig. 2 for probe 1F8/F29. Drastic size differences were observed among the hybridizing bands of distinct stocks as well as between the two presumptively homologous chromosomal bands of a single stock (Table 1). Unexpectedly, the distribution of chromosomal sizes across the stocks did not appear to correspond to a progressive continuum of variation. Instead, with all probes, most hybridizing bands appeared to fall either into a bigger or into a smaller size mode, with little if any intermediate chromosomal sizes between

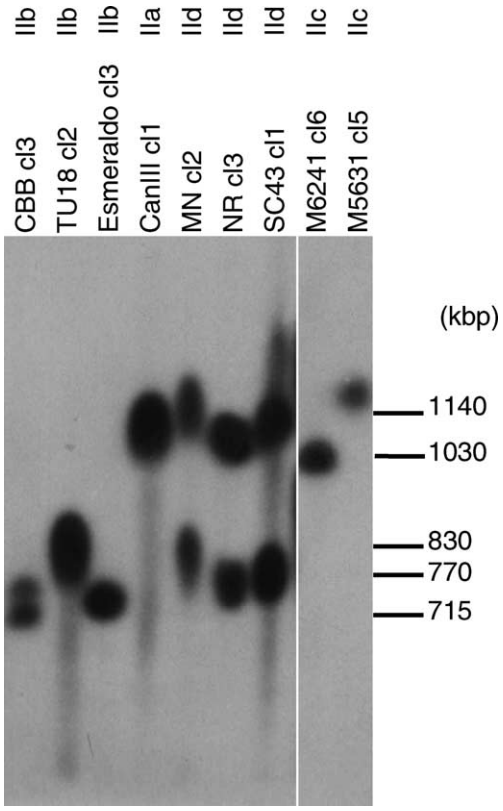

Fig. 2. Chromosomal hybridization patterns obtained with probe 1F8/F29. The size-distribution of the chromosomal bands illustrates the existence of two distinct size modes, with limited size variation among chromosomes of each mode, but with no intermediate sizes between the two modes. Stocks of the hybrid lineage IId show two chromosomal bands in distinct modes, with stocks of the putative parental lineages IIb and IIc individually showing chromosomal bands in the corresponding modes. With this probe, the hybridization profile of clone IIe-CL Brener was very similar to that of clone IId-NR cl3 (Table 2).

them, as illustrated in Fig. 2. The distribution of chromosomal sizes is given in Fig. 3. The statistical significance of a distribution into several modes was demonstrated for all chromosomes (see M & M). The modes are indicated by the arrows in Fig. 3. Bimodal patterns were found for probes Tc2 ($P = 0.001$), 1F8/F29 ($P = 0.003$), CA7:12 ($P = 0.003$) and CA7:32 ($P = 0.005$), and a trimodal pattern was found for probe FFAg6 ($P = 0.04$), the third mode corresponding to the exceptional bands observed in the compression zone for stocks IId-Bug2148 cl1 and IId-Bug2149 cl10 (Table 1, Fig. 3). For probe P19, the significance of the bimodal distribution was borderline ($P = 0.056$) but increased ($P = 0.047$) when the eccentric chromosomes of stocks IIe-CL Brener and IIe-Tulahuen cl2 (Table 1, Fig. 3) were excluded from the analysis.

Modal distribution appeared very stable and chromosomal alterations resulting in a mode change seem to be rare within a given lineage, with the exception of lineage IIe (Table 1). We investigated whether some stocks were mode-heterozygous, i.e. possessed presumptively homologous chromosomes distributed into distinct size modes. Remarkably, stocks of lineage IId and IIe all showed four mode-heterozygous chromosomes, with the only exception of stock IIe-Tulahuen cl2 (three mode-heterozygous chromosomes; Table 1). In contrast, stocks of the other lineages showed none or only one such chromosomal pattern (Table 1), even though in these lineages, two chromosomal bands (mostly of the same mode) were observed with one to four probes (Table 1).

Fig. 3 shows the positions of chromosomal bands of IIb-Esmeraldo cl3, IIc-M6241 cl6, IId-MN cl2 and IIe-CL Brener, taken as examples of their respective lineages. When considering lineages IId and IIe, the modal distribution of several chromosomal bands was consistent with the hypothesis of a hybrid origin of these lineages (Fig. 3). For example, in stock IId-MN cl2, chromosomal pairs 1F8/F29, FFAg6 and P19 are each distributed in two distinct modes, with the putative parental chromosomal bands of lineages IIb and IIc in the corresponding modes. The same was true for clone IIe-CL Brener, with probes 1F8/F29, FFAg6 and CA7:32 (Fig. 3). However, data obtained with several probes were not consistent with the direct result of a hybridization event: for example, IId-MN cl2 and IIe-CL Brener showed one non-parental mode with probes CA7:12 and P19, respectively.

4. Discussion

T. cruzi is considered to exhibit a predominantly clonal population structure, as determined mainly on the basis of linkage disequilibrium tests (Tibayrenc et al., 1986; Tibayrenc and Ayala, 1988). Although the clonal model is compatible with occasional sex (Tibayrenc et al., 1990), the possible occurrence of rare events of genetic exchange at the evolutionary scale cannot be determined using solely population genetics approaches.

While this study was under way, Machado and Ayala (2001) showed that sequence data from two nuclear genes, dihydrofolate reductase-thymidilate synthase and trypanothione reductase, and a portion of the maxicircle DNA, provide convincing evidence of genetic exchange and hybridization in *T. cruzi*. Here, we show that comparison of the molecular phylogenies of rRNA and CYb, as well as molecular karyotype data, also challenge the view of a purely clonal evolution of *T. cruzi*. Indeed, the present sequence data are fully compatible with the hybrid nature of lineages IId and IIe. First, stocks of lineage IIe appeared to have a composite genetic constitution, with CYb sequences very similar or identical to those of stocks IIc-M6241 cl6 and IIc-X109/2, respectively, but with nuclear rRNA promoter sequences very similar to those of lineage IIb. Second, stock IId-MN cl2 showed heterogeneity of its rRNA promoter sequences, which were very similar to either those of lineage IIb or those of lineage IIc.

About 110 copies of the rRNA cistrons exist in the *T. cruzi* genome (Castro et al., 1981). As in other organisms

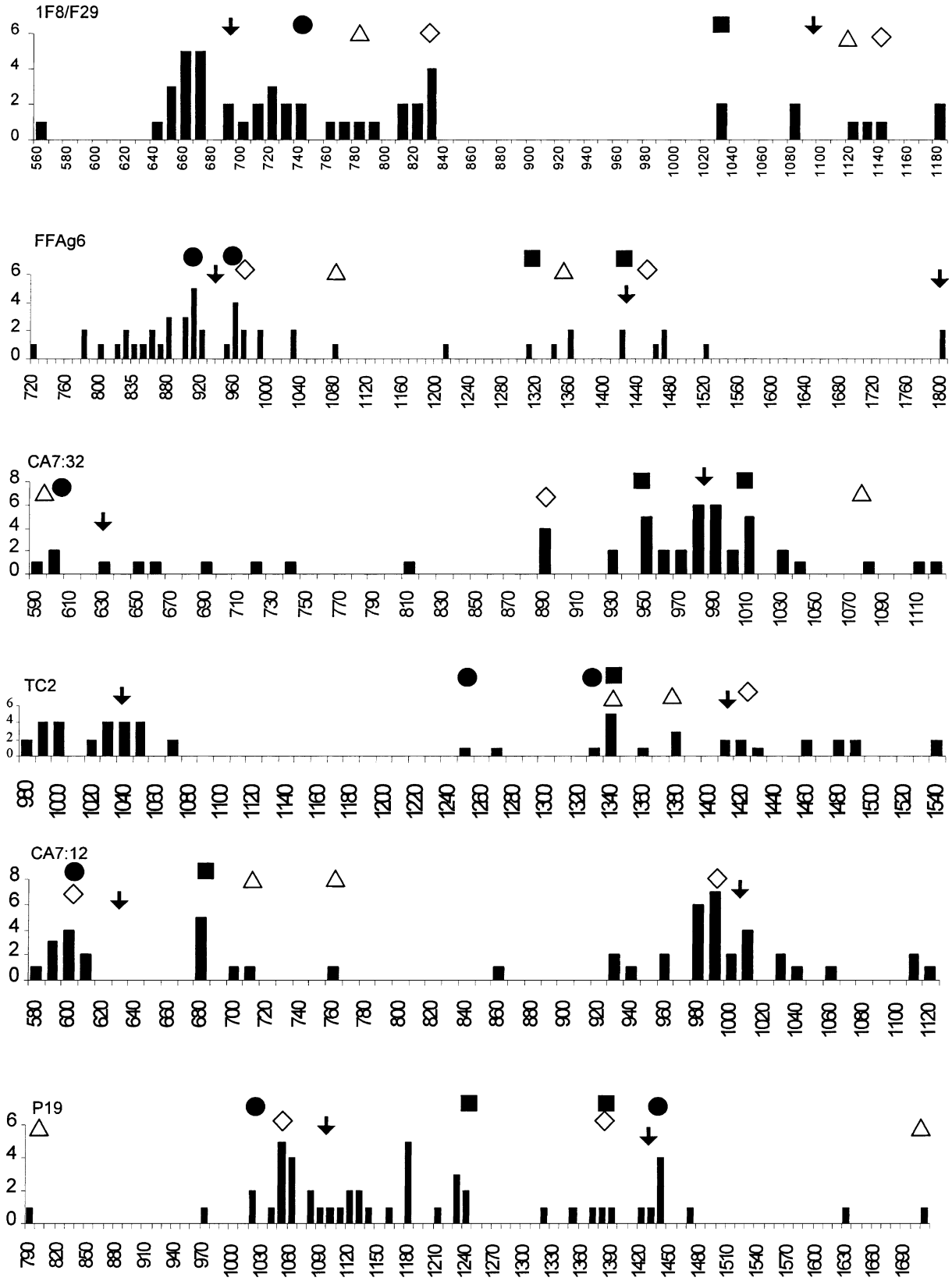

Fig. 3. Size-distribution of the chromosomal bands obtained with the six probes belonging to distinct linkage groups for 25 *T. cruzi* stocks. Band size categories are given in kpb. The *Y*-axis gives the number of chromosomal bands found for each size category. A bimodal distribution was statistically demonstrated with all probes except FFAg6 which showed as trimodal distribution. The central position of the modes is indicated by arrows (↓). The symbols above the histograms indicate the chromosomal sizes of stocks IIb-Esmeraldo cl3 (●), IIc-M6241 cl6 (■), IId-MN cl2 (◇) and IIe-CL Brener (△). Considering the modal distribution of chromosomes, data obtained with probes 1F8/F29 and FFAg6, in particular, suggest a hybrid composition of IId-MN cl2 and IIe-CL Brener with parental genotypes corresponding to representatives of lineage IIb and IIc.

(Hillis and Dixon, 1991), intra-strain homogeneity among the rRNA copies has been found for the transcribed sequences (Stevens et al., 1999) as well as for the rRNA cistron promoter region (Nunes et al., 1997), a fact possibly attributable to gene conversion among the multiple copies and generally conferring to rRNA sequence data its validity for phylogenetic analysis (Hillis and Dixon, 1991). Similarly, maxicircle DNA has been shown to be homogeneous within a single *T. cruzi* cell (Simpson, 1987).

Incongruence between the two gene genealogies is highly significant and cannot be attributed to homoplasy. Instead, it can easily be explained by a hybrid origin of lineages IId and IIe, each after hybridization between parental genotypes related to lineages IIb and IIc. Following hybridization, loss of lineage IIc nuclear rRNA copies by gene conversion would explain the pattern observed in lineage IIe. In this lineage, absence of lineage IIb maxicircle DNA in lineage IIe can possibly be explained by uniparental inheritance, as was observed in *T. brucei* (Sternberg et al., 1989; Gibson and Stevens, 1999). Instead, for lineage IId, one must infer conversion of lineage IIb rRNA copies by lineage IIc copies to account for the pattern observed in most stocks. We cannot exclude completely the alternative hypotheses (i) that an ancestral polymorphism of rRNA and/or CYb sequences was differentially homogenized in the distinct *T. cruzi* lineages, or (ii) that paralogous copies of the rRNA promoter sequences were sequenced in distinct strains of this study. However, we strongly favor the hybridization hypothesis. Other studies have shown that rRNA sequences can be homogenized rapidly after hybridization (Hillis and Dixon, 1991; Ganley and Scott, 1998; Fuertes Aguilar et al., 1999). However, in some stocks of lineage IId, the gene conversion may not be complete. Indeed, in contrast to the rRNA sequence homogeneity found in other stocks of lineage IId, stock IId-MN cl2 still possesses the two parental rRNA promoter sequences. Second, RFLP data also indicate the presence, in some stocks of lineage IId including MN cl2, of a small amount of rRNA copies sharing structural similarity with the rRNA genes of lineage IIb (Souto et al., 1996). This data are consistent with a hybrid origin of lineage IId, with different degrees of subsequent homogenization in distinct representative strains.

In order to investigate evidence of hybridization on other components of the genome, we analyzed the chromosomal size variation of six probes corresponding to linkage groups distinct from that encoding the rRNA genes (Henriksson et al., 1995; Cano et al., 1995). In agreement with previous work (Gibson and Miles, 1986; Engman et al., 1987; Aymerich and Goldenberg, 1989; Henriksson et al., 1990, 1993, 1995; Wagner and So, 1990), we found extensive chromosomal size variation, with one or two hybridization bands per stock in agreement with the previously proposed diploidy hypothesis of *T. cruzi* (Tibayrenc et al., 1981; Lanar et al., 1981; Gibson and Miles, 1986). Unexpectedly, we observed that chromosomal size variation was distributed into discrete size-modes, indicating that intermediate-sized chromosomes are less likely to occur. Within each mode, the observed pattern of continuous size variation is compatible with variation in the copy number of repeated sequences or gene families by progressive amplification–deletion events (Henriksson et al., 1995). However, the multimodal size-distribution can be explained by (i) long-term continuous size variation in independently-evolving evolutionary lines, with loss of intermediate size chromosomes by genetic drift or natural selection, or (ii) by drastic evolutionary events such as amplification/deletions of large chromosomal portions.

Interestingly, our data on chromosomal size variation in *T. cruzi* provide support to the hybridization hypothesis. First, with two (in lineage IId) and three (in IIe-CL Brener) chromosome-specific probes, two chromosomal bands belonging to two distinct size modes were observed, with the corresponding chromosomal modes being observed individually either in lineage IIb or in lineage IIc stocks. Although the alternatives hypotheses of homoplasy of chromosomal size variation or ancestral polymorphism cannot be ruled out, the hybridization hypothesis seems to us much more likely to account for the distribution of chromosomal size classes in these four lineages. In addition, the probes used belong to conserved linkage groups (Henriksson et al., 1995), and radical size variations can, therefore, not be attributed to translocations of the probe target sequences. The differences between lineage IIe stocks CL Brener and Tulahuen cl2, as well as their aberrant P19 chromosomal bands, may be explained by the continuous laboratory cultivation of these two long-standing reference stocks (McDaniel and Dvorak, 1993). Second, in concordance with the sequence data, chromosomal data also pointed to lineage IIb on the one hand, and to stock IIc-M6241 cl6 on the other hand, as the best plausible parental stocks for both putative hybrid lineages (Table 1). Reexamination of MLEE and RAPD data obtained on larger strain sample sets (Brisse et al., 2000a; Barnabé et al., 2000) also indicates that lineages IIb and IIc represent the best combinations of putative parental genotypes (not shown). Stocks of lineage IId and IIe also show a hybrid allelic composition based on sequence data of the two single-copy nuclear genes recently analyzed (Machado and Ayala, 2001). The non-parental characters observed in our study could be due to the fact that the hybrid lineages are of ancient hybrid origin and have undergone subsequent evolution, and/or that the actual parental genotypes could be related, but not identical, to those analyzed here.

Whether hybridization follows Mendelian rules in *T. cruzi*, as seems to be the case in *T. brucei* (Gibson and Stevens, 1999), will have to be elucidated in experimental crosses. The putative parental lineages identified here might represent good candidates for mating trials such as those reported recently for extrachromosomal material (Stothard et al., 1999). Because the two hybrid lineages have very similar pairs of plausible parental genotypes, it is difficult to determine if both lineages are derived from the same hybridization event or if they have originated by two independent hybridization

events implicating the same or slightly different parental genotypes.

In contrast to the possible natural hybrid genotypes previously described in *T. cruzi* (Bogliolo et al., 1996; Carrasco et al., 1996) and related species (Gibson and Stevens, 1999), the hybrid lineages described here are geographically widespread, being found mainly in Brazil, Bolivia, Peru, Chile and Paraguay, and almost exclusively in humans and in the domestic vector *T. infestans* (Barnabé et al., 2000). It has been proposed, based on historical and epidemiological records, that *T. infestans* has spread during the last centuries, largely through the action of man, from an initial focus in Bolivia or Peru toward the South, and north again into Brazil (Schofield et al., 1982). Since the parental lineages IIb and IIc were also found in Bolivia (Barnabé et al., 2000), this dispersal scenario could account for the present geographic distribution of the two hybrid lineages. The fact that the *T. cruzi* genome project reference strain CL Brener has a hybrid composition has important practical consequences, such as a high expected amount of heterozygosity, as previously underlined (Brisse et al., 1998; Machado and Ayala, 2001).

It is important to stress that, although this study provides clear evidence for it, hybridization must be rare among *T. cruzi* lineages given their clear genetic distinctness (Brisse et al., 2000a; Barnabé et al., 2000). Moreover, given the nearly-fixed heterozygosity observed at several MLEE loci in lineages IId and IIe (Barnabé et al., 2000), hybridization was followed by a preponderant clonal propagation and evolution of both hybrid lineages, since segregated genotypes would have been produced by genetic recombination. In addition, homologous chromosome structural divergence is considered strong confirmation of long-term asexual reproduction (Birky, 1996). Given its occurrence in stocks of all six *T. cruzi* lineages (Table 2), genetic recombination is probably very restricted in all of them, as was also suggested by considerable linkage disequilibrium between MLEE (Tibayrenc et al., 1981, 1986; Tibayrenc and Ayala, 1988; Barnabé et al., 2000) and RAPD data (Tibayrenc et al., 1993). However, even rare events of sexual recombination may have profound evolutionary implications on long-term evolution of *T. cruzi*. For example, they would eliminate all problems related to Muller's ratchet.

Our sequence data also provide important information regarding the phylogenetic relationships among *T. cruzi* lineages, since they did not support a subdivision of *T. cruzi* into two primary phylogenetic lineages as previously proposed (Tibayrenc, 1995; Souto et al., 1996; Nunes et al., 1997). Instead, taking the hybrid groups apart, the genealogies separate three (CYb) or four (rRNA) groups of sequences, corresponding to the four MLEE/RAPD lineages I, IIa, IIb and IIc (Brisse et al., 2000a; Barnabé et al., 2000). The discrepancy is explained by the clear divergence of lineage IIb with both CYb and rRNA data, showing that lineage II (Tibayrenc,

Table 2
Sizes of the chromosomal bands (in kbp) obtained after hybridization with six distinct chromosome-specific probes[a]

Lineage	Stock	Tc2	1F8/F29	FFAg6	CA7:12	CA7:32	P19
T. cruzi I	X10 cl1	I_{1030}	I_{660}	I_{800}/I_{860}	II_{1040}/II_{1120}	II_{1040}/II_{1120}	I_{1080}/I_{1160}
T. cruzi I	OPS22	I_{1050}	I_{650}	I_{720}/I_{830}	II_{1010}	II_{1010}	II_{1320}/II_{1415}
T. cruzi I	OPS21 cl11	I_{1070}	I_{780}	I_{820}/I_{910}	II_{1030}	II_{1030}	I_{1180}/I_{1240}
T. cruzi I	133 79 cl7	I_{1000}	I_{660}/I_{690}	I_{780}/I_{830}	II_{1005}	II_{1005}	I_{1045}
T. cruzi I	P209 cl1	I_{990}	I_{670}	I_{880}/I_{945}	II_{960}	II_{960}	I_{1015}/I_{1210}
T. cruzi I	P11 cl3	I_{1040}	I_{705}	I_{895}	II_{990}	II_{990}	I_{1135}/I_{1230}
T. cruzi I	SP104 cl1	I_{1050}	I_{700}	I_{910}	II_{990}	II_{990}	I_{1180}/II_{1430}
T. cruzi I	CUTIA cl1	I_{980}	I_{670}/I_{715}	I_{880}	II_{980}	II_{980}	I_{1080}/I_{1180}
T. cruzi I	CUICA cl1	I_{1000}	I_{660}/I_{715}	I_{835}/I_{910}	II_{980}	II_{980}	I_{1035}/I_{1110}
T. cruzi I	Esquilo cl1	I_{990}	I_{640}/I_{690}	I_{850}/I_{920}	II_{980}	II_{980}	I_{1055}/I_{1120}
T. cruzi I	GAMBA cl1	I_{1030}	I_{670}/I_{725}	I_{860}/I_{960}	II_{990}	II_{990}	I_{1045}/I_{1100}
T. cruzi I	SO34 cl4	I_{1040}	I_{670}/I_{725}	I_{870}/I_{965}	II_{995}	II_{995}	I_{1055}/I_{1120}
T. cruzi I	LGN	I_{1020}	I_{555}/I_{650}	I_{780}/II_{1360}	II_{930}	II_{930}	I_{1045}
T. cruzi IIa	CAN III cl1	II_{1540}	II_{1080}	II_{1470}	I_{590}	I_{735}/II_{810}	I_{113}/I_{1230}
T. cruzi IIb	CBB cl3	II_{1270}/II_{1365}	I_{715}/I_{760}	I_{905}/I_{985}	I_{600}	I_{630}/I_{690}	I_{970}/II_{1390}
T. cruzi IIb	TU18 cl92	II_{1410}	I_{820}	I_{960}	I_{610}	I_{660}/I_{720}	I_{1090}/II_{1440}
T. cruzi IIb	Esmeraldo cl3	II_{1250}/II_{1330}	I_{740}	I_{900}/I_{955}	I_{600}	I_{600}	I_{1020}/II_{1440}
T. cruzi IIc	M6241 cl6	II_{1340}	II_{1030}	II_{1310}/II_{1420}	I_{680}	II_{945}/II_{1010}	I_{1240}/II_{1380}
T. cruzi IId	MN cl2	II_{1430}	I_{830}/II_{1140}	I_{970}/II_{1460}	I_{590}/II_{990}	II_{890}	I_{1055}/II_{1370}
T. cruzi IId	NR cl3	II_{1380}	I_{770}/II_{1080}	I_{920}/II_{1415}	I_{580}/II_{940}	II_{890}	I_{1055}/II_{1350}
T. cruzi IId	SC43 cl1	II_{1490}	I_{830}/II_{1180}	I_{990}/II_{1520}	I_{680}/II_{1110}	II_{970}	I_{1180}/II_{1470}
T. cruzi IId	Bug 2148 cl1	II_{1480}	I_{830}/II_{1180}	I_{1030}/III_{1800}[b]	I_{680}/II_{1110}	II_{950}	I_{1180}/II_{1440}
T. cruzi IId	Bug 2149 cl10	II_{1460}	I_{830}/II_{1130}	I_{1030}/III_{1800}[b]	I_{680}/II_{1060}	II_{950}	I_{1130}/II_{1440}
T. cruzi IIe	Tulahuen cl2	II_{1420}	I_{810}	II_{1210}/II_{1340}	I_{700}/II_{860}	I_{645}/II_{1110}	I_{1230}/II_{1630}
T. cruzi IIe	CL Brener	II_{1340}/II_{1380}	I_{790}/II_{1115}	I_{1080}/II_{1360}	I_{710}/I_{760}	I_{590}/II_{1080}	I_{790}/II_{1710}

[a] The chromosomal bands are distributed into two size modes I and II (see text).
[b] Correspond to a third mode of FFAg6 size-distribution; these bands were in the compression zone of the gel and were given a 1800 kbp size.

1995; Brisse et al., 2000a) is not monophyletic. This may be explained by insufficient resolution of MLEE and RAPD at the higher clustering levels, due to their too fast molecular clock. Interestingly, data from other genes also indicate the existence of three (maxicircle data) or four (nuclear data) distinct primary lineages (Machado and Ayala, 2001; Kawashita et al., 2001). Comparison of the phylogeny based on rRNA promoter sequence (Fig. 1) with those based on 18S rDNA and the D7 region of the 24Sα rDNA (Kawashita et al., 2001) suggest a correspondence between lineages IIa and IIc (Brisse et al., 2000a) with groups Z3B and Z3A (Kawashita et al., 2001), respectively. Altogether, these results suggest that *T. cruzi* is at least composed of (i) four primary phylogenetic lineages corresponding to MLEE/RAPD lineages I, IIa, IIb and IIc, and (ii) two hybrid lineages corresponding to MLEE/RAPD lineages IId and IIe. Thus, the results are in agreement with the grouping into six genetic lineages as described previously (Brisse et al., 2000a). The close similarity of maxicircle sequence data from lineages IIa and IIc may be due to ancient horizontal transfer of maxicircle molecules between them or, alternately, to a low rate of nucleotide substitution. An unrooted genealogy of a nuclear intergenic region (Robello et al., 2000) shows only three major branches but, importantly, lineage IIa was not represented in this study.Our estimate of the divergence date of the main lineages of *T. cruzi* (10.6 ± 2.1 Myr) is comparable to that (10.45 ± 2.27 Myr) estimated based on the maxicircle region encompassing parts of the genes cytochrome oxydase subunit II and NADH dehydrogenase subunit I, using *T. brucei* as an outgroup and fixing its divergence time with *T. cruzi* to 100 Myr (Machado and Ayala, 2001). These divergence dates must be taken with caution given the hypotheses they are based on and given that other genes and outgroups provide distinct estimates (Machado and Ayala, 2001; Kawashita et al., 2001). Still, they provide a quantitative confirmation of the huge phylogenetic heterogeneity previously reported for this taxon (Miles et al., 1978) and, in the absence of fossil records, will help reconstructing the evolutionary history of *T. cruzi* lineages with respect to the evolution of their host and vectors and geological data.

Acknowledgements

We thank Dr. Li Min Wen for assistance in preparation of the clones of the rRNA promoter segment for some of these *T. cruzi* strains. The contribution of ED was the one 2002-016 of the Institut des Sciences de l'Evolution de Montpellier (UMR 5554 CNRS). SB acknowledges financial support from the Eijkman–Winkler Center of the University Medical Center Utrecht, Utrecht, The Netherlands.

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