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TRYPANOSOMA CRUZI: EVALUATION OF A RAPD SYNAPOMORPHIC FRAGMENT AS A SPECIES-SPECIFIC DNA PROBE

Bruno Oury, Nathalie Dutrait, Brigitte Bastrenta, and Michel Tibayrenc

Unité Mixte de Recherche CNRS-ORSTOM 9926, Laboratoire de Génétique Moléculaire des Parasites et des Vecteurs, Centre de Recherche ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), BP 5045, F-34032 Montpellier cédex 1, France

ABSTRACT: A methodological approach is proposed to select rapidly DNA sequences characterized by a well defined specificity and potentially interesting to be used as diagnostic probes or as taxonomic and phylogenetic markers. A fragment amplified from a diversified sample of *Trypanosoma cruzi* stocks by the RAPD (random amplified polymorphic DNA) method with the A8 primer, previously found to be monomorphic in all stocks, was separated in 2 fragments using polyacrylamide electrophoresis. RFLP (restriction fragment length polymorphism) analysis of the 750-bp fragment common to all stocks revealed some sequence heterogeneity within the *T. cruzi* species, whereas hybridization experiments showed a high homology between fragments amplified from different *T. cruzi* stocks. These results suggest that sequence analysis will allow the design of internal primers to be used as probes to target specific taxonomic levels (clone, family of related clones, or species) and for diagnosis.

Natural populations of *Trypanosoma cruzi,* the agent of Chagas' disease, have a clonal structure as previously demonstrated by extensive population genetic studies (Tibayrenc et al., 1986). This model does not imply that genetic recombination is absent, but rather indicates that such genetic recombination is not frequent enough for altering significantly the uniparental propagation of the parasite and, consequently, its clonal evolution. Clones are stable in space and time, and their perenniality permits their use as epidemiological markers. The accumulation of genetic differences among the clonal lineages has an impact on some biological and medical properties of clones or families of related clones (Laurent, 1994; Revollo, 1995). It is, therefore, useful to identify clones in epidemiological surveys or experimental studies with reliable and easy-to-use markers.

At present, *T. cruzi* stocks are characterized by 3 main methods: (1) multilocus enzyme electrophoresis (MLEE) on 22 loci (Tibayrenc et al., 1986); (2) amplification of hypervariable sequences in kinetoplast DNA minicircles (Sturm et al., 1989; Veas et al., 1991; Brenière et al., 1992); and (3) random amplified polymorphic DNA (RAPD) (Tibayrenc et al., 1993).

MLEE is the most widely used method, but it presents 2 drawbacks. First, it is time-consuming and, second, parasites must be amplified in culture after their isolation from chagasic patients, mammalian hosts, or vectors. This introduces a bias, because a selection of genotypes may occur during the culture step. Although amplification of hypervariable sequences from kinetoplast DNA minicircles allows characterization of stocks directly in biological samples, several steps are required during the procedure: hypervariable sequences must be amplified, then labeled before being used as probes to hybridize southern blots of amplified DNA from reference stocks (Brenière et al., 1995). RAPD is a recently developed method that has been used to characterize many different organisms (Welsh and McClelland, 1990; Williams et al., 1990; Tibayrenc et al., 1993). It is a relatively straightforward procedure, but requires meticulous sample processing to avoid contamination with foreign DNA. RAPD analysis of *T. cruzi* has shown that some RAPD fragments are specific for a given level of phylogenetic divergence (a clone, a family of related clones, or the whole species) (Tibayrenc et al., 1993). In particular, it was observed that the A8 primer generated a 1-banded pattern monomorphic in all *T. cruzi* stocks and was different from the patterns observed for other species of parasitic protozoa. The present study was designed to explore the value of this A8 fragment as a taxonomic and epidemiologic tool.

MATERIALS AND METHODS

Parasite stocks

A total of 6 *T. cruzi* stocks was studied (Table I). They were selected to be representative of the extensive genetic variability of the parasite and correspond to clonal genotypes 17, 19, 27, 30, 32, and 39 described by Tibayrenc et al. (1986) (Fig. 1). Stocks corresponding to 14 other parasitic protozoa taxa (Table I) were included in the study in order to evaluate the species specificity of the A8 fragment.

Parasite culture

Trypanosoma cruzi epimastigotes were grown in LIT medium supplemented with 10% inactivated fetal bovine serum (FBS) at 28 C, as described Castellani et al. (1967). *Trypanosoma cruzi marenkellei* epimastigotes were grown in the same conditions as *T. cruzi* stocks whereas *Trypanosoma rangeli* epimastigotes were obtained in NNN biphasic medium at 28 C (Tibayrenc and Le Ray, 1984). *Leishmania* promastigote cultures were kept at 28 C in RPMI-1640 medium supplemented with 10% inactivated FBS for *Leishmania braziliensis* and *Leishmania guyanensis* or 20% FBS for *Leishmania infantum, Leishmania major,* and *Leishmania tropica.* Procyclic forms from *Trypanosoma brucei* and *Trypanosoma congolense* were grown in Cunningham medium supplemented with 20% inactivated FBS at 28 C according to Cunningham (1977). *Plasmodium falciparum* stock was kept in continuous culture using the method described by Trager and Jensen (1976).

Preparation of cell pellets

For trypanosomatids, cultures were harvested at the late log phase and washed 3 times in phosphate-buffered saline (PBS: 1.75 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4). After the last high-speed centrifugation (15,000 *g*), the supernatant was removed and parasites were frozen in liquid nitrogen to be stored and to facilitate cell lysis.

For *Plasmodium falciparum,* cultures were harvested at parasitemia levels of about 5–10%. Infected red blood cells were washed 3 times in PBS. Intracellular parasite forms were released by lysis of host erythrocytes with 0.15% saponin for 15 min on ice and recovered by centrifugation (15,000 *g*). Red cell walls and hemoglobin were discarded by several washings in large volumes of PBS until the supernatant was clear. Parasite pellets were frozen in liquid nitrogen.

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TABLE I. List of the parasite stocks* used in this study.

Species	Stock reference
Trypanosoma cruzi	X10 cl1 (17)
Trypanosoma cruzi	P209 cl1 (19)
Trypanosoma cruzi	CanIII cl1 (27)
Trypanosoma cruzi	ESMERALDO (30)
Trypanosoma cruzi	Tu18 cl2 (32)
Trypanosoma cruzi	SC43 cl1 (39)
Trypanosoma cruzi marenkellei†	B3
Trypanosoma rangeli	Basel
Trypanosoma brucei brucei	MTRG/UG/66/Eatro 1125
Trypanosoma brucei gambiense	MHOM/CI/86/DAL 967
Trypanosoma brucei rhodesiense	MHOM/ZM/74/058 clA3
Trypanosoma congolense forest form‡	G/GB/86/ANR3
Leishmania major	MHOM/SU/73/5ASKH
Leishmania tropica	MHOM/SU/74/K27
Leishmania infantum	MHOM/MA(BE)/67
Leishmania chagasi	MHOM/BR/74/PP75
Leishmania mexicana	MNYC/BZ/62/M379
Leishmania braziliensis	MHOM/BR/75/M2502
Leishmania guyanensis	MHOM/BR/78/M5378
Plasmodium falciparum	SGE-1

* Numbers in parentheses after *T. cruzi* stock names refer to the genotype numbering established by Tibayrenc et al. (1986) on the basis of 15 isoenzyme loci (see also Fig. 1).

† A trypanosome isolated from bats in South America that is morphologically identical to *T. cruzi* (Baker et al., 1978).

‡ See Gashumba et al. (1988).

DNA preparation

Parasite pellets (100 mg) were resuspended in 300 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium dodecyl sulfate [SDS]) and incubated for 1.5 hr at 37 C with RNase A (100 µg/ml) and for 2 hr at 50 C with proteinase K (100 µg/ml). After 2 phenol and 3 chloroform extractions, DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ® water. DNA concentration was measured by spectrophotometry at 257 nm. Genomic DNA samples were stored at −20 C.

DNA amplification (RAPD)

Amplification reactions were performed in a volume of 100 µl according to Williams et al. (1990). Briefly, genomic DNA samples (10 ng) were used as template for polymerase chain reaction (PCR) in buffer containing 0.2 µM of the A8 primer (5′-GTGACGTAGG-3′; Operon Technologies Kit A), 4 × 100 µM dNTP, 1.5 mM $MgCl_2$, and 1.5 U Taq polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). The amplification program was achieved on a PTC-100-60 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA) and consisted of 45 cycles at 94 C for 1 min, 36 C for 1 min, and 72 C for 2 min and a final elongation step at 72 C for 7 min. Amplified DNA was kept at 4 C before use.

DNA electrophoresis

Amplification products were separated by electrophoresis in 1.6% agarose gels with TAE buffer (40 mM Tris-acetate, pH 7.4, 1 mM EDTA) at 5 V/cm or in 5% polyacrylamide gels with TBE buffer (45 mM Tris-borate, pH 7.5, 1 mM EDTA) at 5 V/cm. DNA fragments were visualized by ethidium bromide staining.

Elution of DNA

The 750-bp fragment from each stock was retrieved from a precut polyacrylamide gel slice by electroelution using the electroeluter 422® (Bio-Rad, Richmond, California) under conditions recommended by the supplier. Briefly the elution was done at constant current (15 mA/glass tube). After 1 phenol, 1 phenol–chloroform, and 1 chloroform extractions,

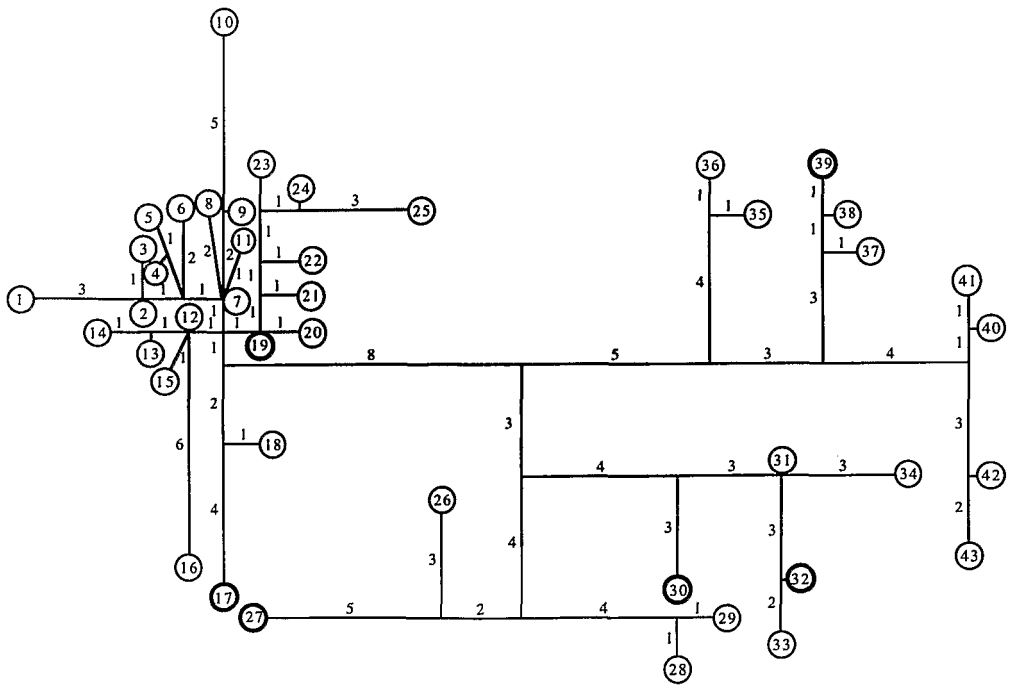

FIGURE 1. An unrooted Wagner network depicting the phylogenetic relationships among 43 *Trypanosoma cruzi* clonal genotypes (circled numbers) characterized for 15 isoenzyme loci (Tibayrenc et al., 1986). Genotypes used in this study are circled in bold. The lengths of the branches are proportional to the evolutionary distances among the genotypes.

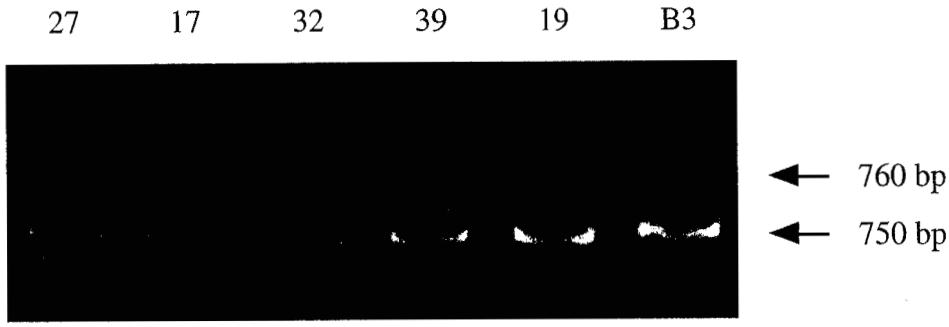

FIGURE 2. Ethidium bromide-stained 5% polyacrylamide gel showing the discrimination of the synapomorphic A8 fragment into 2 bands, respectively, 750 bp and 760 bp length. Fifty nanograms of A8 fragment were loaded in each slot. The numbers correspond to the clonal genotypes (see Fig. 1 and Table I) of the stocks from which the corresponding A8 fragments have been amplified.

DNA fragment was precipitated in 70% ethanol and redissolved in sterile MilliQ™ water. DNA concentration was determined (as described).

Restriction fragment length polymorphisms

In order to obtain enough material for RFLP analysis, the 750-bp fragment was reamplified with the A8 primer under the same conditions as described above. Reamplified DNA was purified by chromatocentrifugation through a Quick Spin Column Sephadex™ (Boehringer Mannheim) according to the manufacturer's instructions. Eluted DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ™ water. The DNA concentration was measured (as described). The 750-bp fragment (500 ng) was digested with each (1 U) of the following restriction enzymes: Acc I, Alu I, Bam HI, Eco RI, Hae III, Hha I, Hind III, Hpa II, Kpn I, Pst I, Rsa I, Sal I, Sau3A, Sca I, Sma I, and Xba I, under conditions recommended by the suppliers. Digested DNA (250 ng) was analyzed after electrophoresis on agarose gels, as described above.

DNA probe labeling

Amplified DNA products were purified by chromatocentrifugation (as described). Purified DNA was precipitated in 70% ethanol and redissolved in sterile MilliQ™ water. The DNA concentration was measured (as described) and labeled with ^{32}P by nick translation (Boehringer Mannheim).

Southern blot and dot blot

Amplified DNA was separated by electrophoresis in 1.6% agarose gels and transferred onto nylon membranes (Hybond N+™; Amersham, Buckinghamshire, U.K.) according to the alcalin method described by the manufacturer. Dot blot was performed as follows: genomic DNA (1 μg) was denatured by heating at 95 C for 10 min followed by rapid cooling in ice water for 10 min. After addition of 1 volume (100 μl) of cold 20× SSC (0.3 M tri-sodium citrate, 3 M NaCl, pH 7.0), samples were applied onto a nylon membrane, using a minifold vacuum filtration ("dot blot") apparatus (BRL, Bethesda, Maryland). The membrane was then laid onto 3MM filter paper prewetted with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min before transfer onto filter paper soaked in neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM EDTA) for 1 min. The membrane was then air dried and DNA fixed by placing the membrane onto 3MM filter paper soaked in 0.4 M NaOH for 30 min. The membrane was finally rinsed in 5× SSC for 1 min under gentle agitation, air dried, and stored in the dark until used.

Hybridization

Filters were prehybridized in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml herring sperm DNA (20× SSPE: 0.2 M NaH_2PO_4, 3.6 M NaCl, 20 mM EDTA, pH 7.7) at 65 C for 2 hr before hybridization at 65 C overnight in the presence of 10^6 cpm/ml of ^{32}P nick-translated 750-bp A8 fragment (specific activity: 10^8 cpm/μg of DNA). Filters were washed twice at room temperature with 2× SSPE, 0.1% SDS for 10 min, once at 65 C with 1× SSPE, 0.1% SDS for 15 min. Higher stringency conditions were achieved by washing the filters at 65 C with 0.5× SSPE, 0.1% SDS or 0.1× SSPE, 0.1% SDS for 15 min. Filters were autoradiographed at −70 C with intensifying screens.

RESULTS

Tibayrenc et al. (1993) have previously reported that amplification with the A8 primer generated a 1-banded pattern that was identical for all *T. cruzi* stocks screened. *Trypanosoma cruzi marenkellei,* although exhibiting a more complex overall pattern, exhibited the same band as *T. cruzi* stocks. In this study, polyacrylamide gel electrophoresis of A8 primer-amplified DNA revealed 2 different profiles (Fig. 2): the P209 cl1, CAN-III, ESMERALDO (not shown), and SC43 cl1 *T. cruzi* stocks, as well as the *T. cruzi marenkellei* stock, exhibited 2 fragments of 750 bp and 760 bp, respectively, whereas the X10 cl1 and TU18 cl2 *T. cruzi* stocks showed the 750-bp fragment only.

In order to determine whether the 750-bp fragment, which is common to all *T. cruzi* stocks plus the *T. cruzi marenkellei* stock, is homogeneous, it was submitted to RFLP analysis. Among the 16 restriction enzymes tested, Hae III, Hha I, Hpa II, Pst I, and Sau3A gave 2 different restriction patterns that were specific for *T. cruzi* and *T. cruzi marenkellei* stocks. Polymorphism detected by Pst I is shown in Figure 3a. Rsa I revealed a polymorphism among the *T. cruzi* stocks: the 750-bp fragment from the X10 cl1 and P209 cl1 stocks was digested into 2 subfragments of 550 bp and 200 bp (Fig. 3b). For the other *T. cruzi* stocks, the 750-bp fragment remained undigested.

The homology between the different 750-bp fragments was assessed by hybridizing a southern blot of amplified products with the A8 fragment taken from the SC43 cl1 stock (genotype 39) as a probe. High stringency hybridization conditions revealed a difference of signal intensity between the *T. cruzi* stocks and the *T. cruzi marenkellei* stock, whereas the signal intensity was quite similar among all *T. cruzi* stocks (Fig. 4).

Dot blot hybridization of the A8 fragment from the *T. cruzi* SC43 cl1 stock, with genomic DNA from a panel of parasites belonging to various species (Table I) revealed no signal for *T. brucei* s.l., *T. congolense, Leishmania* spp., and *P. falciparum. Trypanosoma rangeli* DNA was weakly recognized, whereas the signal intensity was strong for *T. cruzi* and *T. cruzi marenkellei* stocks (Fig. 5a). Hybridization with the A8 fragment from *T. cruzi marenkellei* produced no signal for *T. brucei* s.l., *T. congolense, Leishmania* spp., and *P. falciparum.* Signal intensity was high for homologous DNA, whereas it was lower for *T. cruzi* and *T. rangeli* DNA (Fig. 5b).

DISCUSSION

Trypanosoma cruzi and *T. cruzi marenkellei* showed similar amplification patterns with the A8 primer. However, RFLP anal-

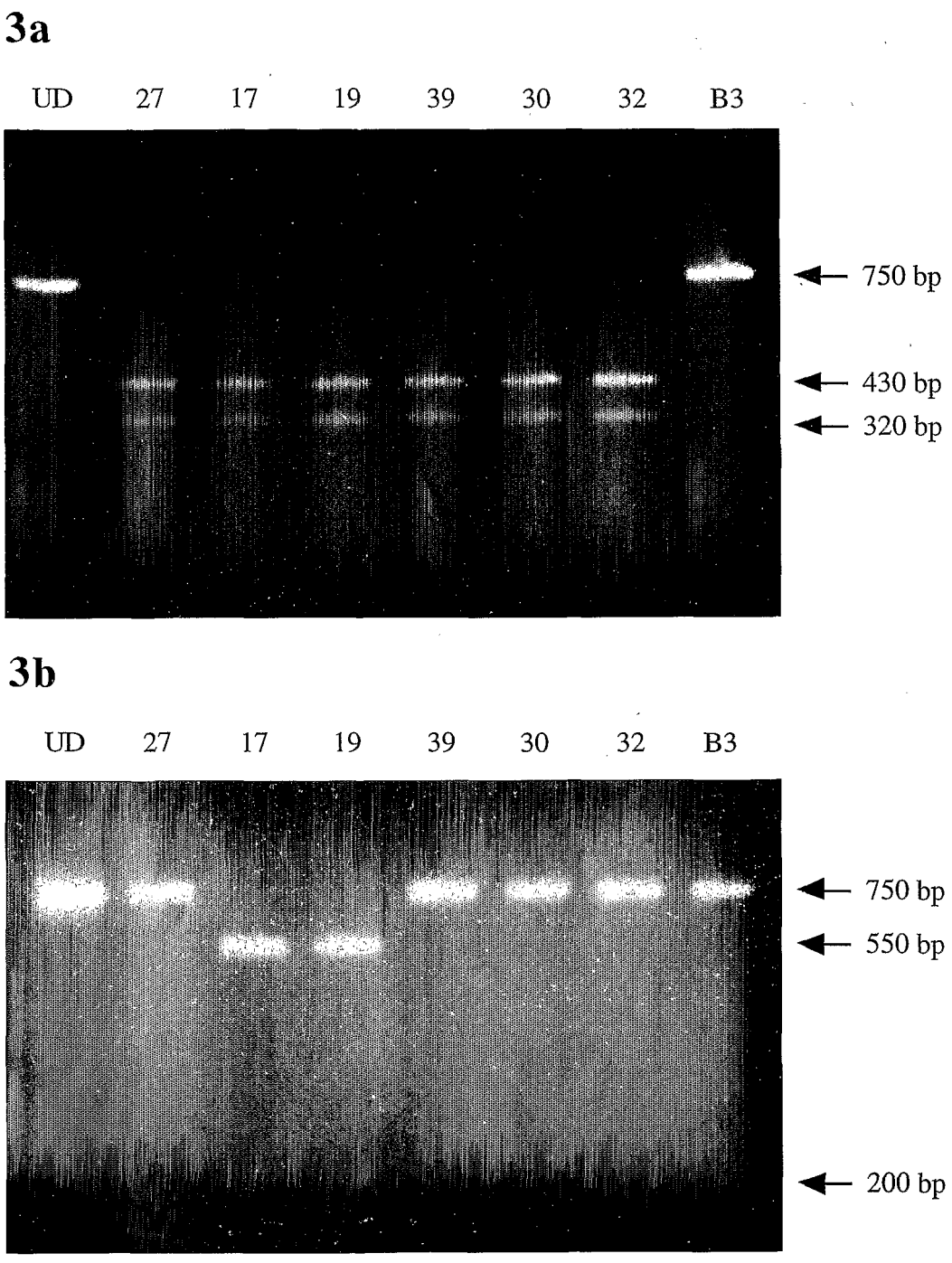

FIGURE 3. RFLP patterns of the 750-bp A8 fragment obtained from different *Trypanosoma cruzi* stocks and from *Trypanosoma cruzi marenkellei*. The numbers correspond to the clonal genotypes (see Fig.1 and Table I). UD is the undigested A8 fragment amplified from the SC43 cl1 and used as control DNA. **a:** Digestion with PstI distinguished *T. cruzi marenkellei* from all *T. cruzi* stocks. **b:** Digestion with RsaI revealed a polymorphism among *T. cruzi* stocks.

ysis of the common 750-bp fragment produced a unique species-specific pattern for 5 enzymes, a result consistent with their known phylogenetic relationship. RFLP analysis of the A8 fragment among the *T. cruzi* stocks also revealed a polymorphism with the enzyme Rsa I. This enzyme distinguished stocks with the genotypes 17 and 19 (Tibayrenc et al., 1986) from the other *T. cruzi* stocks. This corroborates the known picture of *T. cruzi* subspecific variability, which clearly shows that this taxon is subdivided into 2 main phylogenetic lineages (Tibayrenc, 1995).

The variability detected by RFLP confirms that the size identity of PCR fragments does not imply that the sequences involved are necessarily identical. However, this heterogeneity

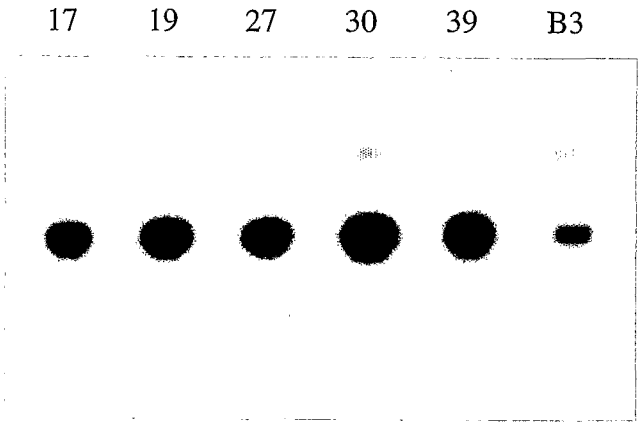

FIGURE 4. Southern blot of the 750-bp A8 fragment (25 ng) from various *Trypanosoma cruzi* stocks and from *Trypanosoma cruzi marenkellei* hybridized with the ^{32}P nick-translated 750-bp A8 fragment from the *T. cruzi* stock SC43 (clonal genotype 39; see Fig. 1 and Table I). The numbers correspond to the clonal genotypes. Signal intensity was similar for all *T. cruzi* stocks and lower for *T. cruzi marenkellei.*

has no consequence for hybridization experiments; the A8 fragment amplified from the stock SC43 (genotype 39) hybridizes on fragments amplified from all other *T. cruzi* stocks with a good affinity. This suggests the existence of a high sequence homology between the different fragments.

Given the relative weakness of signal intensity in hybridizing *T. cruzi* genomic DNA, it seems that the A8 fragment exists in only 1 copy or a limited number of copies in the parasite genome. Now it is generally believed that random amplified fragments correspond to highly or moderately repetitive sequences in the genome. At least for the A8 fragment of *T. cruzi,* our results do not corroborate this hypothesis.

Various DNA sequences have been proposed as diagnostic probes or taxonomic markers for *T. cruzi,* e.g., highly repeated sequences (195 bp) (Moser et al., 1989; Russomando et al., 1992; Brenière et al., 1993), kinetoplast minicircle DNA sequences (Sturm et al., 1989; Avila et al., 1991, 1993; Veas et al., 1991; Brenière et al., 1992; Britto et al., 1993; Wincker et al., 1994), intergenic spacer (IGS) or nontranscribed spacer (NTS) sequences (Dietrich et al., 1990; González et al., 1994), 18S rRNA genes (Hernández et al., 1990; Clark, 1992; Souto and Zingales, 1993; Clark and Pung, 1994), or 24S rRNA genes (Benavides et al., 1993). These markers were generally selected with a goal of species identification and did not address the problem of subspecific variability of *T. cruzi.*

In this study, we used information obtained from previous population genetic studies on *T. cruzi* to provide a convenient framework to rapidly select RAPD fragments exhibiting a given phylogenetic specificity (species, subspecies, natural clone).

Future work will involve looking for other potentially interesting RAPD fragments in the species *T. cruzi* and sequencing the 750-bp A8 fragment in several natural clones of the parasite. This will make it possible to select primers for the identification of *T. cruzi* or given subdivisions of the species without culturing the parasite.

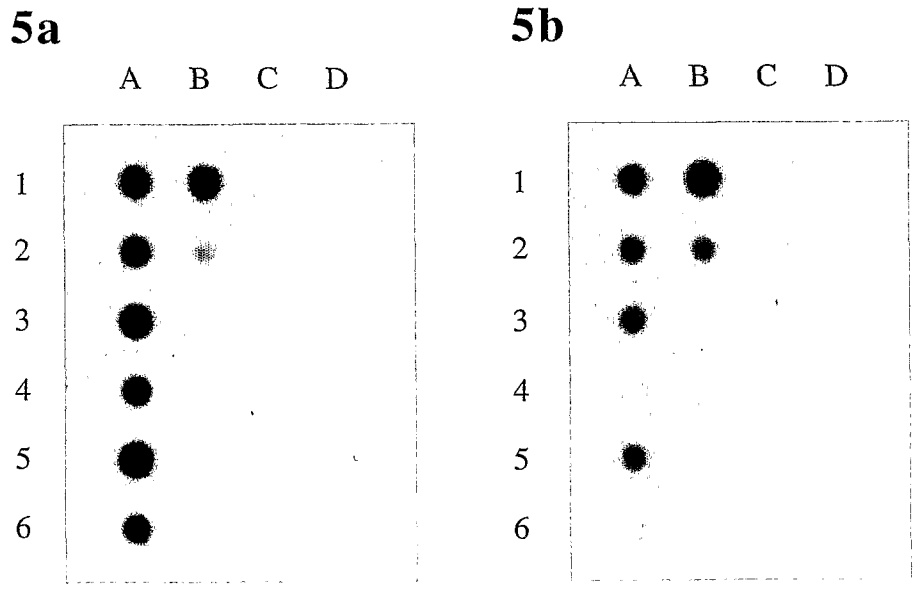

FIGURE 5. Dot blot of genomic DNA (1 μg). Column A: *Trypanosoma cruzi* stocks: (1) clonal genotype 17; (2) genotype 19; (3) genotype 27; (4) genotype 30; (5) genotype 32; (6) genotype 39. Column B: (1) *Trypanosoma cruzi marenkellei*; (2) *Trypanosoma rangeli*; (3) *Trypanosoma brucei brucei*; (4) *Trypanosoma brucei gambiense*; (5) *Trypanosoma brucei rhodesiense*; (6) *Trypanosoma congolense* (forest variant). Column C: (1) *Leishmania major*; (2) *Leishmania tropica*; (3) *Leishmania infantum*; (4) *Leishmania chagasi*; (5) *Leishmania mexicana*; (6) *Leishmania braziliensis.* Column D: (1) *Leishmania guyanensis*; (2) *Plasmodium falciparum*; (3–6) none. **a:** Hybridization with the ^{32}P nick-translated 750-bp A8 fragment from *T. cruzi* stock SC43 (genotype 39). *Trypanosoma cruzi* and *T. cruzi marenkellei* DNA were well recognized while signal intensity was weak for *T. rangeli* DNA. No signal was detected on other trypanosomatids and *P. falciparum.* **b:** Hybridization with the ^{32}P nick-translated 750-bp A8 fragment from *T. cruzi marenkellei.* Signal intensity was higher on homologous *T. cruzi marenkellei* DNA than on *T. cruzi* and *T. rangeli* DNA. None of the DNA from other parasites was recognized.

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