Identification of six *Trypanosoma cruzi* lineages by sequence-characterised amplified region markers[*]

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Received 9 March 2000; received in revised form 23 June 2000; accepted 7 July 2000

Abstract

Six discrete phylogenetic lineages were recently identified in *Trypanosoma cruzi*, on the basis of multilocus enzyme electrophoresis and random amplified polymorphic DNA (RAPD) characterisation. The objective of the present study was to develop specific PCR-based markers for the identification of each of the six lineages. Eighty-seven *T. cruzi* stocks representative of all the lineages were characterised by RAPD with three primers, resulting in the identification of three fragments that were specifically amplified in the given sets of lineages. After cloning and sequencing these fragments, three pairs of sequence-characterised amplified region (SCAR) primers were designed. After PCR amplification using the SCAR primers, the initial polymorphism was retained either as the presence or absence of amplification, or as size variation between the PCR products. Although most PCR products, taken individually, were distributed across several lineages, the combination of the three SCAR markers resulted in characteristic patterns that were distinct in the six lineages. Furthermore, *T. cruzi* lineages were distinguished from *Trypanosoma rangeli*, *T. cruzi marinkellei* and *T. cruzi*-like organisms. The excellent correspondence of these new PCR markers with the phylogenetic lineages, allied with their sensitivity, makes them reliable tools for lineage identification and strain characterisation in *T. cruzi*. The approach described here could be generalised to any species of microorganism harbouring clear-cut phylogenetic subdivisions. © 2000 Elsevier Science B.V. All rights reserved.
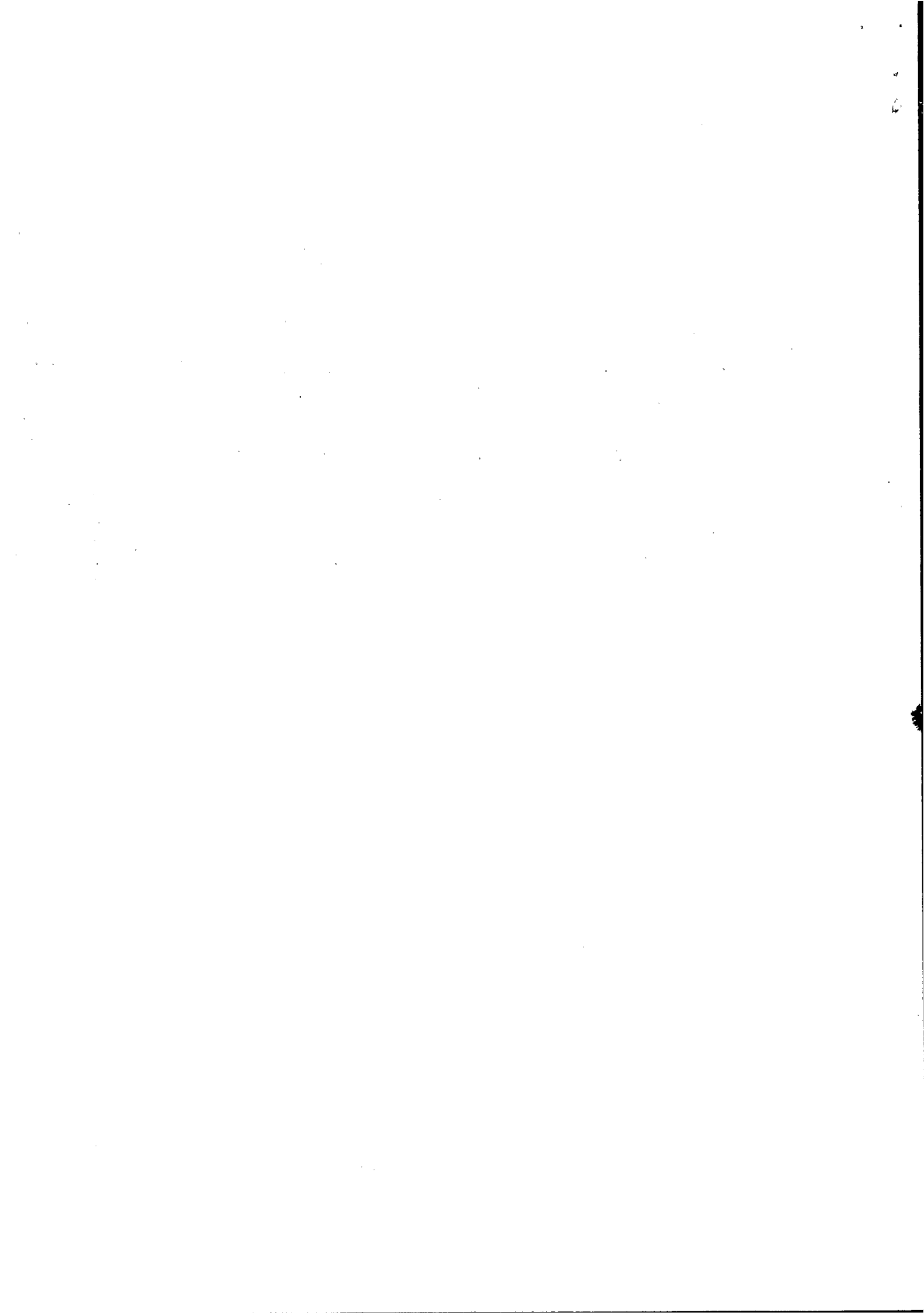
1. Introduction

Trypanosoma cruzi, the etiologic agent of Chagas' disease, exhibits a high degree of genetic polymorphism, as revealed by multilocus enzyme electrophoresis (MLEE) [1,2], schizodeme analysis [3], DNA fingerprinting [4], random amplified polymorphic DNA (RAPD) [5,6], karyotype variability [7,8], microsatellites [9] and variability of the ribosomal RNA (rRNA) genes [10–12] and mini-exon genes [10,13]. The mode of reproduction of this parasite is thought to be predominantly clonal [2,14], although genetic recombination might also take place occasionally [15–17].

Various independent descriptions of the infraspecific phylogenetic diversity of T. cruzi have been proposed, based on an increasing number of strains and genetic markers. Three principal zymodeme groups, called Z1, Z2 and Z3 were first described [1], whereas 43 zymodemes, corresponding to clonal genotypes or clonets, were identified in another study [2]. The clustering analysis of MLEE and RAPD data of a representative set of these clonets [6] later suggested the existence of two major phylogenetic lineages within T. cruzi [18]. This was subsequently corroborated by the dimorphism of the 24Sα rRNA and mini-exon genes [10,11], although it has to be noted that the nomenclature was inverted, since the rRNA and mini-exon lineages 1 and 2 [10] correspond to the MLEE and RAPD lineages 2 and 1 [18], respectively. Miles' Z1 group fell within MLEE/RAPD lineage 1, while Z2 and Z3 fell within lineage 2 [18]. In an attempt to harmonise the nomenclature of T. cruzi strains, MLEE/RAPD lineages 1 and 2 have recently been proposed to be designated as T. cruzi I and T. cruzi II, respectively, although Z3 was not definitely included in T. cruzi II, since the agreement is not total on its phylogenetic status [19]. Both groups are highly heterogeneous. Recently, a set of 434 T. cruzi isolates with broad geographic and ecological origins and representative of the two major lineages, was characterised by the means of 22 isoenzyme loci [20], and a subset of these stocks was characterised with 20 RAPD primers [21], which yielded the identification of five lower phylogenetic subdivisions, designated 2a–2e, within the second major MLEE/RAPD lineage, whereas no clear subdivision was found within the first lineage. These subdivisions are characterised by numerous specific MLEE and RAPD characters and are believed to correspond to phylogenetic lineages that have diverged predominantly by long-term clonal evolution [2,20,21]. Thus, considering T. cruzi I and the five subdivisions of T. cruzi II, six phylogenetic lineages can be distinguished in T. cruzi. In order to take into account both these recent findings and the new proposal on T. cruzi strains nomenclature, we will designate MLEE/RAPD lineages 1 and 2a–2e as lineages I and IIa–IIe, respectively. Significant differences in clinically relevant biological parameters have been reported between isolates belonging to some of these lineages, both in experimental studies [22–25] and in human [26], showing the relevance of infraspecific strain characterisation and the need to take as the unit of analysis the lesser genetic subdivisions of T. cruzi rather than solely the major lineages I and II. Although numerous lineage-specific MLEE and RAPD markers are available [20,21], both the techniques require the production of a substantial amount of parasites before characterisation, and are, therefore, not fast and might involve elimination of particular genotypes that are not adapted to culture conditions. In order to obtain faster and more sensitive characterisation tools, we developed PCR-based markers for the specific identification of the six T. cruzi phylogenetic lineages, following an approach previously applied to the development of sequence-characterised amplified regions (SCAR) genetic markers [27].

2. Materials and methods

2.1. Parasite stocks

Table 1 gives the 93 trypanosome stocks under study. The detailed origin of the stocks is available upon request. All the stocks were previously characterised by multilocus enzyme electrophoresis at 22 loci [20] and by RAPD with 10–20 different primers [21]; Brisse, (unpublished). The sample includes one *T. rangeli* stock, two *T. cruzi marinkellei* bat trypanosomes [28], and three stocks of *T. cruzi*-like organisms shown to be genetically distinct from *T. cruzi* according to

MLEE analysis [29]. Nineteen stocks were cloned in our laboratory by micromanipulation and verification under the microscope, and are indicated in Table 1 by the letters 'cl' followed by a clone number. Stocks were cultured in liver infusion tryptose medium containing 10% foetal calf serum (FCS) and 50 μg.ml^{-1} gentamycin.

2.2. RAPD and PCR amplifications

Preparation of the samples and RAPD analysis were carried out as previously described [17,21]. The three primers used are given in the legends of Fig. 1. PCR amplifications were performed with

Table 1
PCR characterisation of the trypanosome stocks under study

Taxon	PCR products obtained with primer pair[a]:			Stocks
	p3/p6	P9/p10	p7/p8	
T. rangeli	NA	NA	NA	LDG
T. cruzi marinkellei	NA	2	NA	M1117
T. cruzi marinkellei	NA	1	NA	B3
T. cruzi-like	NA	NA	4	A276, A87
T. cruzi-like	NA	2	4	A83
T. cruzi I	NA	2	4	SC13, Davis2, STP3.1, OPS21 cl11, Tehuentepec cl2, OPS4, X10 cl1, P209 cl1, 133-79 cl7, LGN, Cuica cl1, SO34 cl4, Nazca, SP1, X39501, AP, 422
T. cruzi I	ND[b]	2	4	458, 31R16, A102, A105, A269, A82, Bp-48, CID, Ep243, Ev-113, Ev-41, Ev-77, Honduras 1968, PB6, QUE, R112, R174, RTD, SC1, ZAC, USA Armadillo, USA Dog Y
T. cruzi IIa	NA	2	2	CanIII cl1, 10R26, Saimiri 3
T. cruzi IIa	NA	2	2 and 4	Ep-255, Ep-272
T. cruzi IIa	NA	2	NA	DogT, STC33R, STC5R
T. cruzi IIb	NA	1	5	Esmeraldo cl3
T. cruzi IIb	NA	1	4	AAB2, MSC2, SO50, TU18 cl2, MAS1 cl1, CBB cl3, MCV2, VOV2, Bug2152
T. cruzi IIc	1	2	3 and 4	CM17
T. cruzi IIc	1	2	1	CM25, M6241 cl6, M5631 cl5, 85/847, X110/8, X9/3, X109/2, Brazil NIH 1954
T. cruzi IId	1	2	3 and 5	92.80 cl1, P274, MN cl2, Kundera, JSR6, SC43 cl1, NR cl3, BMS, CA34, AG-0
T. cruzi IId	1	2	4 and 5	NT
T. cruzi IIe	1	1 and 2	4	Tulahuen 1954, Tulahuen cl2, Tula Yale, CL Brener, Guateque, 50/1, P69/8, SABCHO 109a, VINCHO 195, X57/3

[a] NA: no amplification; '*x* and *y*' means that both product x and product y were observed. For each pair of primers, each distinct PCR product was designated by a number, with number 1 corresponding to the largest product
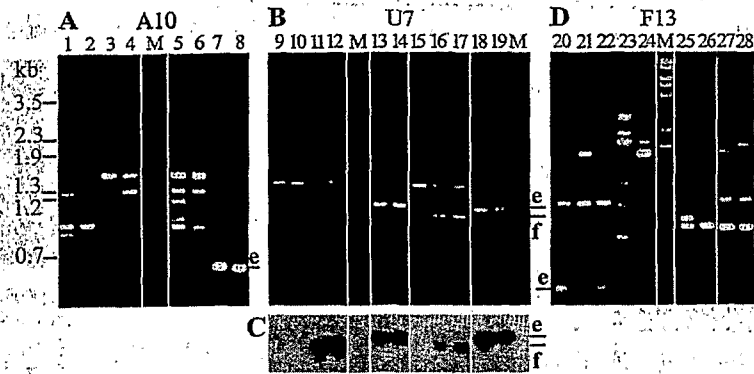
[b] ND: not determined

Fig. 1. (A) RAPD amplifications obtained with primer A10 (5'-GTGATCGCAT-3'). The lanes correspond to the following stocks (the lineage to which they belong is given in parentheses): 1, X-39501 (I); 2, AP (I); 3, CanIII cl1 (IIa); 4, 10R26 (IIa); 5, MCV2 (IIb); 6, VOV2 (IIb); 7, CM17 (IIc); 8, CM25 (IIc). The fragment labelled 'e' (designated A10-e) was exclusively amplified in all stocks of lineages IIc, IId and IIe, and homology across the stocks was confirmed by molecular hybridisation experiments (not shown). Profiles obtained in all stocks of lineages IIc, IId and IIe were similar to those shown for representatives of lineage IIc. (B) RAPD amplifications obtained with primer U7 (5'-CCTGCTCATC-3'). The lanes correspond to the following stocks (the lineage to which they belong is given in parentheses): 9, X-39501 (I); 10, AP (I); 11, CanIII cl1 (IIa); 12, 10R26 (IIa); 13, MCV2 (IIb); 14, VOV2 (IIb); 15, X110/8 (IIc); 16, P274 (IId); 17, MN cl2 (IId), 18, CL Brener (IIe); 19, Guateque (IIe). The fragment labelled 'e' (designated U7-e) was exclusively amplified in all stocks of lineages IIb and IIe. Fragment labelled 'f' was exclusively amplified in all stocks of lineages IIa and IId. (C) Autoradiogram of the gel lanes of panel B after transfer onto a nylon membrane and hybridisation with the cloned fragment U7-e from stock CL Brener used as a probe, showing the homology of fragments U7-e across stocks of lineages IIb (lanes 13 and 14) and IIe (lanes 18 and 19), and their homology with fragment U7-f seen in stocks of lineages IIa (lanes 11 and 12) and IId (lanes 16 and 17), and in some stocks of lineage I (not shown). (D) RAPD amplifications obtained with primer F13 (5'-GGCTGCAGAA-3'). The lanes correspond to the following stocks (the lineage to which they belong is given in parentheses): 20, SP1 (I); 21, X-39501 (I); 22, AP (I); 23, CanIII cl1 (IIa); 24, 10R26 (IIa); 25, Esmeraldo cl3 (IIb); 26, AAB2 (IIb); 27, BMS (IId); 28, Tulahuen 1954 (IIe). The fragment labelled 'e' (designated F13-e) was exclusively amplified in all stocks of lineage I, and homology across the stocks was confirmed by molecular hybridisation experiments (not shown). Lanes marked with M correspond to phage lambda DNA digested with BstEII; the size of the marker bands are indicated on the left. Photographs of the amplification profiles obtained for 48 T. cruzi stocks are available upon request.

three pairs of SCAR primers, each derived from the sequence of a distinct RAPD fragment. Primers p3 (5'-GTG ATC GCA GCA AGT AGT CC) and p6 (5'-GTG ATC GCA GGA AAC GTG A) were designed from the sequence of RAPD fragment A10-e from clone CL Brener (Fig. 1). Primers p7 (5'-GGC TGC AGA ACC AAT AAG AAA TG) and p8 (5'-GGC TGC AGA AAG GGA AAT CCG AAG) were derived from the sequence of the RAPD fragment F13-e from stock SP1 (Fig. 1). Primers p9 (5'-CCT GCT CAT CCT CGT GCT TTA CGG) and p10 (5'-TGC TCA TCG ACA ACA TTT TAT TGG) were designed from the sequence of fragment U7-e from clone CL Brener (Fig. 1). For each pair, the two PCR primers correspond to the sequence of both ends of the RAPD fragment, and the first ten nucleotides of each PCR primer

correspond to the original RAPD primer, except for primer p10, which begins at position 3 of the U7 RAPD primer. PCR amplifications were performed in a final volume of 60 µl containing 0.9 units Taq Polymerase (Boehringer, Mannheim, Germany), 100 µM each dNTP, 200 nM of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, and 20 ng template DNA. Annealing temperatures were as follows, 65°C for the primer pair p3 and p6, 50°C for the pair p7 and p8, and 55°C for the pair p9 and p10. Thirty-five cycles (denaturation, 1 min at 94°C; annealing, 1 min at the appropriate temperature; elongation, 2 min at 72°C) were followed by a final elongation step of 7 min at 72°C. Amplification was performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA, USA). PCR products were analysed by electrophoresis in 1.6%

agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide and visualised by ultraviolet light.

2.3. Characterisation of the RAPD and PCR fragments

For cloning, RAPD and PCR DNA fragments were separated on low-melting agarose, the gel slice containing the fragment of interest was cut out, and the DNA was purified using the Wizard DNA purification kit (Promega, France) and cloned into the pGEM-T Easy vector (Promega, France). The sequence of the fragments was determined in both directions with the M13 forward and reverse primers using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and an ABI 373 automated sequencing apparatus. For genomic characterisation, 2 µg of the total genomic DNA from the parasites were digested for 2 h with 20 units of restriction enzyme (New England Biolabs, Saint Quentin en Yvelines, France). The DNA fragments were separated in a 0.8% agarose gel in TAE buffer at 45 V for 22 h, transferred onto nylon membranes (Hybond N$^+$ Amersham) as previously described [30], and hybridised with random prime labelled DNA probes (Boehringer, Mannheim, Germany). Last post-hybridisation washing was in 0.1 × sodium chloride/sodium citrate buffer (SSC 20 × : 3 M NaCl, 0.3 M Na$_3$ citrate·2H$_2$O, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) at room temperature. A10-e and U7-e probes were hybridised on total genomic DNA cut with enzymes KpnI, BamHI and SmaI; probe F13-e on total DNA cut with enzymes BsaWI, SmlI and HincII.

3. Results

3.1. RAPD analysis with primers A10, U7 and F13

A collection of 87 T. cruzi stocks, representative of the six T. cruzi phylogenetic lineages described recently on the basis of RAPD and MLEE [20,21], was selected (Table 1). In addition, six stocks previously characterised as T. rangeli, T. cruzi marinkellei [28] and T. cruzi-like [29] were included for comparison. All the stocks were submitted to RAPD analysis with primers A10, U7 and F13 (Fig. 1). Some RAPD fragments appeared particularly relevant for lineage characterisation, since they were observed in all the representatives of some T. cruzi lineages, but in none of the others. Three such fragments were selected for further analysis (Fig. 1); fragment A10-e, amplified in all stocks of lineages IIc, IId and IIe; fragment U7-e, amplified in all the stocks of lineages IIb and IIe; and fragment F13-e, present in all the stocks of lineage I. Molecular hybridisation experiments were performed to control their sequence homology across the T. cruzi stocks, using each of the three cloned RAPD fragments as radioactive probes. Probes A10-e and F13-e hybridised only with the corresponding RAPD fragment in all the stocks in which they were originally observed (not shown). Probe U7-e hybridised with the RAPD fragment U7-e but, additionally, hybridised with a smaller fragment, U7-f, observed in stocks of lineages IIa and IId (Fig. 1).

3.2. PCR characterisation of the parasites

In order to use the lineage-specific RAPD fragments to develop SCAR markers, we followed an approach previously described [27,31]. After cloning and sequencing the RAPD fragments, oligonucleotides corresponding to the sequence of their two extremities were synthesised and used as PCR primers (see Section 2).

When using primers p3 and p6, derived from fragment A10-e, a single PCR product (called fragment p3/p6-1) of the expected size (657 bp) was obtained in all stocks of lineages IIc–IIe, corresponding to those in which the original RAPD fragment was observed (Table 1). No amplification product was observed in any stock belonging to the other T. cruzi lineages and to the other species (Table 1).

Fig. 2 shows the results obtained after PCR amplification (hereafter called p9/p10 PCR) with primers p9 and p10, derived from the sequence of RAPD fragment U7-e. Depending on the stock, two PCR products of distinct sizes, product p9/p10-1 (approximately 1170 bp) and product p9/
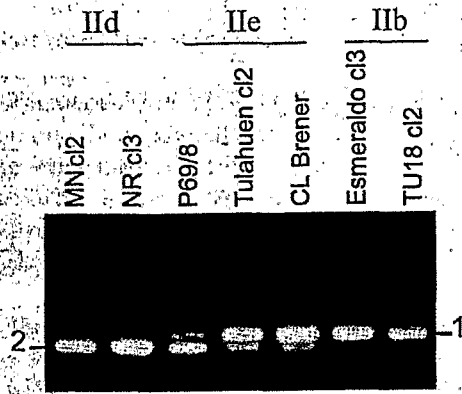
Fig. 2. PCR amplifications obtained with the SCAR primers p9 and p10, derived from the RAPD fragment U7-e. Two PCR products, p9/p10-1 (approximately 1170 bp) and p9/p10-2 (approximately 1080 bp), were observed across the stocks. Product p9/p10-1 is of equal size to RAPD fragment U7-e (Fig. 1), whereas the product p9/p10-2 is of equal size to RAPD fragment U7-f (Fig. 1). Stocks of lineage IIe were characterised by the presence of both PCR products. The additional DNA fragments observed with low intensity in some stocks were not reproducible, and were therefore not taken into account in the analysis. They could correspond to PCR artefacts or non-specific amplification products.

served. As revealed by side-by-side electrophoresis control experiments, the size of the PCR products p9/p10-1 and p9/p10-2 corresponded to that of the RAPD fragments U7-e and U7-f, respectively (data not shown). Results of the PCR characterisation of the 93 stocks using primers p9 and p10 are given in Table 1. Within a given lineage, all the stocks showed the same PCR profile. Interestingly, lineages I, IIa, IIc and IId were characterised by the presence of product p9/p10-2 alone, and could, therefore, be distinguished from lineage IIb, in which the PCR product p9/p10-1 was observed, and from lineage IIe, in which both the PCR products were always observed. In non-*T. cruzi* stocks, we observed either PCR product p9/p10-1, or PCR product p9/p10-2, or no amplification (Table 1).

Primers p7 and p8, derived from the sequence of RAPD fragment F13-e, yielded somewhat more complex PCR amplification results, as five PCR products of different sizes could be distinguished (Fig. 3). Additionally, a sixth PCR product of low intensity was observed in some stocks of lineage IIb, such as Mas1 cl1 (Fig. 3), but the presence of this product was not highly reproducible, and it was, therefore, discarded

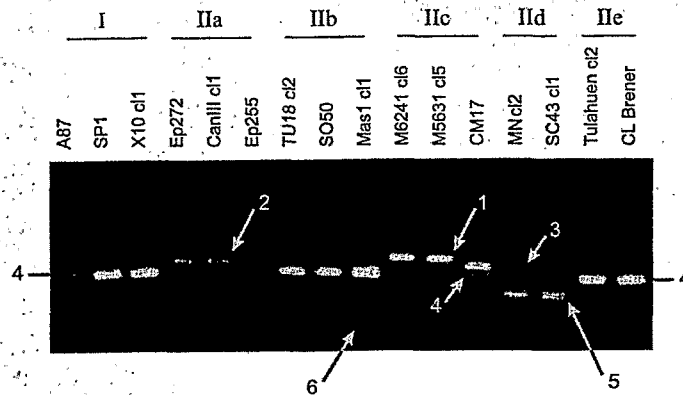p10-2 (approximately 1080 bp), were observed. In stocks of lineage IIe, both products were ob-

Fig. 3. PCR amplifications obtained with primers p7 and p8, derived from the RAPD fragment F13-e. Six p7/p8 PCR products, labelled 1–6, were observed across the stocks. Product p7/p8-1 can be observed in stocks M6241 cl6 and M5631 cl5. Product p7/p8-2 was amplified in stocks of lineage IIa. Product p7/p8-3 was amplified in stocks CM17, MN cl2 and SC43 cl1. Product p7/p8-4 was observed in stocks of lineages I, IIb, and IIe, and in stocks CM17, Ep272 and Ep255. Product p7/p8-5 was amplified in stocks MN cl2 and SC43 cl1. Product p7/p8-6 was observed in stock Mas1 cl1. In addition, products p7/p8-1, p7/p8-5 and p7/p8-6 were observed with low intensity in some stocks of lineage IIe, IIb and IIb, respectively, but were not reproducible in these lineages and therefore not included in the analysis. Sizes of the PCR products are as follows, as revealed by sequencing: p7/p8-1, 474 bp; p7/p8-2, 455 bp; p7/p8-3, 443 bp; p7/p8-4, 410 bp; p7/p8-5, 350 bp; p7/p8-6, 248 bp. Product p7/p8-4 is of equal size to RAPD fragment F13-e (Fig. 1). Distinction of products p7/p8-1 and p7/p8-2 was straightforward in side-by-side control electrophoresis.

from the analysis. The PCR profiles obtained for the 93 stocks were useful for the distinction of the *T. cruzi* lineages (Table 1). All stocks of lineages I and IIe, and most of the stocks of lineage IIb, showed the PCR product p7/p8-4. Interestingly, they could be distinguished from the stocks of lineage IId, characterised by the simultaneous presence of the PCR products p7/p8-3 and p7/p8-5, from lineage IIc (p7/p8-1, or both p7/p8-3 and p7/p8-4 in stock CM17), and from lineage IIa (p7/p8-2, or both p7/p8-2 and p7/p8-4, or no amplification). The *T. cruzi*-like stocks were characterised by the presence of PCR product p7/p8-4, although with a lower intensity than in lineages I, IIb and IIe (A87, Fig. 3). No p7/p8 PCR product was observed in *T. rangeli* and *T. cruzi marinkellei*.

3.3. Molecular characterisation of the PCR products

In order to understand the molecular basis of the size polymorphism of PCR products p9/p10 and p7/p8, the sequence of the different products was established in several stocks (see Note on sequence accession numbers). For both p9/p10 and p7/p8 characterisations, the PCR products of different sizes proved to be homologous and their sequences could be easily aligned, except for insertion/deletions events, to which the size polymorphism could therefore be attributed. A single deletion of 90–97 bp, according to the stocks, was observed in the p9/p10-2 sequence, as compared with the sequence of RAPD fragment U7-e, which corresponds to PCR product p9/p10-1. PCR products p7/p8 differed among them by the presence or absence of ten small insertion/deletions, of a size ranging from 5 to 15 nucleotides, scattered over the entire length of the longest product sequenced, p7/p8-1 from stock M6241 cl6. Additionally, PCR products p7/p8-4 from stocks CL Brener, SP1 and TU18 cl2 proved to have the same pattern of insertions/deletions.

The distribution, in the genome of *T. cruzi*, of sequences corresponding to p3/p6, p9/p10 and p7/p8 PCR products was investigated by southern blotting experiments (Fig. 4). With each RAPD fragment, a number of hybridising bands were observed in the six *T. cruzi* lineages. When the cloned RAPD fragment A10-e was used as probe, two to four hybridising bands were observed, depending on the *T. cruzi* stock analysed, with the three enzymes tested. Differently, when the cloned U7-e RAPD fragment was used as probe, only one hybridising genomic fragment was observed in all lineages after digestion with the restriction enzymes *Kpn*I and *Bam*HI, and only two hybridising bands of equal intensity were observed after digestion with the enzyme *Sma*I (Fig. 4), for which a restriction site was present within the sequence of U7-e RAPD fragment and p9/p10-2 PCR product. Finally, 10–15 genomic fragments showing different intensities were observed after hybridisation with the cloned RAPD fragment F13-e. No open reading frame was found in the sequence of the three RAPD fragments A10-e, U7-e and F13-e, and none of them showed a significant degree of similarity to sequences deposited in public sequence databases.

4. Discussion

4.1. PCR-based identification of the six T. cruzi lineages

The objective of the present study was to develop a PCR-based identification scheme of the six discrete *T. cruzi* genetic lines [20,21]. When they are considered together, the three PCR assays p3/p6, p9/10 and p7/p8 make it possible to assign any *T. cruzi* stock of our test sample to its corresponding lineage, since each combined profile was observed in a unique lineage. This was true even if most PCR products taken individually, showed overlapping distribution over several lineages, since these distributions appeared to complement one another. Indeed, the p3/p6 PCR discriminated between lineages I, IIa and IIb on one hand and lineages IIc, IId and IIe on the other hand, whereas with the p9/p10 PCR, lineage IIe was distinguished from lineages IIc and IId, and lineage IIb was distinguished from lineages I and IIa. Finally, the p7/p8 characterisation allowed, at the same time, the discrimination of lineage I from lineage IIa, and of lineage IIc from
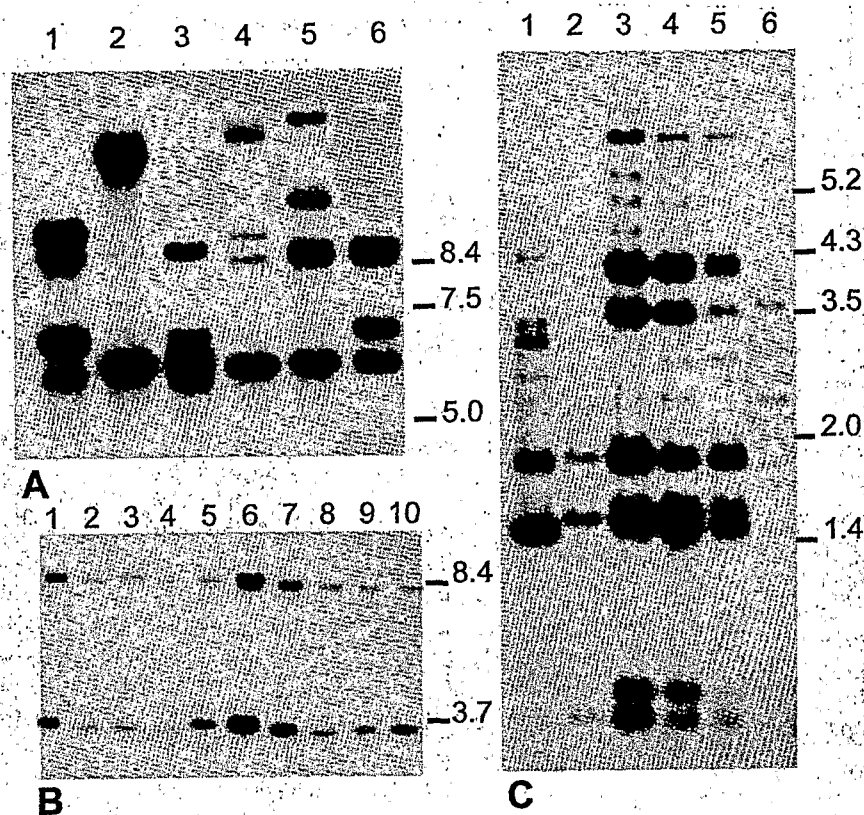
Fig. 4. Hybridisation of the probes corresponding to the three RAPD fragments A10-e, U7-e and F13-e on total DNA of representative stocks of each of the six *T. cruzi* lineages. (A) Hybridisation with probe A10-e on *Bam*HI-digested DNA. The lanes correspond to the following stocks (the lineage to which they belong is given in parentheses): 1, Tehuentepec cl2 (I); 2, CanIII cl1 (IIa); 3, Esmeraldo cl3 (IIb); 4, M6241 cl6 (IIc); 5, MN cl2 (IId); 6, Tulahuen cl2 (IIe). (B) Hybridisation with probe U7-e on *Sma*I-digested DNA: 1, Cuica cl1 (I); 2, Tehuentepec cl2 (I); 3, CanIII cl1 (IIa); 4, Saimiri3 (IIa); 5, Esmeraldo cl3 (IIb); 6, TU18 cl2 (IId); 7, M6241 cl6 (IIc); 8, MN cl2 (IId); 9, Tulahuen cl2 (IIe); 10, CL Brener (IIe). (C) Hybridisation with probe F13-e on *Bsa*WI-digested DNA: 1, X10 cl1 (I); 2, CanIII cl1 (IIa); 3, TU18 cl2 (IIb); 4, MN cl2 (IId); 5, CL Brener (IIe); 6, M6241 cl6 (IIc). Molecular sizes are indicated in kb.

lineage IId. The fact that individual PCR products show an overlapping distribution across the lineages can be explained either by different rates of evolution of individual characters along phylogenetic lines, by convergence or reversion of character states (homoplasy), and/or by genetic recombination, which does not seem to be completely absent in *T. cruzi* [15,16,20,21].

Valuable identification methods for *T. cruzi* lineages should combine sensitivity and ability to discriminate between lineages. Although we did not optimise their sensitivity in the present work, serial dilution experiments showed that the three

PCR assays could be performed with as little as 20 pg of total parasitic DNA, which corresponds approximately to 200 parasite cells.

Currently, one of the most sensitive PCR assays for *T. cruzi* identification relies on amplification of the highly repetitive kinetoplast DNA minicircles [32–34], and combined hybridisation with specific probes can achieve identification of *T. cruzi* groups that correspond to lineage IId and to a subset of stocks belonging to lineage I [35,36]. Kinetoplast DNA probes that would be specific for the other lineages of *T. cruzi* remain to be developed, however. Similarly, the relevance for

lineage identification of other sensitive characterisation methods [37–39] remains to be evaluated. Other valuable T. cruzi identification tests described are PCR characterisation of the 5' end of the 24Sα rRNA genes and mini-exon genes, which achieve distinction of the two major T. cruzi lineages and of some of the smaller lineages within major lineage II [10,13]. However, some reference stocks belonging to lineage IIc such as M6241 cl6, and some stocks of lineage IId, show lineage I PCR profiles based on 24Sα rRNA genes [10], and these characterisation tests do not discriminate between all smaller lineages of lineage II. Therefore, the PCR tests described in the present study represent the best presently available tools for T. cruzi lineage identification.

4.2. Molecular characterisation of RAPD markers

Although RAPD markers have been widely used for characterisation of parasites [6], they have rarely been investigated at the molecular level and to our knowledge, never in T. cruzi. Understanding the molecular basis of RAPD polymorphism is important to interpret it better in genetic and evolutionary studies.

Since, as revealed by the southern blots results, the sequence corresponding to each RAPD fragment was present in all lineages, the PCR amplifications obtained with the SCAR primers provide information on the molecular basis of the initial RAPD polymorphism. Similar to observations made previously [27], the polymorphism obtained after PCR amplification was either retained as the presence or absence of amplification of the band, as was the case with the p3/p6 PCR assay, or appeared as size polymorphism in the p9/p10 and p7/p8 assays. Results with these two last markers show that the RAPD polymorphism of fragments U7-e and F13-e was caused by mismatches in one or a few nucleotides in the priming sites, and that these mismatches were tolerated by the longer SCAR primers. Differently, the RAPD polymorphism of the A10-e fragment must be attributed either to a more extensive sequence divergence of the RAPD priming sites, and/or to divergence of the nucleotide sites of the SCAR primer itself, preventing the SCAR primers to hybridise effi-

ciently to their priming sites. As revealed by the hybridisation experiments, the A10-e sequence was also present in lineages I, IIa and IIb, and using the SCAR primers p3 and p6 at lower annealing temperatures, a p3/p6-1 PCR product was observed also in some stocks outside lineages IIc, IId and IIe (data not shown). These findings indicate that the p3 and p6 priming sites are still present in lineages I, IIa and IIb, although they are modified enough to result in a negative amplification.

Results obtained after southern blot analysis of total DNA with probe U7-e strongly suggest that the U7 sequence is present at a single location in the genome. Therefore, PCR products p9/p10-1 and p9/p10-2, as well as RAPD fragments U7-e and U7-f, may represent two alleles of a single genetic locus, and this locus would thus represent an interesting genetic marker for T. cruzi genetics. Interestingly, the presence of both PCR products in lineage IIe, each of them being individually observed either in lineage IIb or in lineage IIc, is consistent with the hypothesis of a hybrid origin of lineage IIe previously proposed [17,20,21]. Differently, the sequence corresponding to the p7/p8 PCR products probably represent a family of sequences with varying degrees of repetition in the genome of T. cruzi, and which have been subjected to several insertion/deletion evolutionary events. Finally, the sequence corresponding to the A10-e RAPD fragment appears to correspond to a repeated sequence with a low copy number in the T. cruzi genome. In other organisms, RAPD fragments corresponding to either repeated or unique genomic sequences were also described [40].

To our knowledge, this study represents the first in which RAPD fragments are exploited to develop specific PCR diagnostic tools for genetic subdivisions of a unicellular parasite. Many micro-organisms, including protists, fungi and bacteria, prove to be phylogenetically subdivided into discrete genetic lines [41–43], which have been proposed to be called 'discrete typing units' or DTUs [41]. Since specific RAPD fragments are easy to identify in any species of micro-organism and can be obtained without prior sequence knowledge of the organism, the approach de-

scribed here could be easily generalised, either for described species or subspecies, or for phylogenetic lineages of particular interest.

Acknowledgements

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