MAJOR ARTICLE

Multilocus Polymerase Chain Reaction Restriction Fragment–Length Polymorphism Genotyping of *Trypanosoma cruzi* (Chagas Disease): Taxonomic and Clinical Applications

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Background. *Trypanosoma cruzi*, the agent of Chagas disease, is subdivided into 6 discrete typing units (DTUs); their identification is important to understand clinical pleomorphism and track sylvatic DTUs that might (re-)invade domestic foci of the disease and jeopardize the running control programs.

Methods. The genetic polymorphism of 12 loci was analyzed by multilocus polymerase chain reaction restriction fragment–length polymorphism (PCR-RFLP) analysis (MLP analysis) in a sample representative of the diversity within *T. cruzi*. We paid particular attention to genes involved in host-parasite relationships, because these may be prone to polymorphism as an adaptive answer to the immune selective pressure.

Results. The results of MLP analysis were shown to agree with the current multilocus enzyme electrophoresis– and random amplified polymorphic DNA–based classification of *T. cruzi* in 6 DTUs, thereby providing a taxonomic validation of our method. Our data supported hypotheses of genetic recombination within *T. cruzi*. We demonstrated direct applicability of PCR-RFLP analysis to blood of mammal hosts and intestine content of vector insects. Domestic DTUs were encountered in wild animals, and, reciprocally, sylvatic DTUs were encountered in humans, raising questions about changes of transmission patterns.

Conclusions. MLP analysis represents a new alternative to existing molecular methods for *T. cruzi* typing. It might offer an invaluable support to clinical and epidemiological studies and to control programs.

Trypanosoma cruzi is the etiologic agent of Chagas disease, which affects ~20 million people in Latin America [1]. *T. cruzi* has a broad host range and infects wild and domestic mammals, thus giving rise to a large reservoir of parasites. The parasites are transmitted by several species of bloodsucking reduviid bugs as well as through blood transfusion, passage by infected women to offspring, and even organ transplantation. Chagas disease presents a short acute phase followed by a lifelong chronic phase characterized by clinical pleomorphism [2].

Natural populations of *T. cruzi* are characterized by a great genetic diversity, and this led to a series of classification attempts [3–5]. The most recent and commonly accepted classification is based on random amplified polymorphic DNA (RAPD) and multilocus enzyme electrophoresis (MLEE) and distinguishes 2 major lineages (called TCI and TCII, the second lineage being further divided into 5 phylogenetic subdivisions: IIa, IIb, IIc, IId, and IIe). The term "discrete typing unit" (DTU) was adopted to designate a set of stocks that are genetically more similar to each other than to any other stock; the existence of 6 DTUs in total was proposed [5–7]. *T. cruzi* presents a clonal replication mode [8], but it has been hypothesized that the evolutionary emergence of some DTUs could have been the result

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of recombination: (i) with DTU IIa and IIc likely originating from a fusion between ancestral DTU I and IIb strains [9] and (ii) with IId and IIe probably resulting from the hybridization of strains similar to DTU IIb with DTU IIc [7, 9].

Identification of the different DTUs is highly relevant for clinical and epidemiological studies. On the one hand, it allows exploration of the possible link between the genetic diversity of *T. cruzi* and the pleomorphism in severity and symptoms of the disease that is observed in different geographic regions [10–12] and/or chemotherapeutical failure [13]. On the other hand, considering the association of specific DTUs to sylvatic or domestic cycles [1, 6], it permits the molecular tracking of sylvatic *T. cruzi* populations, which might constitute a potential reservoir of reinfestation in the domestic transmission cycle (which is specifically targeted by the currently running control programs). In this context, there is a need for simple, sensitive, and discriminatory molecular methods allowing a direct application in host samples. In *Leishmania,* another Trypanosomatid, we recently showed that polymerase chain reaction multilocus restriction fragment–length polymorphism (PCR-RFLP) analysis (MLP analysis) constituted an adequate answer to this need [14, 15].

The objective of the present study was to apply MLP analysis to 12 gene loci in a sample representative of the diversity of *T. cruzi*. We particularly focused on genes involved in host-parasite relationships (antigens and others) because these may be prone to polymorphism as an adaptive answer to the immune selective pressure [16]. Results were compared with those obtained by RAPD and MLEE, respectively, for taxonomic validation and practical applications for molecular epidemiological studies of Chagas disease were considered. The proof-of-evidence for the direct applicability of our method on host samples was demonstrated.

MATERIALS AND METHODS

Parasite strains and host samples. Twenty parasite strains of *T. cruzi* representative of the 6 major DTUs and 1 strain of *T. cruzi marinkellei* (table 1), all previously typed by MLEE were selected for this study. DNA was isolated from epimastigote cultures as described elsewhere [6].

Blood samples from 10 humans and 21 other mammals (4 *Euneomys chinchilloides,* 4 *Abrothrix olivaceus,* 3 *Thylamys elegans,* 5 *Phyllotis darwini,* and 5 *Capra hircus*) and intestinal contents from 19 of the vector *Mepraia spinolai* were previously obtained in the fourth region of Chile, an area where Chagas disease is hyperendemic. All samples were shown by kDNA amplification [13, 17, 18] to contain *T. cruzi*. Informed consent was obtained from patients or their parents or guardians. Human experimentation guidelines of the Instituut voor Tropische Geneeskunde were followed. Ethics clearance was obtained from the ethics committee of the Facultad de Medicina, Universidad de Chile, Santiago, Chile.

PCR assays. Twelve loci (2 complete [coding and noncod-

Table 1. Stocks of *Trypanosoma cruzi* used in the present study to assess genetic polymorphism.

Sample	Stock	Country	Locality	Host	DTU
1	OPS21 cl11	Venezuela	Macuayas	Human	*T. cruzi* I
2	P209 cl1	Bolivia	Sucre	Human	*T. cruzi* I
3	Florida C1D12	US	Gainesville	*Triatoma sanguisuga*	*T. cruzi* I
4	SP104 cl1	Chile	Combarbalá	*Triatoma spinolai*	*T. cruzi* I
5	CanIII cl1	Brazil	Belém	Human	*T. cruzi* IIa
6	Stc35R	US	St. Catherines Island	*Procyon lotor*	*T. cruzi* IIa
7	Tsang_5	US	Bullooch Co.	*Triatoma sanguisuga*	*T. cruzi* IIa
8	IVV cl3	Chile	Cuncumen	Human	*T. cruzi* IIb
9	MAS1 cl1	Brazil	Brasilia	Human	*T. cruzi* IIb
10	Tu18 cl93	Bolivia	Tupiza	*Triatoma infestans*	*T. cruzi* IIb
11	M5631 cl5	Brazil	Selva Terra, Island of Marajo	*Dasypus novemcinctus*	*T. cruzi* IIc
12	85/847	Bolivia	Alburquerque	*Dasypus novemcinctus*	*T. cruzi* IIc
13	X110/8	Paraguay	Makthlawaiya	*Canis familiaris*	*T. cruzi* IIc
14	RN-PCR-0	Chile	Santiago	Human	*T. cruzi* IId
15	Sc43 cl93 cl1	Bolivia	Santa Cruz	*Triatoma infestans*	*T. cruzi* IId
16	SAXP5b	Chile	Elqui	Human	*T. cruzi* IId
17	MN cl2	Chile	Ilapel	Human	*T. cruzi* IId
18	CL Brener	Brazil	Rio Grande do Sul	*Triatoma infestans*	*T. cruzi* IIe
19	X157/7	Paraguay	Makthlawaiya	*Canis familiaris*	*T. cruzi* IIe
20	VMV4	Chile	Francia	*Triatoma infestans*	*T. cruzi* IIe
21	M1117	Brazil	Para	*Phyllostomus hastatus*	*T. cruzi marinkellei*

NOTE. The molecular characterization of the stocks is based on multilocus enzyme electrophoresis data. DTU, discrete typing unit.

ing] sequences of *kmp11* [19] and *1f8* [20], 9 coding sequences of *TcCRT* [21], *gp72* [22], *Tcgp63-II* [23], *SAPA* [24], *fl-160* [25], *hsp70, hsp60* [26], *cruzipain* [27], *sa85-1* [28], and 1 intergenic sequence of *hsp70*) were selected for the analysis. The target sequences differed in copy number and chromosome location [19–28]. The corresponding sequences were retrieved from GenBank and were aligned using ClustalW (version 1.8; available at: http://www.ebi.ac.uk/clustalw) (table 2). Conserved regions from the aligned sequences were chosen for primer design (table 2). Optimal parameters for primer selection were verified in silico using the software Primer Premier (version 5.0; Premier Biosoft International). Amplification reactions were performed in a final volume of 50 μL containing 1× Taq DNA polymerase buffer, 200 μmol/L of each deoxynucleoside triphosphate, 10 pmol of each primer, 0.5 U of Taq DNA polymerase (Eurogentec), 1.5 mmol/L $MgCl_2$, and 20 ng of template DNA. The reaction mixture was amplified in an MJ Research PTC-100 cycler using the following PCR conditions: *(a)* for *hsp60, hsp70inter, 1f8, SAPA, Tc-CRT, gp72, sa85-1, fl-160,* and *kmp11,* we initially heated the lid at 94°C for 2 min and then ran 33 cycles consisting of denaturation for 30 s at 94°C, annealing for 1 min at 65°C, extension for 2 min at 72°C, and a final extension step of 8 min at 72°C; *(b)* for *hsp70intra, cruzipain,* and *Tcgp63-II,* we used the same PCR conditions as in *(a),* but the annealing temperature was set at 60°C. We used 2 μL of the PCR products and negative controls for the analysis on 2% agarose gel; the rest was stored at 4°C.

PCR-RFLP analysis. After PCR assays, the products were precipitated in ethanol, dried, and resuspended in 10 μL of water for restriction enzyme digestion. The restriction enzymes were chosen using the program Webcutter (available at: http://www.firstmarket.com/cutter/cut2.html); only those enzymes that cut at least 3 times were considered (table 2), as a way to maximize the obtention of different patterns among the distinct *T. cruzi* groups. Different sequences per target gene (as far as possible pertaining to different DTUs) were analyzed for this purpose. The assay was performed in a final volume of 10 μL using 10 U of each restriction enzyme, as recommended by the manufacturer (MBI-Fermentas; New England Biolab), and the reaction was stopped using EDTA (0.5 mol/L [pH 8.0]). Nine microliters of PCR-RFLP products were analyzed by electrophoresis in 3% small fragment agarose gels, and 1 μL was analyzed in microchips (2100 Bioanalyzer capillary electrophoresis system; Agilent Technologies; using Labchips 500, 1500, and 7500). The capillary electrophoresis system offers the advantages of a high-sensitivity, discriminatory power [29], min-

Table 2. Primers and restriction enzymes used for each target gene.

Gene	Accession no.	Oligonucleotide	Sequence (5′→3′)	Fragment size, ~bp	Restriction enzymes	Enzyme for 21-strain analysis
cruzipain	AF265226	CP2*sen* CP3*anti*	ATGGCAGGGTGTACGAGA AGCCGCAGAAGAACTCAA	1100	*Alu* I, *Acy* I, *Bse*LI, *Csp6*I, *MspA1*I, *Hpa*II, *Bsa*JI, *Eco*RII	*Hpa*II
Tcgp63-II	AY266318	GP*sen* GP*anti*	TGCTGCGTCTCTGCTTCTGTT GTTGGTGTAATACTGCTGCCC	1400	*Bse*XI, *Taq*I, *Taq*I, *Hpa*II, *Csp6*I	*Taq*I
1f8	X02838	1F8*sen* 1F8*anti*	CTGGAGTTCCGTCTGATGCTG CAACAAAGTCCTCGGAGCCCT	950	*Bsa*JI, *Hpy8*I, *Taq*I, *Hpa*II, *Cac8*I, *Alw21*I	*Alw21*I
kmp11	AJ000077	KMP*sen* KMP*anti*	CACGCTCTCCCCTGAAATGAA TGCTCTCGTCGGGTTTGTCC	560	*Hpa*II, *Hpy8*I, *Taq*I, *Bse*XI, *Taq*I	*Hpa*II
hsp60	X67473	HP1*sen* HP1*anti*	CGCCGTTTTTCTCAGACACA TTCTCGTTCACCTCCACCTC	1500	*Bsa*JI, *Bse*XI, *Bsh1236*I, *Hae*III, *Alu*I, *MspA1*I, *Taq*I	*Hae*III
hsp70inter	M26595	H70*intsen* H70*intanti*	CAAGAAGACGATTACGAGTGCCG CACGAGTAAGTTGTGCCGAGAT	700	*Alu*I, *Csp6*I, *Hpa*II, *MspA1*I, *Mva*I, *Sau96*I, *Taq*I, *Taq*I	*Csp6*I
hsp70intra	M26595	H70*sen* H70*anti*	GACGGTGCCTGCCTACTTCAA CCGCCCATGCTCTGGTACATC	1400	*Alu*I, *Bse*XI, *Taq*I, *MspA1*I, *BsuR*I, *Bsa*JI, *Cac8*I, *Nla*III	*Bse*XI
SAPA	L38463	TS1*sen* TS1*anti*	ATGTGGACGGGGTGATGGTTG AGAAGTGGGAGATGTCAGGCG	1600	*Alu*I, *Bsh1236*I, *Bsa*JI, *Hpa*II, *Mva*I, *Sau96*I, *Taq*I	*Hpa*II
gp72	M65021	GP72*sen* GP72*anti*	GCGGACAGTGCCAACAACCT CGCCGAACTTCCAACCATCAG	1200	*Alu*I, *Bse*XI, *Bsh1236*I, *Crf13*I, *Hae*III, *Hpa*II, *MspA1*I, *Taq* I	*Taq* I
sa85-1	X53545	SA1*sen* SA4*anti*	CTTGGTAAAGCGGAGGGA ATTCAGTGGGCGGTTGTA	1500	*Bsa*JI, *Csp6*I, *Hae*III, *Hpa*II, *Sau96*I	*Bsa*JI
TcCRT	AF107115	Cal2*sen* Cal2*anti*	GGTTTTACGCCTTGTCTACTGCC CCTCCTTATCACGGTCACCCTTTT	1000	*Bse*LI, *Alu*I, *Hae*III, *Csp6*I, *Hpa*II, *Taq*I, *Taq*I	*Hae*III
fl-160	X70948	Fl*sen* Fl*anti*	GCACTTTTACATTGGTGGGGACG GATGTTGCGTTGGACTCCGATG	890	*Alu*I, *Bsh1236*I, *Csp6*I, *Hae*III, *Hga*I, *MspA1*I, *Hpa*II, *Taq*I, *Bsa*JI	*Hpa*II

NOTE. "*Sen*" refers to the forward primer, and "*anti*" to the reverse primer. The size of the fragment expected in each case was calculated using the available corresponding GenBank sequences.

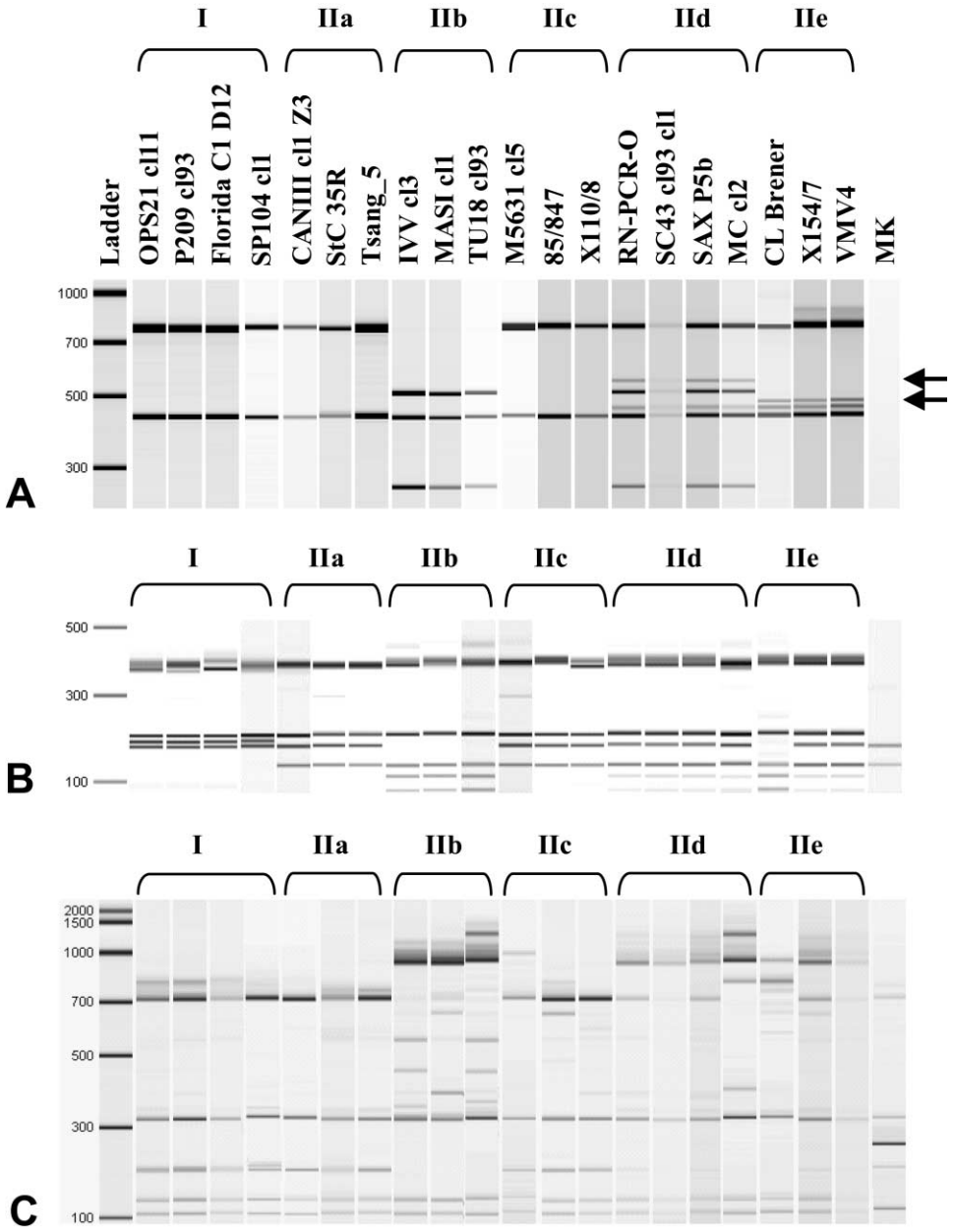

Figure 1. Polymerase chain reaction restriction fragment–length polymorphism analysis by capillary electrophoresis (size in base pairs). *A, gp72* gene, analyzed with *Taq*I. Arrows indicate fragments that distinguish discrete typing unit (DTU) IId from DTU IIe. *Trypanosoma cruzi marinkellei* does not show any amplification of this target. *B, 1f8* gene, analyzed with *Alw21*I. *C, gp63* gene, analyzed with *Tai*I.

imal sample consumption, and speed. All the assays were performed in duplicate to reduce possible errors in data acquisition caused by incomplete digestions. In a first stage, we considered 6 representative *T. cruzi* strains of the 6 DTUs and 1 strain of the out-group *T. cruzi marinkellei* for screening with the selected markers and all the restriction enzymes. For the final MLP analysis, we choose 1 enzyme by marker (with the most differentiation power among the 6 DTUs of *T. cruzi*) and applied the 12 assays to the 21 reference strains (table 2).

RESULTS

PCR-RFLP assays and reference strains. DNA from 21 strains pertaining to the 6 DTUs of *T. cruzi* together with the out-group (*T. cruzi marinkellei*) (table 1) was used for PCR amplification of the 12 target loci. Amplification products corresponded to the expected length, as calculated on the basis of the primer annealing sites in the reported sequences (table 2).

In a first step, PCR amplicons of the 7 *T. cruzi* and *T. cruzi*

Table 3. Polymerase chain reaction restriction fragment–length polymorphism assays evidencing inter–discrete typing unit (DTU) differences.

Gene	Restriction enzyme	DTU I	DTU IIa	DTU IIb	DTU IIc	DTU IId	DTU IIe	*Marinkellei*
1f8	*Alw21*I	**190**, 200	[a]no 80, 140, 180, 200	**80**, **140**, **no 180**, **200**	[a]no 80, 140, 180, 200	[b]80, 140, 180, 200	[b]80, 140, 180, 200	no 80, 140, 180, **no 200**
gp72	*Taq*I	[c]440, 760 (no other fragment)	[c]440, 760 (no other fragment)	**no 760**	[c]440, 760 (no other fragment)	**580**	**480**	Not amplified
hsp70 intra	*Bse*XI	[d]no 310, 330, 400	**310**, 400	[d]no 310, 330, 400	[d]no 310, 330, 400	[d]no 310, 330, 400	[d]no 310, 330, 400	**320**, **no 40**0
hsp70 intra	*Bsp*143 II	[e]120, 330	[e]120, 330	[e]120, 330	**450**	[e]120, 330	[e]120, 330	[e]120, 330

NOTE. Data are sizes in base pairs, unless otherwise indicated. Out of several fragments constituting each restriction pattern (except when indicated), we reported only the diagnostic characters: single fragments or the presence/absence of different fragments. Patterns preceded by the same superscript letter in the same row indicate DTUs with similar characters. DTU-specific features are indicated in bold type.

marinkellei reference strains were cleaved with all the enzymes summarized in table 2 (hereafter referred to as the "7-strain analysis"). After cleavage with restriction enzymes, 1 or more fragments were obtained. In most cases, the sum of the restriction fragments' size was similar to the amplicon's size or was a multiple of it (figure 1*A*). The latter feature is expected for multicopy genes characterized by different sequence variants or for heterozygous genotypes as often observed among hybrid DTUs IId and IIe (figure 1*A*). For some multicopy genes (with a copy number >50) like *SAPA, Tcgp63-II, fl-160, sa85-1,* and *cruzipain,* several bands were generated. To eliminate the possibility that the observed patterns resulted from incomplete digestion, we performed a second test, and we obtained the same results (data not shown). All target genes evidenced polymorphism among the 7 tested strains, but its extent varied according to the target gene and/or the restriction enzyme used. The highest polymorphism was generally observed in target sequences with a copy number >50 and with the enzymes *Bsh1236*I, *Hpa*II, *Hae*III, and *Taq*I. For many markers, the restriction patterns of hybrid DTUs IId and IIe were the superposition of the pattern of their putative parents (IIb and IIc, respectively) (figure 1*B*).

In a next step, the 20 *T. cruzi* strains together with the *T. cruzi marinkellei* reference strain were submitted to PCR-RFLP analysis using the restriction endonucleases showing the highest discriminatory power in the 7-strain analysis. A specific atten-

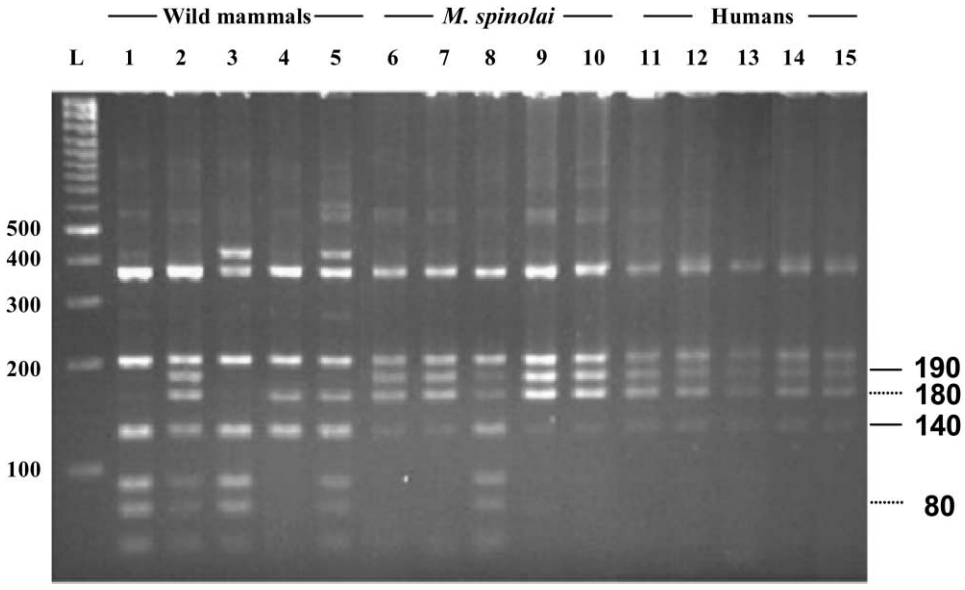

Figure 2. Application of marker *1f8/Alw21*I to host samples. *Lanes 1–5,* blood samples from wild mammals: *Phyllotis darwini, Octodon degu,* and the peridomestic *Capra hircus, Abrothrix olivaceus,* and *Euneomys chinchilloides,* respectively. *Lanes 6–10,* intestinal content of the wild vector *Mepraia spinolai. Lanes 11–15,* blood samples from humans. All the samples originated from the same area in Chile, where Chagas disease is endemic. L, ladder.

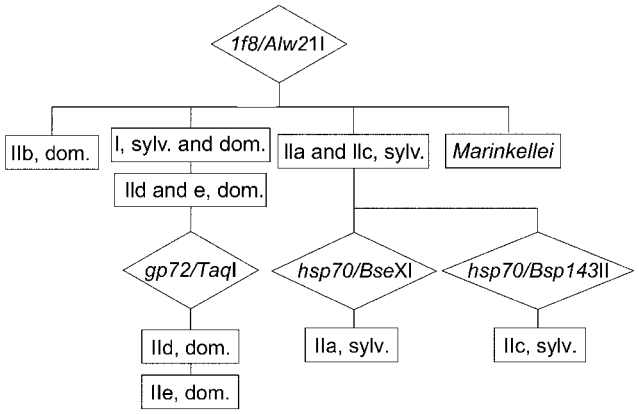

Figure 3. Proposed algorithm for the identification of *Trypanosoma cruzi* discrete typing units, using successive polymerase chain reaction restriction fragment–length polymorphism assays. Domestic (dom.) or sylvatic (sylv.) associations are as described elsewhere [6].

tion was paid to inter- and intragroup differences. On the one hand, several PCR-RFLP assays evidenced patterns that were conserved within a same DTU and differed between different DTUs. The most informative DTU-diagnostic features are summarized in table 3: (i) some single fragments were DTU specific (e.g., the *gp72*/*Taq*I 580- and 480-bp fragments specific to the hybrid DTUs IId and IIe, respectively, which were not observed in their putative parents IIb and IIc) (figure 1*A,* arrows, and table 3, bold) and (ii) some combination of features were unique (e.g., with *1f8*/*Alw21*I, all tested strains of DTUb showed bands of 80, 140, 180, and 200 bp) (figure 1*B* and table 3, bold). A single PCR-RFLP assay allowed typing several DTUs in a "single shot," and, in case of ambiguities, the combination of a second assay (or, if needed, a third one) allowed a complete typing: for example, DTUs IIa and IIc could not be resolved with *1f8*/*Alw21*I (both presenting 140- and 180-bp fragments in the absence of the 80-bp one) (figure 1*B* and table 3) but could be distinguished with *hsp70intra*/*Bse*XI (IIa: 310 bp) (table 3). On the other hand, intra-DTU polymorphism, relevant for a fine molecular tracking of *T. cruzi,* was evidenced with some markers as *gp63* (figure 1*C*), *SAPA, fl-160,* and *sa85-1* cleaved with the restriction enzymes *Tai*I, *Bsh1236*I, *Hga*I, and *Hpa*II, respectively. With the combination of these markers, we can discriminate a specific genotype for most strains.

Direct application of PCR-RFLP analysis in host samples. The clinical applicability of 1 marker (*1f8*/*Alw21*I) was tested in different host samples. As shown in figure 2, mammals (*P. darwini, Octodon degu, C. hircus, A. olivaceus,* and *E. chinchilloides*) revealed the highest diversity of patterns (4 patterns in 5 samples). Some likely corresponded to single infections: IIb (80-, 140-, and 200-bp bands but no 180-bp band) (lanes 1 and 3); IIa or IIc (140-, 180- and 200-bp bands but no 80-bp band) (lane 4); and IId or IIe (80-, 140-, 180-, and 200-bp bands) (lane 5). In the latter case, we cannot exclude a coinfection with IIb, because this would give a pattern similar to the one of IId or IIe. Another mammal contained a mixed infection: mixture of I (190-bp band) and IId or IIe (80-, 140-, 180-, and 200-bp bands) (lane 2). Two patterns were encountered among the *M. spinolai* samples: one was identical to the mammal sample 2 (lane 8; mixture of I and IId or IIe), and the other was identical to the single pattern encountered among humans (lanes 6, 7, 9, and 10, for insects; and lanes 11–15, for humans) and corresponded to a mixture of DTUs I (190-bp band) and IIa or IIc (140-, 180-, and 200-bp bands but no 80-bp band).

DISCUSSION

This study presents the multilocus PCR-RFLP (MLP) analysis of the genetic polymorphism of 12 loci in a sample representative of the diversity within *T. cruzi.* Our results were analyzed in the perspective of the classification of *T. cruzi* but also in terms of practical applications for clinical and epidemiological purposes.

MLP analysis was shown to agree with the current MLEE- and RAPD-based classification of *T. cruzi* in 6 DTUs [5–7]. Several of our PCR-RFLP assays evidenced inter-DTU differences, and, with 1–3 assays, it was possible to type the 6 DTUs. We recommend to start with *1f8*/*Alw21*I and to follow with *gp72*/*Taq*I (discrimination between DTU IId/IIe) or *hsp70*/*Bse*XI/*Bsp143*II (differentiation between DTU IIa/IIc, respectively), according to the results (algorithm in figure 3). Other PCR-RFLP assays revealed intra-DTU differences, useful for a finer parasite genotyping. Last, but not least, we presented the proof-of-evidence for the direct applicability of PCR-RFLP analysis to host samples. Clear patterns were observed with DNA directly extracted from human blood, animal reservoir, and insects, and, with a single assay (*1f8*/*Alw21*I), it was already possible to discriminate sylvatic DTUs from the others. Taken together, these features demonstrate the relevance of MLP analysis to the molecular epidemiological study of Chagas disease. This was already illustrated in the present study by the application of the single marker *1f8*/*Alw21*I to samples originating from the fourth region of Chile, an area where Chagas disease is hyperendemic. A great diversity of patterns was observed among wild animals (figure 2). In 4 of 5 samples, we encountered DTUs generally associated with the domestic transmission cycle in extreme south regions (IIb, IId, or IIe [6]). In contrast, human samples were quite homogeneous, and all showed the same mixed pattern, composed of DTU I (encountered in domestic and sylvatic transmission cycles [6] and DTUs IIa or IIc (both associated with a sylvatic transmission cycle [6]). The vector, *M. spinolai,* clearly showed parasite patterns encountered in humans and in wild animals, suggesting its implication in both transmission cycles. This confirms previous studies indicating that although *M. spinolai* is fundamentally a sylvatic

vector, it may approach human settings, feeding on blood of peridomestic mammals and in some cases of humans, thus becoming a potentially dangerous species in *T. cruzi* transmission [30].

The observation of "domestic" DTUs in wild animals and, reciprocally, of "wild" DTUs in humans raises questions on the changes in transmission patterns of *T. cruzi*. This phenomenon should be verified by a more-extensive study, but, if it were confirmed, it could jeopardize the currently running control programs focusing on the domestic vector.

The finding of coinfection of vertebrate and invertebrate hosts with different DTUs constituted another interesting result. This phenomenon was already documented in previous studies in Chile (42% of mixed infections among humans [31]) and Bolivia (22%–35% in *T. infestans* [32]). Experimental studies showed that mixed *T. cruzi* infections might have a great impact on the biological properties of the parasite in the host, emphasizing the importance of considering the possible occurrence of natural mixed infections in humans and their consequences on the biological aspects of ongoing Chagas disease [33]. PCR-based methods are most adequate to document these mixed infections, because strain selection was shown to occur through laboratory manipulation of initially mixed populations of *T. cruzi* [34, 35].

In recent years, a series of new genotyping methods were reported for epidemiological monitoring of Chagas disease. Sequence-characterized amplified region markers analysis allowed identification of the different DTUs when 3 different markers were applied together, but, to our knowledge, it was not evaluated in clinical samples [5]. More recently, real time PCR was applied to genotyping in chronically infected human tissues but could only differentiate the 2 main lineages, *T. cruzi* I and *T. cruzi* II [36]. A main complication of *T. cruzi* typing is the frequent occurrence of mixed infections involving different DTUs [13, 31]. In many cases, molecular methods allow to identify the composition of the mixture, except when the combination of the genetic features of the mixture is the same as that of hybrid genotypes like IId and IIe. In that case, some specific bands observed only in hybrid genotypes and not in their putative parents (e.g., *gp72*/*Taq*I) revealed that our method could resolve these ambiguities.

The variation of symptoms in Chagas disease remains unexplained. Peculiar differential geographical patterns of variation were described by Dias [12]. This geographical heterogeneity suggests that genetic variation of the host, the parasite, or both is important in establishing the clinical type of the disease [37, 38]. Considering the immunopathological characteristics of Chagas disease [2], the battery of genes here selected—most of them being involved in host-parasite relationships—might allow exploring in the future the possible association between parasite diversity and clinical pleomorphism. This is supported by a preliminary phenetic analysis done with the 7 strains and all restriction enzymes, because this suggests that different genetic information might become available when considering polymorphism of each gene separately (different topology of single-gene trees; authors' unpublished data) An adequate, well-documented clinical sample should be constituted, MLP analysis should be performed with more restriction enzymes, and we would recommend to apply univariate as well as multivariate analysis for testing any association with a given clinical form.

In conclusion, MLP analysis represents a new alternative to existing molecular methods for *T. cruzi* typing. It is simple, allows discrimination at various levels (DTUs and strains), and may be directly applied to host tissues. Our method might thus offer an invaluable support to epidemiological studies and control actions, particularly to track the emergence of new transmission cycles that might jeopardize control programs.

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