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Probing Population Dynamics of *Trypanosoma cruzi* during Progression of the Chronic Phase in Chagasic Patients[▽]

Daniella Alchaar D'Ávila,[1] Andréa Mara Macedo,[2] Helder Magno Silva Valadares,[2] Eliane Dias Gontijo,[3] Ana Maria de Castro,[5] Carlos Renato Machado,[2] Egler Chiari,[1] and Lúcia Maria Cunha Galvão[1,4]*

Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil[1]; Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil[2]; Departamento de Medicina Preventiva e Social, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil[3]; Programas de Pós-Graduação em Ciências da Saúde e Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal do Rio Grande do Norte, Natal, Brazil[4]; and Departamento de Microbiologia, Imunologia, Parasitologia e Patologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil[5]

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Our research aimed to characterize the genetic profiles of 102 *Trypanosoma cruzi* isolates recently obtained from 44 chronic chagasic patients from different regions of the states of Minas Gerais and Goiás in Brazil. At least two isolates were obtained from each patient at different times in order to study the parasite population dynamics during disease progression in the chronic phase. The isolates were characterized molecularly by genotyping the 3′ region of the 24Sα rRNA, the mitochondrial cytochrome oxidase subunit 2 (COII) gene, and the intergenic region of the spliced leader intergenic region (SL-IR) gene. Seventy-seven isolates were analyzed for nine microsatellite loci. The data presented here show a strong correlation between the *T. cruzi* lineage II (*T. cruzi* II) and human infection in these regions of Brazil. Interestingly, isolates from two patients were initially characterized (by rRNA genotyping) as *T. cruzi* I and hybrid strains, but subsequent analyses of the COII and SL-IR genes confirmed that those isolates belonged to *T. cruzi* III and a hybrid group, respectively. Our results confirm the risk of misclassifying *T. cruzi* isolates on the basis of analysis of a single molecular marker. The microsatellite profiles showed that different isolates obtained from the same patient were genetically identical and monoclonal. Exceptions were observed for *T. cruzi* isolates from two patients who presented differences for the SCLE11 locus and also from two other patients who showed amplification of three peaks for a microsatellite locus (TcAAAT6), implying that they were multiclonal. On the basis of the findings of the studies described here, we were not able to establish a correlation between the clinical forms of Chagas' disease and the genetic profiles of the *T. cruzi* isolates.

The protozoan *Trypanosoma cruzi* is both phenotypically and genotypically heterogeneous. Strains of *T. cruzi* exhibit large differences in growth rate, histotropism, antigenicity, pathogenicity, their infectivity of potential insect vectors, drug susceptibility, and DNA content. *T. cruzi* causes Chagas' disease, which is characterized by a variable clinical course that ranges from the absence of symptoms to severe disease with cardiac involvement and/or digestive tract damage. This variability has been attributed both to differences in the host response and to the genomic heterogeneity of the parasite (2, 7, 12, 23, 25, 27, 31, 33, 34, 42, 44).

T. cruzi is a diploid organism. Its genome has recently been sequenced; however, most aspects of its population structure and evolution remain to be elucidated (17). Despite their high degrees of genetic variability, *T. cruzi* isolates can be classified into two major phylogenetic lineages: *T. cruzi* lineage I (*T. cruzi* I) and *T. cruzi* II (4). These lineages are very divergent, as revealed by several biological and molecular markers, including isoenzymes, 24Sα rRNA, the miniexon gene, and microsatellite polymorphisms, among others (4, 10, 35, 36, 39, 42, 45, 50).

There are two different transmission cycles for *T. cruzi.* The sylvatic cycle involves wild triatomine species and nonhuman vertebrate hosts. The domestic cycle involves home-dwelling triatomines and primarily humans and household animals. In the Southern Cone, *T. cruzi* I and *T. cruzi* II strains predominantly belong to distinct ecological environments: the sylvatic and domestic transmission cycles of Chagas' disease, respectively (8, 49). In Brazil, *T. cruzi* II strains appear to be responsible for tissue lesions in Chagas' disease, while human cases of infection caused by *T. cruzi* I strains are rare and are usually asymptomatic (8, 20, 50). However, growing evidence has revealed that *T. cruzi* I is more associated with clinical cases of Chagas' disease than was initially realized, especially in the northern part of South America (3, 15, 41). Recently, the existence of a third ancestral lineage, *T. cruzi* III, was proposed, but the epidemiological importance of these parasites in humans remains to be determined (21).

Many research groups have tried to correlate the genetic variability of the parasite with the clinical manifestation of the disease, but they were unsuccessful (6, 16, 22, 23, 26, 49). In

* Corresponding author. Mailing address: Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627 Caixa Postal 486, 31270-901, Belo Horizonte, Minas Gerais, Brazil. Phone: 55-31-34092866. Fax: 55-31-34092970. E-mail: address: galvao@icb.ufmg.br.

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FIG. 1. Map of Brazil showing the different regions of the country. The MG and GO states (patient recruitment areas) are located in the southeast and central west regions of Brazil, respectively.

light of the histotropic-clonal model proposed for Chagas' disease in the beginning of the 1980s, at least two explanations can be devised. (i) With regard to the hypothesis of *T. cruzi* subpopulation selection, vertebrate hosts (particularly humans) may act as selective biological filters as the host immune response selects a subset of the parasitic clones present in the original infecting parasite population. In addition, tissue tropism can differ between parasitic clones. Therefore, parasites isolated from patients' blood and maintained under laboratory conditions for analyses can differ from those parasites directly involved in the specific tissue lesions (1, 2, 22, 27, 30, 33, 36, 43). (ii) Different *T. cruzi* life-cycle stages are represented in an infected host. At the time of parasite isolation from host blood, one particular clone could predominate over others, resulting in the isolation of an underrepresentative parasite population (13).

In order to address the last possibility, we analyzed the genetic profiles of 102 *T. cruzi* populations isolated on different occasions from 44 chronic chagasic patients from two different regions of endemicity of the states of Minas Gerais (MG) and Goiás (GO) in Brazil.

MATERIALS AND METHODS

Origin and clinical evaluation of patients. In total, 102 *T. cruzi* isolates were obtained from chronic chagasic patients in different regions of endemicity of the states of MG and GO, located in the southern and central parts of Brazil, respectively (Fig. 1). At least two isolates were obtained from each patient at different times in order to study parasite population dynamics during clinical disease progression in the chronic phase. Among these isolates, 83 were obtained from 35 patients from the state of MG and 19 were obtained from 9 patients from the state of GO. All patients included in this study were selected by use of the following criteria: positive results by at least two serological tests performed by using different techniques and clinical examination with two-view chest X rays, 12-lead electrocardiogram (ECG), and Doppler echocardiogram. Patients with other heart conditions were excluded. The clinical classification of the patients was based on the Chagas' Disease Consensus (2005) (5).

Twenty-seven patients (61%) were classified as having the indeterminate clinical form of the infection by a series of tests (the presence of specific immunoglobulin G antibodies; either positive or negative results by the parasitological methods; the absence of ECG abnormalities; and regular heart, esophagus, and colon sizes, as determined by X ray). Seventeen patients (39%) had the cardiac clinical form of Chagas' disease on the basis of measurements of heart function by using ECG and radiographs as well as Doppler echocardiography. In six patients, *T. cruzi* was isolated before and after specific treatment. Information on the geographic origin, age, sex, and clinical form of disease for each patient and on the *T. cruzi* isolates recovered from each patient are given in Table 1. This work and all procedures were carried out with the informed consent of the participants and was approved by two independent research ethics committees: those from the Universidade Federal de Minas Gerais (087/99), Belo Horizonte, MG, and the Hospital São Salvador, Goiânia, GO, Brazil.

Isolation and culture of *T. cruzi*. A total of 102 *T. cruzi* isolates were obtained by hemoculture from 44 chronic chagasic patients during the period from 1996 to 2006 (14). The patients were examined, and their blood was collected for parasite isolation by hemoculture more than once. We found a blood culture positivity rate of 66% (102/154 patients) for the patients examined at different times. Therefore, the letters in the isolate number indicate the number of attempts to isolate the organism that were made. In order to minimize parasite selection, the cultures were maintained in individual tubes in the laboratory for a short period of time and the parasites were cultivated by two successive passages in liver infusion tryptose medium. The cultures were washed three times in Krebs Ringer-Tris, pH 7.3, by centrifugation at 2,000 × *g* for 15 min at 4°C and were then stored at −20°C for DNA extraction. Genomic DNA from the cultured parasites was obtained by the phenol-chloroform method and was used as the template for the PCR assays (27).

Microsatellite PCR assay. In the present study, nine microsatellite loci, identified as SCLE10, SCLE11, MCLE01, MCLF10, MCLG10, TcTAT20, TcAAT8, TcATT14, and TcAAAT6, were amplified by PCR (36, 45). The PCRs were performed as described previously (45).

Allele sizes. To determine the allele sizes, 1 to 3 μl of each of the PCR fluorescent products was analyzed in the 6% denaturing polyacrylamide gel of an automated laser fluorescent DNA sequencer (GE Healthcare, Milwaukee, WI), and the fragments were compared to fluorescent DNA fragments of 50 to 500 bp by using Allelelocator software (GE Healthcare) to determine their sizes.

24Sα rRNA gene amplification. Amplification of the D7 domain of the 24Sα rRNA gene was achieved by PCR with primers D71 (5′-AAGGTGCGTCGAC AGTGTGG-3′) and D72 (5′-TTTTCAGAATGGCCGAACAGT-3′) by following the protocols described previously (39).

Polymorphism analysis of the mitochondrial COII gene. Amplification of the mitochondrial cytochrome oxidase subunit 2 (COII) gene was performed with primers Tcmit-10 (5′-CCATATATTGTTGCATTATT-3′) and Tcmit-21 (5′-TT GTAATAGGAGTCATGTTT-3′), designed to amplify a fragment of 375 bp from *T. cruzi* maxicircle DNA (21). Each PCR was performed with a mixture with a total volume of 20 μl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100 (10× Buffer I0; Phoneutria, Belo Horizonte, MG, Brazil), 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Phoneutria), 250 μM of each deoxynucleoside triphosphate, 0.3 μM of each primer, and 3 μl of DNA template (3 ng/μl) covered with mineral oil. Amplification was performed in a PTC-100 thermocycler (MJ Research) by using the following protocol: 5 min denaturation at 95°C and then 40 cycles of 45 s denaturation at 95°C, annealing for 45 s at 48°C, and primer extension for 1 min at 72°C. On the basis of the restriction map of COII sequences, the AluI restriction endonuclease was chosen for use in restriction fragment length polymorphism (RFLP) analysis of the mitochondrial COII gene. After PCR amplification, the amplicons were subjected to enzyme digestion for 16 h, according to the instructions provided by the manufacturer (Promega). The digested products were analyzed by silver staining, after separation on 6.0% polyacrylamide electrophoresis gels.

Amplification of the intergenic region of SL-IR genes. The amplification of the spliced leader intergenic region (SL-IR) genes was achieved with primers TcIII forward (5′-CTCCCCAGTGTGGCCTGGG-3′) and UTCC reverse (5′-CGTA CCAATATAGTACAGAAACTG-3′). The PCR was performed in a total volume of 15 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 3 μM of each primer, 0.5 U *Taq* DNA polymerase platinum (Invitrogen, Carlsbad, CA), and 1 μl of total DNA (3 ng/μl). The PCR strategy with the SL-IR gene was devised to distinguish populations belonging to *T. cruzi* III (amplicons of 200 bp) from *T. cruzi* I, *T. cruzi* II, and hybrid strains, which present fragments of approximately 150 to 157 bp

TABLE 1. Geographic origin, sex, age, clinical forms, specific treatment. and characteristics of *Trypanosoma cruzi* isolates from 44 chronic chagasic patients from MG and GO states in Brazil[a]

Isolate no.[b]	Birth locality	Sex	Age (yr)	Date (day/mo/yr) of HC and mo after first isolation	Clinical form	Specific time of treatment (day/mo/yr) and mo AT
003a* 003b**	Janaúba, MG	Female	27	08/07/1997 10 mo	Indeterminate	20/09/1997 8 mo
005a 005b	Teófilo Otoni, MG	Male	39	24/09/1996 18 mo	Indeterminate	01/07/1998
012a* 012b** 012g**	Santana de Pirapama, MG	Male	51	30/09/1997 7 mo 62 mo	Indeterminate	04/04/1998 1 mo 59 mo
013a 013c 013e	Piranga, MG	Female	38	29/10/1997 42 mo 73 mo	Indeterminate	—/10/04
019a* 019c** 019e**	Carbonita, MG	Male	45	24/06/1997 53 mo 99 mo	Cardiac	26/10/1997 49 mo 95 mo
029a 029b 029c	Itaipé, MG	Female	33	12/03/1999 7 mo 33 mo	Indeterminate	
044a 044d	Turmalina, MG	Male	33	17/09/1996 93 mo	Indeterminate	
045a 045c	Coração de Jesus, MG	Female	27	17/06/1998 30 mo	Indeterminate	
050b* 050f** 050h**	Claro de Poções, MG	Female	39	01/04/1998 58 mo 84 mo	Indeterminate	08/04/1998 58 mo 84 mo
053a 053d 053e	Januária, MG	Male	25	17/06/1997 62 mo 90 mo	Indeterminate	
058a 058c 058d	Novo Cruzeiro, MG	Male	44	05/08/1998 73 mo 83 mo	Cardiac	
065b 065c 065d	Felixlândia, MG	Female	46	05/12/2000 16 mo 57 mo	Indeterminate	
079a 079d	Hematita, MG	Female	54	24/06/1997 58 mo	Cardiac	
083a 083c	Ferros, MG	Female	49	15/04/1998 67 mo	Cardiac	
090a 090b	Montes Claros, MG	Female	54	22/04/1998 30 mo	Indeterminate	
092a 092b 092c	Teófilo Otoni, MG	Female	35	28/05/1999 12 mo 30 mo	Cardiac	
094a 094b	Alvorada de Minas, MG	Female	31	25/06/1999 22 mo	Cardiac	
103a 103c	Santa Maria Suaçuí, MG	Female	37	06/05/1998 59 mo	Indeterminate	
105a 105b 105d	São Francisco, MG	Female	26	12/11/1997 23 mo 56 mo	Indeterminate	
109a 109b	Novo Cruzeiro, MG	Female	44	16/04/1999 49 mo	Indeterminate	
111a 111c 111d	Araçuaí, MG	Female	43	19/08/1998 57 mo 73 mo	Indeterminate	

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TABLE 1—*Continued*

Isolate no.[b]	Birth locality	Sex	Age (yr)	Date (day/mo/yr) of HC and mo after first isolation	Clinical form	Specific time of treatment (day/mo/yr) and mo AT
115b 115c	Brasília de Minas, MG	Female	33	12/04/2000 34 mo	Indeterminate	
116a* 116b* 116e* 116f**	Joaquim Felício, MG	Female	20	23/04/1999 14 mo 56 mo 84 mo	Indeterminate	 03/03/2004 25 mo
120a 120b	Porteirinha, MG	Male	30	15/04/1998 28 mo	Indeterminate	
132a 132d	Planalto de Minas, MG	Male	42	24/06/1997 70 mo	Indeterminate	
138a 138c	Teófilo Otoni, MG	Female	22	24/06/1998 75 mo	Indeterminate	
146a 146b	Itamarandiba, MG	Male	30	01/10/1996 8 mo	Indeterminate	
154a 154c	Itaipé, MG	Male	38	12/04/2000 42 mo	Indeterminate	
162a 162b	Congonhas do Norte, MG	Female	35	04/10/2000 37 mo	Indeterminate	
038 045	Pains, MG	Female	53	10/12/1996 4 mo	Indeterminate	
012B $012B_2$	Capelinha, MG	Female	56	13/09/2005 12 mo	Cardiac	
016B $016B_2$	Santana de Pirapama, MG	Female	29	20/09/2005 12 mo	Cardiac	
024B $024B_2$	Carbonita, MG	Male	49	04/10/2005 12 mo	Cardiac	
010 079	Ubaí, MG	Female	33	14/09/1998 3 mo	Cardiac	—/09/1999
177 205	Matipó, MG	Male	42	18/05/1999 2 mo	Indeterminate	—/11/1999
157 187 384	Cabeceiras, GO	Female	40	29/03/1999 2 mo 26 mo	Indeterminate	
038 063	Fazenda Nova, GO	Male	50	05/10/1998 2 mo	Cardiac	30/04/1999
001 074	Itumbiara, GO	Male	75	09/09/1998 3 mo	Indeterminate	
021C 044	Formosa, GO	Male	57	17/09/1998, 2 mo	Cardiac	—/08/1999
008 061	Córrego do Ouro, GO	Male	45	11/09/1998 2 mo	Cardiac	—/04/1999
006 147B	Campestre de Goiás, GO	Female	39	10/09/1998 6 mo	Cardiac	—/04/1999
036 059	Anícuns, GO	Female	50	01/10/1998 2 mo	Cardiac	—/10/2000
145 186	Itapuranga, GO	Female	59	25/02/1999 3 mo	Cardiac	—/12/99
160* 399**	Nova Veneza, GO	Male	25	31/03/1999 27 mo	Cardiac	—/01/2000 17 mo

[a] HC, hemoculture; AT, after treatment; mo, number of months after first isolation and specific treatment; —, day unknown.
[b] At least two *T. cruzi* isolates were obtained from the same patient before (*) and after (**) specific treatment.

(10). The PCR products were analyzed by electrophoresis in 6.0% polyacrylamide gels, and the DNA fragments were visualized by silver staining.

Construction of phylogenetic trees. To make inferences from phylogenetic tree, we assumed a stepwise mutation model for microsatellite evolution and used the minimum number of mutational steps necessary to transform one genotype into the other as a measure of the genetic distance between any two strains (37, 46). The microsatellite multilocus patterns were transformed into characters of 0 and 1 by using the FACTOR program from the PHYLIP (Phylogeny Inference Package) software package, version 3.67 (18, 19). These data were then used to construct unrooted Wagner parsimony trees by using the MIX program, also from the PHYLIP software package. The significance levels of the branching in the Wagner network were determined by bootstrapping (1,000 reiterations) with the program SEQBOOT from the PHYLIP software package. The relationships between the *T. cruzi* isolates were estimated by the use of phenograms representing microsatellite data. Additionally, we generated a genetic distance matrix from the previous data that had been transformed into 0's and 1's and submitted it to processing with the Treecon software program for Windows (47). In this case, the phenograms were constructed by the unweighted pair group method with arithmetic averages (UPGMA). The bootstrap option was used to run 1,000 replicates to obtain confidence estimates for the grouping and taxonomic relatedness (18, 19).

RESULTS

24Sα rRNA polymorphism and mitochondrial genotypes of isolates. A total of 102 *T. cruzi* isolates were analyzed by genotyping of both the 24Sα rRNA and the mitochondrial COII genes. The analyses of the genetic profiles of the *T. cruzi* isolates obtained from the same patient at different times showed a good correlation between these markers. The majority of the *T. cruzi* populations analyzed (98/102) belonged to rDNA group 1 and COII haplotype C, corresponding to *T. cruzi* II (domestic transmission cycle). However, four isolates obtained from two patients, one from MG (isolates 177 and 205) and one from GO (isolates 006 and 147B), were classified as rDNA type 2 and 1/2, respectively (Fig. 2A). Genotyping of the mitochondrial COII genes from these isolates showed that they belonged to haplogroup B, which is related to *T. cruzi* III or hybrid strains (Fig. 2B).

Genotyping of SL-IR genes of *T. cruzi* isolates. We also profiled the *T. cruzi* SL-IR genes or miniexon in order to separate isolates of *T. cruzi* III from those associated with *T. cruzi* I, *T. cruzi* II, or the hybrid group. The majority of the *T. cruzi* populations analyzed (16/20) presented PCR amplification products of 150 to 157 bp, compatible with previously identified *T. cruzi* II major lineages (rDNA group I and COII haplotype C). On the other hand, analysis of the SL-IR gene showed that two isolates from a patient from GO (isolates 006 and 147B, which were COII haplotype B) were related to hybrid strains (amplicons of 150 to 157 bp), while two isolates from a patient from MG (isolates 177 and 205, which were COII haplotype B) were associated with *T. cruzi* III (amplicons of 200 bp) (Fig. 2C).

Microsatellite analyses. PCR strategies targeted to polymorphic microsatellite loci were carried out to address the population composition of the *T. cruzi* isolates analyzed in this study. Seventy-seven isolates were typed for nine microsatellite loci, and typing resulted in the amplification of one (interpreted as homozygous) or two (interpreted as heterozygous) allele peaks for most isolates (data not shown). The microsatellite profiles revealed that the parasite populations isolated from the same patient on different occasions were genetically identical and monoclonal (with amplified alleles of identical sizes), demonstrating the high degree of homogeneity of these

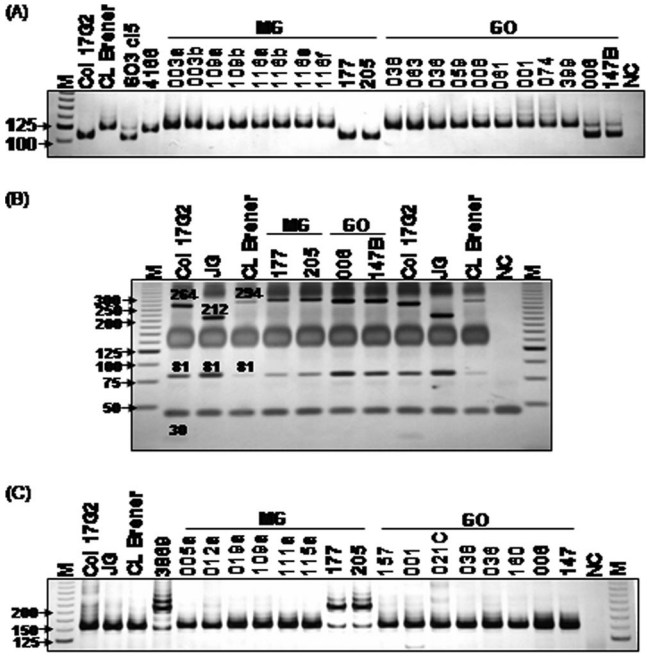

FIG. 2. (A) Representative profiles of 24Sα rRNA of *T. cruzi* isolates from chronic chagasic patients obtained by polyacrylamide gel electrophoresis. Controls were clone Col1.7G2 (amplicon of ~110 bp); rDNA 2 (*T. cruzi* I), consisting of DNA from the *T. cruzi* CL Brener clone (amplicon of ~125 bp); rDNA 1 (*T. cruzi* II), consisting of DNA from the SO3 Cl5 clone (amplicons of 125 and 110 bp), and rDNA 1/2 and strain 4166 (amplicon of ~117/119 bp; associated with strains belonging to zymodeme 3). (B) RFLP analysis of the mitochondrial COII gene in the *T. cruzi* isolates belonging to haplogroup B. Digestion of the DNA with AluI generates three RFLP patterns for the *T. cruzi* strains: restriction fragments of 264, 81, and 30 bp are classified as *T. cruzi* I (mitochondrial haplotype A; control, Col1.7G2 clone), restriction fragments of 212 and 81 bp are classified as *T. cruzi* II (mitochondrial haplotype C; control, strain JG), and restriction fragments of 294 and 81 bp are classified as *T. cruzi* III or hybrid (mitochondrial haplotype B; control, CL Brener clone). (C) The SL-IR gene was used to separate isolates from *T. cruzi* III from those associated with *T. cruzi* I and II and the hybrid group. Controls were the Col1.7G2 clone, strain JG, and the CL Brener clone (fragments of 150 to 157 bp for *T. cruzi* I and II and the hybrid group) and strain 3869 (fragment of 200 bp, associated with strains belonging to *T. cruzi* III). Lanes M, molecular size marker; lanes NC, negative PCR control. Numbers to the left of the gels are in base pairs.

populations, at least at the nuclear genomic level. Exceptions were seen for two isolates from patient 109 (isolates 109a and 109b) and four isolates from patient 116 (isolates 116a, 116b, 116e, and 116f), both from MG, that presented different alleles for the SCLE11 locus (Fig. 3A). These data suggest that the patients were infected with isolates from more than one parasite population. Furthermore, two isolates from patient 115 (isolates 115b and 115c) and three isolates from patient 111 (isolates 111a, 111c, and 111d), both of whom were also from MG, amplified three fragments of different sizes for a single microsatellite locus (TcAAAT6). These data are indicative of polyclonal populations, although we cannot rule out aneuploidy for this locus (Fig. 3B).

Phylogenetic inferences from microsatellite genotyping. From among the 77 *T. cruzi* isolates typed for the nine microsatellite loci, we selected 32 (1 isolate from each patient) to be

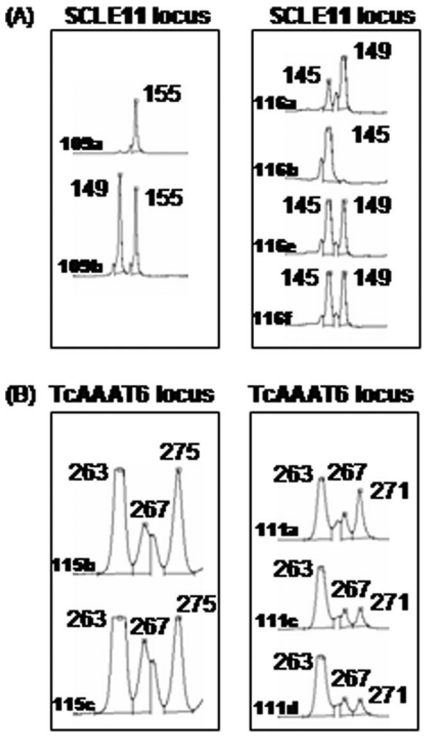

FIG. 3. Representative automated laser fluorescent DNA sequencer electrofluorograms. (A) Microsatellite profiles of *T. cruzi* isolates from the same patient showing differences in the SCLE11 locus; (B) electrofluorogram of microsatellite alleles of *T. cruzi* isolates showing the presence of three peaks for the microsatellite locus TcAAAT6.

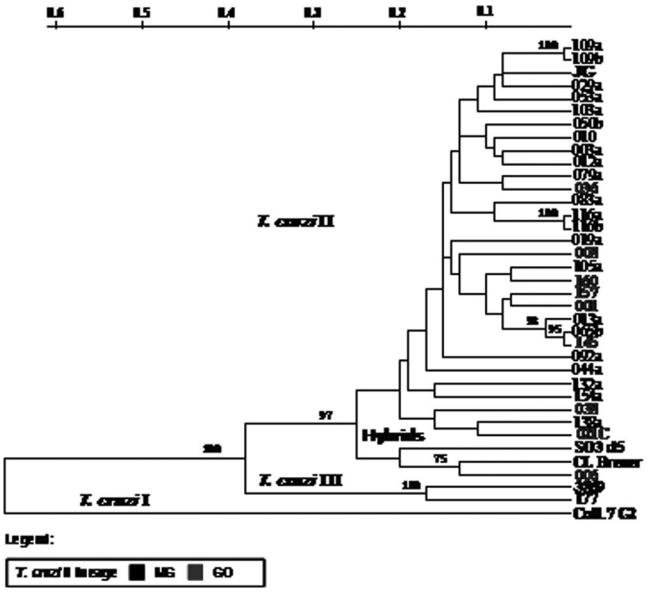

FIG. 4. UPGMA phenogram of 36 *T. cruzi* isolates based on the amplification of nine microsatellite loci. The numbers at the forks indicate the number of times that the branch was observed in 1,000 bootstraps. Bootstrap values (in percent) of less than 70 are not shown.

submitted to maximum-parsimony phylogenetic inferences. For this analysis, we excluded the five isolates from patients 115 (isolates 115b and 115c) and 111 (isolates 111a, 111c, and 111d) because of their multiallelic microsatellite genotypes (Fig. 3B). The well-known SO3 cl5, COL1.7G2, and CL Brener clones and strains JG and 3869 were added to this analysis for comparison. As expected, the microsatellite genotypes of the *T. cruzi* isolates were distant from each other, but despite this feature, we were able to identify one major group (*T. cruzi* lineage II) that strictly correlated the isolates. Isolate 006 (a putative hybrid isolate) was grouped with the hybrid SO3 cl5 and CL Brener clones. Isolate 177, identified here as being related to *T. cruzi* III, was grouped together with strain 3869 (the *T. cruzi* III control strain) and the COL1.7G2 clone (*T. cruzi* I) (data not shown). A very similar topology was obtained with the UPGMA algorithm (Fig. 4). The main difference was that in this tree, isolates 177 and 3869 and clone COL 1.7G2 were set apart. The tree topologies obtained from the microsatellite data for the 32 *T. cruzi* populations did not indicate any specific parasite association in relation to clinical forms or the geographic distribution, sex, or age of the patients.

DISCUSSION

We analyzed the genetic profiles of 102 isolates of *T. cruzi* from 44 chronic chagasic patients with well-characterized clinical profiles. The majority of the patients examined (61%) were defined as having the indeterminate clinical form of Chagas' disease, and 39% were defined as having the cardiac form of Chagas' disease. Isolates of the *T. cruzi* II major lineage were detected in most (42/44) of the patients. Thus, our results are in agreement with previously published data from other areas of Brazil where Chagas' disease is classically endemic, where *T. cruzi* II is mainly associated with severe chronic human infections and is basically the only lineage detected directly in chagasic patients with cardiac and megaesophageal lesions (20, 50).

In contrast to the results obtained in Brazil and Argentina, *T. cruzi* I has been implicated as the major group associated with Chagas' disease in countries like Colombia, Peru, and Venezuela and in Central American countries (3, 24, 32). In Brazil, this lineage has been identified in a few isolates from patients with symptomatic chronic Chagas' disease, particularly those from the Brazilian Amazon, where the illness has been considered emergent and where the illness is also caused by the oral transmission of the parasite (15, 41, 50). However, we cannot discount the possibility that other *T. cruzi* lineages may also be infectious and able to cause illness in humans. In the present work, we found one case caused by *T. cruzi* III and one case caused by a hybrid. These strains were isolated from two chronically infected patients presenting with the indeterminate and cardiac clinical forms, respectively. Moreover, the presence of *T. cruzi* III and hybrid strains has been demonstrated in severe congenital chagasic cases in Argentina and in humans with clinical manifestations of the disease in Brazil (10, 21, 45).

Our findings also demonstrate the necessity of using several molecular markers for the accurate classification of *T. cruzi* populations. Solely on the basis of the dimorphism of the 24sα rRNA gene, for example, one of our isolates from MG could be classified under the *T. cruzi* I major lineage. However, by taking the results of the COII and SL-IR gene analyses to-

gether, we found that this isolate belongs to the *T. cruzi* III group. Other authors have previously demonstrated that the characterization of the *T. cruzi* lineages on the basis of a single marker (either the miniexon or the 24Sα rRNA gene) yields an insufficient resolution and frequently leads to misinterpretation of the epidemiological and evolutionary results (8, 9, 39, 50). Since the dimorphism of the 24Sα rRNA or miniexon genes has been widely used to cluster isolates of *T. cruzi*, a portion of the strains identified in the literature as *T. cruzi* I or *T. cruzi* II may belong to a different lineage or may even misrepresent the total parasite population present in the different tissues of patients. A classic example is strain CL Brener, which was long identified as a *T. cruzi* II clone on the basis of allele sizes of 125 bp and 300 bp amplified from the 24Sα rRNA and miniexon genes, respectively (38, 39, 49). However, it was later clearly demonstrated that CL Brener is in fact a hybrid clone and was probably derived from a hybridization event between *T. cruzi* II (DTU IIb) and *T. cruzi* III (DTU IIc) strains (11, 17, 21, 28). Similarly, it was recently demonstrated that *T. cruzi* II is present in the blood of at least 9% of infected patients in Colombia, although that country has been identified as a typical area of endemicity where *T. cruzi* lineage I is primarily responsible for human cases of Chagas' disease (48).

In addition to the identification of the *T. cruzi* major lineage, we also investigated the stability of the genetic profiles of the isolates obtained at different times from the same patients. We used microsatellite analysis based on nine polymorphic loci, a technique sensitive enough to detect small differences between different clones within a unique isolate (29, 35, 36, 45). We observed differences in the genetic profiles of only six (7.8%) isolates obtained from two patients. In both cases, the differences were detected only for the SCLE11 locus, suggesting that these patients were infected with at least two different, but genetically related, parasite populations.

We also found five (6.5%) isolates that exhibited more than two alleles for a unique microsatellite locus (TcAAAT6). These results probably represent the occurrence of polyclonal infections, although we cannot exclude the possibility of aneuploidy events at this locus. Analysis of the TcAAAT6 locus possesses a high power to discriminate among *T. cruzi* isolates, which increases our capacity to detect multiclonal populations.

The *T. cruzi* microsatellite profiles were also used for phylogenetic investigations for comparison to the polymorphisms of the 24Sα rRNA and COII genes. All of our *T. cruzi* isolates were grouped with the *T. cruzi* II, *T. cruzi* III, or hybrid reference strains. We did not observe any *T. cruzi* stock representative of the *T. cruzi* I lineage, reinforcing the conclusion that this lineage is associated with the sylvatic cycle of transmission of the parasite in the areas of both MG and GO where Chagas' disease is endemic.

Finally, as in many previous studies, we also failed to correlate the variability of the isolated parasite with the geographic distribution, clinical form, or sex or age of the patients. This has been attributed at least in part to the fact that many *T. cruzi* populations are polyclonal, and the different clones may have distinct tissue tropisms (1, 6, 16, 22, 28, 34, 40). Thus, a single isolate from the blood of a patient harboring a mixture of different *T. cruzi* isolates may not represent the whole infecting parasite population (36, 45). Here, we further investigated whether the study of different samples of *T. cruzi* obtained from the same patient at different times could improve the possibility of identifying an association between the genetic profile of the strains and the clinical forms of the disease. We have confirmed the occurrence of different populations simultaneously infecting the same patient, which reinforces the risk of isolate misrepresentation when a single isolate from patient blood is analyzed. Unfortunately, the strategy of repeated isolation of *T. cruzi* from patient blood was insufficient to permit the identification of any association between the genetic aspects of the parasite and the clinical form of Chagas' disease.

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