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ORIGINAL PAPER

Geographical clustering of *Trypanosoma cruzi* I groups from Colombia revealed by low-stringency single specific primer-PCR of the intergenic regions of spliced-leader genes

Ana María Mejía-Jaramillo · Sair Arboleda-Sánchez · Ingrid Bibiana Rodríguez · Carolina Cura · Alexander Salazar · Jesús del Mazo · Omar Triana-Chávez · Alejandro Gabriel Schijman

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Abstract A low-stringency single-primer polymerase chain reaction (LSSP-PCR) typing procedure targeted to the intergenic regions of spliced-leader genes (SL) was designed to profile *Trypanosoma cruzi* I stocks from endemic regions of Colombia. Comparison between SL-LSSP-PCR profiles of parasite DNA from vector faeces and cultures isolated from those faeces showed more conservative signatures than profiles using LSSP-PCR targeted to the minicircle variable regions (kDNA). This was also observed by analysing 15 parasite clones from one stock as well as serial samples of a same stock after in vitro culturing or inoculation into mice. Thus, SL-LSSP-PCR appears more appropriate than kDNA-LSSP-PCR for reliable typing of major *T. cruzi* I groups from in vitro cultured stocks and triatomine faeces. SL-LSSP-PCR grouped 46 of 47 *T. cruzi* I Colombian stocks according to their geographical procedences in four clusters: Cluster Cas from Casanare Department, Cluster Mg from Northern Magdalena department, Cluster Mom from Momposina Depression in Southern Magdalena and finally Cluster NW from northwestern Colombia, including Sucre, Chocó, Córdoba and Antioquia departments. Sequence analysis identified punctual mutations among amplicons from each cluster. Within Cluster Mg, sequence polymorphism allowed association with different sylvatic vector species. Novel SL sequences and LSSP-PCR profiles are reported from *T. cruzi* I infecting *Eratyrus cuspidatus*, *Panstrongylus geniculatus* and *Rhodnius pallescens* vectors.

A. M. Mejía-Jaramillo · S. Arboleda-Sánchez · I. B. Rodríguez · A. Salazar · O. Triana-Chávez
Grupo de Chagas, Universidad de Antioquia,
AA 1226 Medellin, Colombia

J. del Mazo
Centro de Investigaciones Biológicas, (CSIC),
Madrid, Spain

C. Cura · A. G. Schijman
Laboratorio de Biología Molecular de la Enfermedad de Chagas,
INGEBI-CONICET,
Buenos Aires, Argentina

A. G. Schijman (✉)
Vuelta de Obligado 2490,
Buenos Aires 1428, Argentina
e-mail: schijman@dna.uba.ar

Introduction

Trypanosoma cruzi, the etiological agent of Chagas disease, affects about 17 million people from different regions in Central and South America (WHO 2007). This parasitic disease shows a variable clinical course, which ranges from asymptomatic cases to serious chronic stages characterised by low parasitaemias and involving cardiac and/or gastrointestinal disorders (WHO 2002). One of the possible causes of such clinical variability has been attributed to the *T. cruzi* high genetic diversity and multiclonality of the natural populations. This variability has been demonstrated by means of different biochemical and molecular strategies targeted to different genetic markers (Miles et al. 1978; Ready and Miles 1980; Macedo et al. 1992; Steindel et al. 1993; Tibayrenc et al. 1993; Henricksson and Petterson 1996; Umekita and Mota 2000). Analysis of both the D7 domain of the 24Sα ribosomal RNA genes as well as the intergenic regions of spliced-leader RNA (SL-RNA) genes allowed identification of two major phylogenetic groups among the *T. cruzi* isolates, designated as TcI and TcII

(Souto et al. 1996; Fernandes et al. 1998). Five sublineages have been defined within lineage II (named IIa–e), associated to the domestic (IIb, IId and IIe) or wild environments (IIa plus IIc; Brisse et al. 2000).

Lineage I parasites were initially recognised in wild mammals, mainly in marsupials and sylvatic triatomines (Fernandes et al. 1999; Jansen et al. 1999; Ceballos et al. 2006), and more recently detected in humans in Venezuela (Añez et al. 2004), Bolivia (Brenière et al. 2002), Argentina (Burgos et al. 2007, 2008), Chile (Solari et al. 1998) and Colombia (Márquez et al. 1998; Cuervo et al. 2002; Montilla et al. 2002; Triana et al. 1999, 2006; Salazar et al. 2006; Herrera et al. 2007). This lineage is predominant in the Andean region, in Central America and in Mexico (Cortez et al. 2006; Black et al. 2007; Brenière et al. 2007; Samudio et al. 2007). Subdivisions within *T. cruzi* I have been proposed using different approaches (Diosque et al. 2003; Herrera et al. 2007; O'Connor et al. 2007; Britto et al. 2008), but their phylogenetic relationships and epidemiological significance have not been disclosed. In Colombia, it appears as the prevailing lineage associated to both domestic and wild cycles (Salazar et al. 2006). Consequently, the characterisation of *T. cruzi* I genotypes constitutes a fundamental tool for surveys of transmission patterns in these geographical regions. A high genetic variability was revealed using isoenzymatic analysis, schizodemes and low-stringency single-primer polymerase chain reaction (LSSP-PCR) from minicircle sequences of *T. cruzi* I Colombian isolates (Márquez et al. 1998; Jaramillo et al. 1999; Salazar et al. 2006, Britto et al. 2008). However, the high heterogeneity of the kinetoplastid minicircle variable region may preclude distinction of major groups as well as reliable profiling of multiclonal *T. cruzi* I populations when typing is carried out from cultured stocks. In this context, this work reports a LSSP-PCR strategy targeted to a more conserved sequence, the intergenic spacer of the spliced-leader genes, that enables to profile natural parasite populations directly from faeces of triatomines as well as from cultured stocks isolated from a variety of vector and host species from seven departments of Colombia, representing areas of low, medium and high endemicity for Chagas' disease.

Materials and methods

Capture of triatomines for analysis of faecal samples and isolation of *T. cruzi*

Wild *Rhodnius pallescens* bugs were captured from *Attalea butyracea* palms. Faecal samples were collected from adult bugs and suspended in 500 μL phosphate-buffered saline pH 7.2. One aliquot was analysed by optic microscopy to determine *T. cruzi*, another aliquot was used to isolate *T. cruzi* by inoculation into Balb/c mice followed by haemoculture according to Guhl and Nicholls (2001), whereas the remaining material was stored for direct PCR-based characterisation of faecal samples.

Parasite stocks

A total of 47 *T. cruzi* I stocks from different biological origins and geographic areas in Colombia were isolated (Table 1). Parasites were maintained in liver infusion tryptose (LIT) medium at 28°C by passages every 7 days (Camargo 1964). CL Brener and Af1 stocks belonging to *T. cruzi* II lineage were also profiled as controls.

T. cruzi cloning

One isolate from the Momposina Depression within the Magdalena department (Sebas16) was used to obtain clones after culture in agar-LIT-brain heart infusion (BHI)-blood as described elsewhere (Botero et al. 2007): 50 mL of sterile agar pH 7.2, 100 mL LIT with bovine faetal serum (BFS) 10%, 100 mL BHI and 6.25 mL defibrinated blood. Forty epimastigote cells were loaded per petri dish and cultured at 28°C to detect growth of transparent puntiform colonies after around 30 days. Some clones were further incubated in LIT with BFS 10% as described (Camargo 1964). Fifteen clones were analysed by LSSP-PCR.

Nucleic acid extraction from cultured stocks and triatomine faeces

Nucleic acids from epimastigote forms were obtained by means of the salting-out method (Miller et al. 1988). DNA from triatomine faeces was purified using DNAzol reagent (Gibco BRL, USA), as recommended by the manufacturer, using 25 μL of faecal samples as starting material.

PCR amplification of *T. cruzi* kDNA 330 bp fragment and LSSP-PCR

The variable region of the minicircles of kinetoplast DNA was amplified with the primers S35 (5′-AAATAAT GTACGGGT/GGAGATGCATGA-3′) and S36 (5′-GTTCGATTGGGG TTGGTGTAATATA-3′; Sturm et al. 1989). PCR was carried out at a final volume of 50 μL of reaction mix containing 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 1 μL of DNA template (10 ng/μL), 37 pmol of each primer, 200 μM of deoxyribonucleotide triphosphates (dNTPs), 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase. PCR was carried out at an initial

temperature of 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 63°C for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min.

Ten microlitres of the PCR products was run on 1.5% low melting point agarose gel and stained with ethidium bromide. Bands corresponding to 330 bp were cut from the gel and diluted to 1:10 in double-distilled water. One microlitre of the dilution was used as a template for the kinetoplastid DNA-based LSSP-PCR reaction (kDNA-LSSP-PCR; Vago et al. 1996). LSSP-PCR was performed in 25 μL of the final volume using 120 pmol of the S35 primer, 4 U of Taq polymerase, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM Tris–HCl and 0.1% Triton X-100. Amplification was carried out with 3 min of initial denaturing at 94°C, followed by 35 cycles of 94°C for 45 s, 30°C for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min (Pena et al. 1994). Fifteen microlitres of the amplification products from each of the stocks was analysed by ethidium bromide and visualised under UV light (Maniatis et al. 1982). LSSP-PCR for each of the stocks was performed in duplicate.

PCR amplification of the intergenic regions of the SL DNA genes and LSSP-PCR

PCR amplification was performed in 0.2 mL microcentrifuge tubes containing 25 μL of reaction mixture. Primers for amplification of the intergenic region of *T. cruzi* SL RNA genes were: 5′-GTGTCCGCCACCTCCTTCGGGC C-3′ (TC1, group II specific), 5′-CCTGCAGGC ACACGTGTGTGTG-3′ (TC2, group I specific) and 5′-CCCCCCTCCCAGGCCACACTG-3′ (TC, common to groups I and II; Souto et al. 1996). Amplicons of 350 bp for TcI and of 300 bp for TcII were expected. The reaction mixture contained 25 ng of *T. cruzi* DNA, 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 200 μM each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 12.5 pmol each primer, and 0.625 U of Taq DNA polymerase. Amplification was performed on a PTC-100 thermal cycler (MJ Research, USA) programmed for an initial denaturation step of 94°C for 1 min, followed by 27 cycles of 94°C for 30s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min (Souto et al. 1996). Ten microlitres of the PCR products were run on 1.5% low melting point agarose gel in 1× TBE (89 mM Tris borate, 2 mM EDTA [pH 8.3]) and stained with ethidium bromide. Bands corresponding to 300 bp or 350 bp were cut from the gel and, after gel melting, were diluted to 1:10 in double-distilled water. One microlitre of the dilution was used as template for each spliced-leader DNA-based LSSP-PCR reaction (SL-LSSP-PCR). LSSP-PCR was performed in 25 μL of the final volume using 120 pmol of the TC primer, 4 U of Taq polymerase, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM Tris–HCl and 0.1% Triton X-100. Amplification was carried out with 3 min of initial denaturing at 94°C, followed by 35 cycles of 94°C for 45 s, 30°C for 45 s, 72°C for 45 s and a final cycle at 72°C for 10 min (Pena et al. 1994). Fifteen microlitres of the amplification products from each of the stocks was analysed by electrophoresis in 6% polyacrylamide gel (Maniatis et al. 1982) and stained with silver nitrate (Andrade et al. 1999). Each parasite stock was analysed by LSSP-PCR in duplicate.

Analysis of LSSP-PCR signatures

Analysis of the bands was performed using the ImageJ software (Rasband 2004). Bands ranging in size from 50 to 330 bp for kDNA and from 50 to 350 bp for SL-DNA were selected for analysis. Molecular weight markers were used as reference standards to correct the variations in migration among the different gels. The digital profiles of the molecular markers were divided into squares of 10 pixels each, this corresponding to the maximum peak width in most of the *T. cruzi* bands (99%). The gels were considered to be normalised when the peaks corresponding to each of the bands of the molecular weight markers fell in the same vertical square. Similarly, the banding profiles of each *T. cruzi* stock were considered to be equal when they fell in the same vertical square. A data matrix of 0 and 1 was created on the basis of the absence or presence of the peaks for each stock signature, respectively. Finally, genetic distances were analysed using PAUP 4.0 (beta version 4.0b7; Swofford 2002), and a phylogenetic tree was constructed by the neighbor-joining (NJ) method.

Sequence analysis of spliced-leader gene fragments from *T. cruzi* I stocks

Amplicons of the spliced-leader intergenic regions from 20 *T. cruzi* stocks were purified with a DNA purification system kit (Promega, MA, USA). Purified PCR products were sequenced directly in both directions using the Sequenase Kit (USB, USA), according to the manufacturer's protocol. Sequences were determined in an ABI 3700 automated DNA sequencer (Applied Biosystems, USA). Nucleotide sequences data are available in the GenBank under the accession numbers listed in Table 1. Fragments of 205–211 bp were aligned with the homologous sequence of *T. cruzi* I Mg10 stock from the Magdalena department of Colombia (Herrera et al. 2007) using CLUSTAL W software (Thompson et al. 1994). The analysis of polymorphic positions among the aligned sequences was done using the MEGA version 3.1 software.

Table 1 Description of Colombian *T. cruzi* I stocks isolated from different vector and host species captured at different endemic regions

Region	Geographical origin	*T. cruzi* I strain	Biological origin
NW	Antioquia	Amp05	*Canis familiaris*
	Antioquia	Amp07	*P. geniculatus* (S)
	Chocó	Ac17	*R. pallescens* (S)
	Córdoba	B51	*R. pallescens* (S)
	Córdoba	B94	*T. dimidiata* (S)
	Córdoba	B114	*T. dimidiata* (S)
	Córdoba	Sb1	*R. pallescens* (S)
	Sucre	Gal4	*R. pallescens* (S)
	Sucre	Gal34	*R. pallescens* (S)
	Sucre	Gal52	*D. marsupialis*
	Sucre	Ov1	*P. geniculatus* (P)
	Sucre	Ov17	*P. geniculatus* (P)
	Sucre	So5	*R. pallescens* (S)
	Sucre	So8	*R. pallescens* (S)
	Sucre	W3534	*Homo sapiens*
Mg	Magdalena	Mg1	*R. pallescens* (S)
	Magdalena	Mg2	*R. pallescens* (S)
	Magdalena	Mg3	*E. cuspidatus* (S)
	Magdalena	Mg5	*E. cuspidatus* (S)
	Magdalena	Mg6	*P. geniculatus* (S)
	Magdalena	Mg8	*T. dimidiata* (S)
	Magdalena	Mg9	*T. dimidiata* (S)
	Magdalena	Mg10	*T. dimidiata* (S)
	Magdalena	Mg11	*R. pallescens* (S)
Cas	Casanare	Cas7	*R. prolixus* (D)
	Casanare	Cas15	*R. prolixus* (S)
	Casanare	Cas16	*R. prolixus* (P)
	Casanare	Cas18	*D. marsupialis*
	Casanare	HA	*H. sapiens*
Mom	Bolívar	Fer1	*R. pallescens* (S)
	Bolívar	Fer2	*R. pallescens* (S)
	Magdalena	Sebas1	*R. pallescens* (S)
	Magdalena	Sebas2	*R. pallescens* (S)
	Magdalena	Sebas3	*R. pallescens* (S)
	Magdalena	Sebas4	*R. pallescens* (S)
	Magdalena	Sebas5	*R. pallescens* (S)
	Magdalena	Sebas6	*R. pallescens* (S)
	Magdalena	Sebas7	*R. pallescens* (S)
	Magdalena	Sebas8	*R. pallescens* (S)
	Magdalena	Sebas9	*R. pallescens* (S)
	Magdalena	Sebas10	*R. pallescens* (S)
	Magdalena	Sebas11	*R. pallescens* (S)
	Magdalena	Sebas12	*R. pallescens* (S)
	Magdalena	Sebas13	*R. pallescens* (S)
	Magdalena	Sebas14	*R. pallescens* (S)
	Magdalena	Sebas15	*R. pallescens* (S)
	Magdalena	Sebas16	*R. pallescens* (S)

(D) domestic, *(P)* peridomestic, *(S)* sylvatic

Results

Comparison of spliced-leader and kDNA targets for typing *T. cruzi* I cultured stocks

We compared the degree of polymorphism detected by LSSP-PCR targeted to the spliced-leader intergenic region (SL-LSSP-PCR) and the minicircle variable region (kDNA-LSSP-PCR) from *T. cruzi* I DNAs extracted from faeces and from cultured stocks isolated from *R. pallescens* wild triatomines captured at the Momposina Depression department of Magdalena (Mom, Table 1). This characterisation showed a high similarity between the SL-based signatures from faecal samples and those from the corresponding cultured stocks (F and S wells, Fig. 1a), whereas kDNA-based signatures from the same material were polymorphic between most tested paired samples. The latter result points to the multiclonality of natural populations and clonal selection during culture isolation (F-S wells in Fig. 1b) giving rise to dissimilar kDNA-LSSP-PCR signatures. Moreover, we have compared the stability of kDNA- and SL-DNA-based profiles among three cultured stocks (Sebas1, Sebas8 and Sebas15) maintained in different media or after inoculation into mice (Fig. 1c,d). In fact, SL-based profiling rendered identical signatures among aliquots of Sebas1 maintained in LIT or 3N media spanning a period of 7 months (wells 1–4, Fig. 1c), as well as after inoculation into mice (well 5, Fig. 1c). In contrast, kDNA signatures were variable under the same conditions (Sebas1, wells 1–5, Fig. 1d). For Sebas8 and Sebas15 stocks, no polymorphism was observed after 3 and 7 months of growth in LIT using SL or kDNA-LSSP-PCR typing (Fig. 1c,d).

Further analysis was carried out after parasite cloning (Fig. 2). When 15 clones obtained from Sebas16 stock were profiled, SL-LSSP-PCR gave almost identical signatures among them, (Fig. 2a), whereas kDNA-based typing showed higher variability (Fig. 2b). However, when samples from a same-parasite clone maintained in culture after a period of 11 months were compared by SL- and kDNA-LSSP-PCR, conserved signatures were observed using both *T. cruzi* molecular targets, as expected (Fig. 2c,d).

These experiments led us to choose a SL-LSSP-PCR approach for distinguishing major *T. cruzi* I clusters using as source material samples from cultured stocks or triatomine faeces.

SL-LSSP-PCR profiling of *T. cruzi* I stocks from different regions of Colombia

The SL-LSSP-PCR strategy was applied to 47 *T. cruzi* I stocks from seven Colombian departments of low, medium and high endemicity for Chagas disease (Fig. 3). Cultured stocks were obtained from faecal material of different

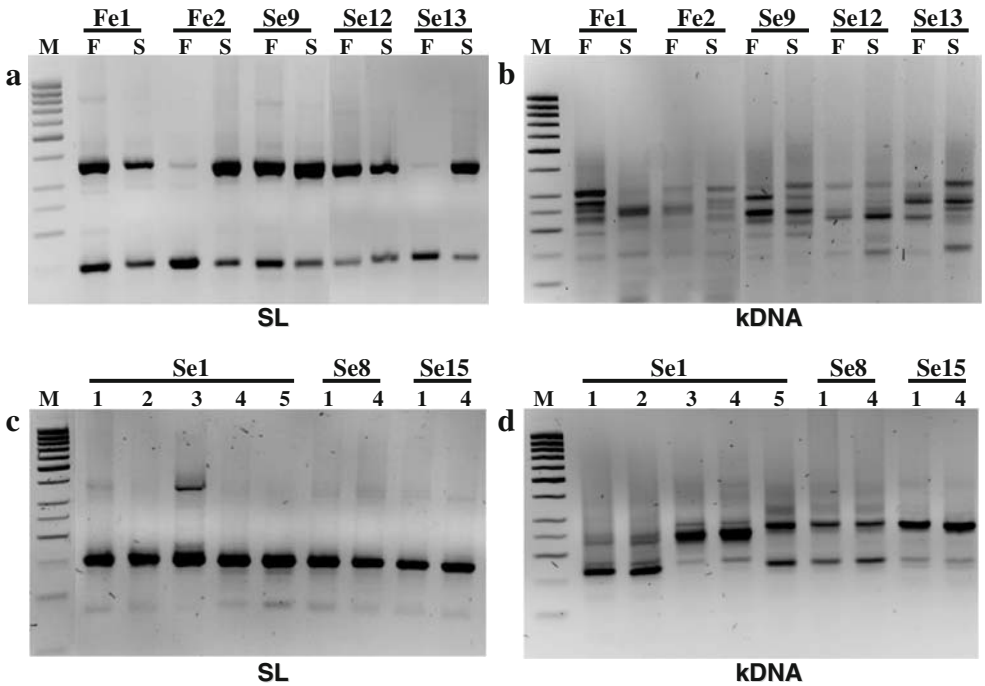

Fig. 1 Three percent Agarose gel electrophoretic analysis of signatures obtained by spliced-leader intergenic region (*SL*)-LSSP-PCR (**a** and **c**) and kinetoplastid (*kDNA*)-LSSP-PCR (**b** and **d**) from *T. cruzi* I populations. **a** and **b** display signatures obtained from faeces (*F*) and after culture isolation of stocks (*S*) from the same faecal material of 5 *R. pallescens* bugs from San Fernando (*Fe*) and San Sebastian (*Se*) localities of the departments of Bolivar and Magdalena, respectively. **c** and **d** represent examples of profiles obtained from different aliquots from the same original stock cultured in LIT medium during 3 months (*1*), 3N medium during 3 months (*2*), 3N during 5 months (*3*), LIT medium during 7 months (*4*), LIT medium during 7 months and reactivated into mice (*5*). *M* 50 bp ladder molecular weight marker

sylvatic and domestic vector species as well as from peripheral blood samples of one domestic reservoir and two Chagas disease patients (Table 1). Figure 4 displays examples of SL-LSSP-PCR signatures from 20 of these *T. cruzi* I stocks as revealed by 6% polyacrylamide gel electrophoresis. The neighbor-joining tree constructed from the mentioned SL-LSSP-PCR signatures shows four clusters associated to their geographical origin (Fig. 5). Among them, Cluster NW includes those stocks isolated from isolates of the northwestern regions of Sucre, Cordoba, Chocó and Antioquia, except the HA stock, isolated from a Chagas disease patient living in the Casanare department. Cluster Cas gathers four out of the five tested parasite stocks from the Casanare department, Cluster Mg includes all nine isolates from the northern region of the Magdalena department, and finally, Cluster Mom groups all the stocks isolated from the Momposina Depression in the southern edge of Magdalena department (Fig. 5).

A 205–211 bp nucleotide sequence was obtained from the SL-PCR products of 20 *T. cruzi* I variants profiled by LSSP-PCR. This alignment distinguished polymorphic sites at 22 positions (Table 2). Cluster NW sequences shared a conserved guanine at position 62 and a conserved cytosine at position 188, whereas those stocks clustered within Cluster Cas shared conserved thymines at positions 62 and 188. Moreover, the stock Cas15, which was isolated from a wild *Rhodnius prolixus* bug showed four substitutions (28, 140, 162 and 183) compared to the Cas stocks that were isolated from domestic and peridomestic *R. prolixus* bugs. In contrast, the sequence from the HA human stock from the same region of the Casanare department shared identity at positions 2 and 112 with stocks Gal52, So5 and So8 isolated from Sucre (Table 2), in agreement with their clustering in the LSSP-PCR-based tree (Fig. 3, Cluster NW). Within Cluster Mg, Mg1, Mg3 and Mg10 stocks showed nucleotide substitutions at positions 2, 186 and 211. In addition, Mg3 presented insertions of adenines at positions 120 and 169, being unique among all 21 aligned sequences. It is worth to note that these stocks were isolated from different sylvatic vector species (Table 1).

Cluster Mom clusters all 18 stocks isolated from sylvatic *R. pallescens* captured at the Momposina Depression in the southern edge of Magdalena department (Figs. 3 and 5).

Discussion

The LSSP-PCR technique allows the detection of single or multiple variations in fragments of target DNA, being applied to the study of genetic diseases (Pena et al. 1994) and genetic characterisation of diverse microorganisms (Villa et al. 1995; Gomes et al. 1997; Oliveira et al. 2003). A high intra-lineage

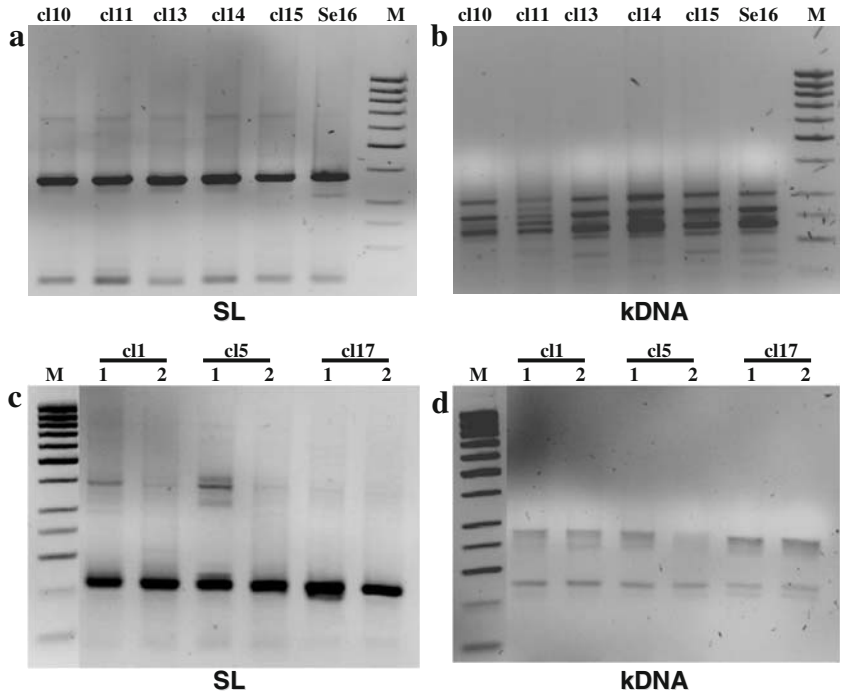

Fig. 2 Three percent Agarose gel electrophoretic analysis of signatures obtained by SL-LSSP-PCR (**a** and **c**) and kDNA-LSSP-PCR (**b** and **d**) from *T. cruzi* I clones obtained from stocks. **a** and **b** display signatures obtained from a cultured stock (*Se16*) and five parasite clones (*cl10–cl15*) obtained from this stock. **c** and **d** display signatures between stocks of the same clone collected in October 2006 (*1*) and September 2007 (*2*). *M* 50 bp ladder molecular weight marker

diversity among circulating *T. cruzi* I isolates in Colombian endemic regions was firstly revealed by means of LSSP-PCR analysis of minicircle signatures (Salazar et al. 2006). However, in the present work, we have detected that kDNA signatures are highly dependent on the source of the parasite DNA used for analysis (Figs. 1 and 2). This must be due to the high variability and mutation rates of minicircle sequences. Alves et al. (1993) observed different isoenzymatic profiles in distinct clones of the *T. cruzi* Y strain that were submitted to long-term in vitro culturing. Later on, it was verified that these changes were followed by alterations in the restriction fragment length polymorphism of the kDNA. This phenomenon was called transkinetoplastid (Alves et al. 1994) and was primarily observed in cloned cells of *Leishmania amazonensis* submitted to drug selection. It consists of fast changes in the kDNA minicircles population leading to different restriction profiles. Thus, LSSP-PCR based on the kDNA variable region can be only recommended for epidemiological studies or characterisation of clonal histotropism when direct analysis of biological or clinical material is available (Vago et al. 1996; Burgos et al. 2005, 2008; Britto et al. 2008). In contrast, the intergenic regions of SL genes displayed conserved LSSP-PCR profiles under the tested scenarios (Figs. 1 and 2). Therefore, aiming to identify major clusters within the *T. cruzi* I populations and to associate them to geographical distribution and/or host preferences, the LSSP-PCR approach based on amplification

Fig. 3 Locations within the Colombian departments where the *T. cruzi* I samples were obtained. *White circles* Momposina Depression (Mom), *dark gray circles* Casanare department (Cas), *light gray circles*, Northwest region (NW), *black circles* Magdalena department (Mg)

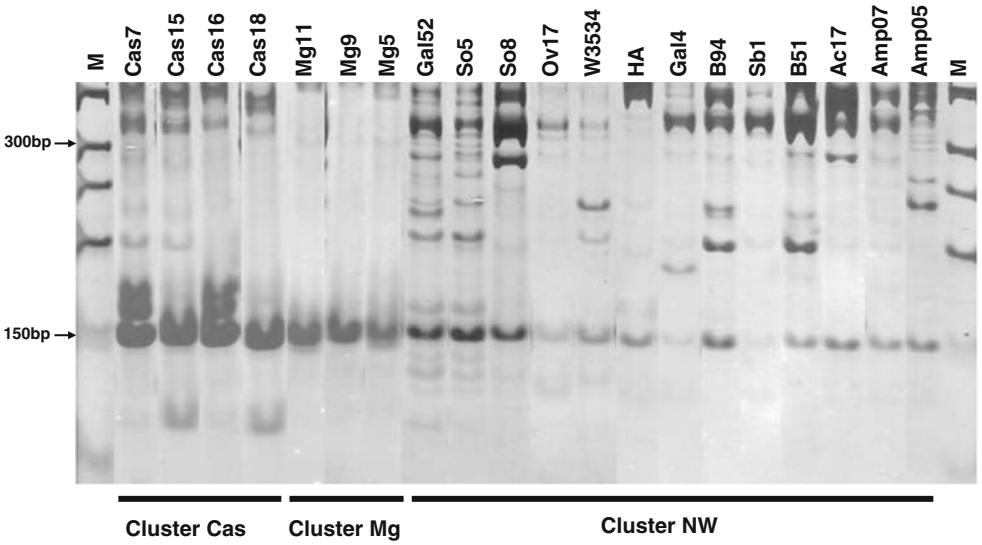

Fig. 4 SL-LSSP-PCR signatures obtained from the SL amplification products of 20 *T. cruzi* I stocks isolated from different regions of Colombia, different triatomine vectors and host species, as revealed by 6% polyacrylamide gel electrophoresis after silver nitrate staining

of the SL intergenic sequence appears more appropriate, especially when samples from in vitro cultured stocks or isolates are being characterised.

Spliced-leader gene sequences have been characterised from a variety of sylvatic and domestic vectors such as *Triatoma infestans*, *Triatoma pallidipennis*, *Triatoma longipennis*, *Triatoma picturata*, *Triatoma barberi* (O'Connor et al. 2007), *Triatoma dimidiata*, *Triatoma venosa* and *R. prolixus* (Herrera et al. 2007). In this report, we describe for the first time SL gene sequences from *T. cruzi* I variants isolated from *R. pallescens*, *Panstrongylus geniculatus* and *Eratyrus cuspidatus* wild specimens. In particular, isolates from *E. cuspidatus* captured in the Magdalena region were identified as *T. cruzi* I by multi-locus enzyme electrophoresis, mini-exon and randomly amplified polymorphic DNA (RAPD) analysis (Dib et al., 2005). In fact, RAPD profiles identified a common *T. cruzi* genotype for parasites isolated from *E. cuspidatus* and humans, pointing towards the significant epidemiological relevance of *E. cuspidatus* in the vectorial transmission cycle of *T. cruzi*. In central and northern regions of Colombia, *E. cuspidatus* occasionally invades houses, whereas in Ecuador and northern Peru, it seems to play no role in human transmission (Aguilar et al. 1999; Cuba et al. 2002). Incrimination of sylvatic triatomine species in the transmission of Chagas' disease underlines the need for depth surveys regarding the ecology of these vectors to improve current control strategies.

SL-LSSP-PCR of 47 *T. cruzi* I isolates allowed us to associate them to the geographical origins of the vector and reservoir species from which they were isolated. Indeed, parasite populations from Casanare are geographically separated from the rest of the tested populations by the eastern branch of the Andean cordillera. Similar clustering among stocks from Casanare were obtained by Márquez et al. (1998); Jaramillo et al. (1999) and Salazar et al. (2006) using other approaches. Moreover, within this cluster, the clustering appears also associated with the habitats from which these parasite populations have been isolated. Recently, it has been reported that *R. prolixus* population from domestic and sylvatic environments are morphologically similar but genetically different (Lopez et al. 2007). Stocks isolated from domestic (Cas7) and peridomestic (Cas16) *R. prolixus* bugs are more related with each other than with Cas15, which was isolated from a sylvatic *R. prolixus* bug. In turn, these three mentioned stocks are more related among them than with one isolate from a *Didelphis marsupialis* specimen captured in the same region (Cas18; Fig. 4). Interestingly, a human isolate from Casanare, designated HA, did not gather within this cluster. Its topology was in agreement with the nucleotide identity detected at several polymorphic positions, with isolates from Sucre (Table 2), suggesting that this individual could have acquired the infection in this region. Nevertheless, the low number of isolates from Casanare does not allow discarding the coexistence of several genotypes in this department.

Other LSSP-PCR cluster included signatures from the northern area of Magdalena (Cluster Mg). Yet again, a geographical barrier, conformed by the Magdalena river, might have reduced the transmission between Magdalena populations and those from the other departments (Fig. 3). Interestingly, Mg1, Mg3 and Mg10 sequence polymorphism allowed association of these stocks with different sylvatic vector species, namely *R. pallescens*, *E. cuspidatus* and *T. dimidiata*, respectively. All stocks from the Momposina Depression gathered together, including those from the departments of Magdalena and Bolivar, isolated from faeces of *R. pallescens* bugs captured in *A. butyracea* palm trees.

On the basis of a microsatellite motif found within the SL intergenic regions of 12 *T. cruzi* I Colombian isolates, Herrera et al. (2007) proposed 4 *T. cruzi* I haplotypes in this

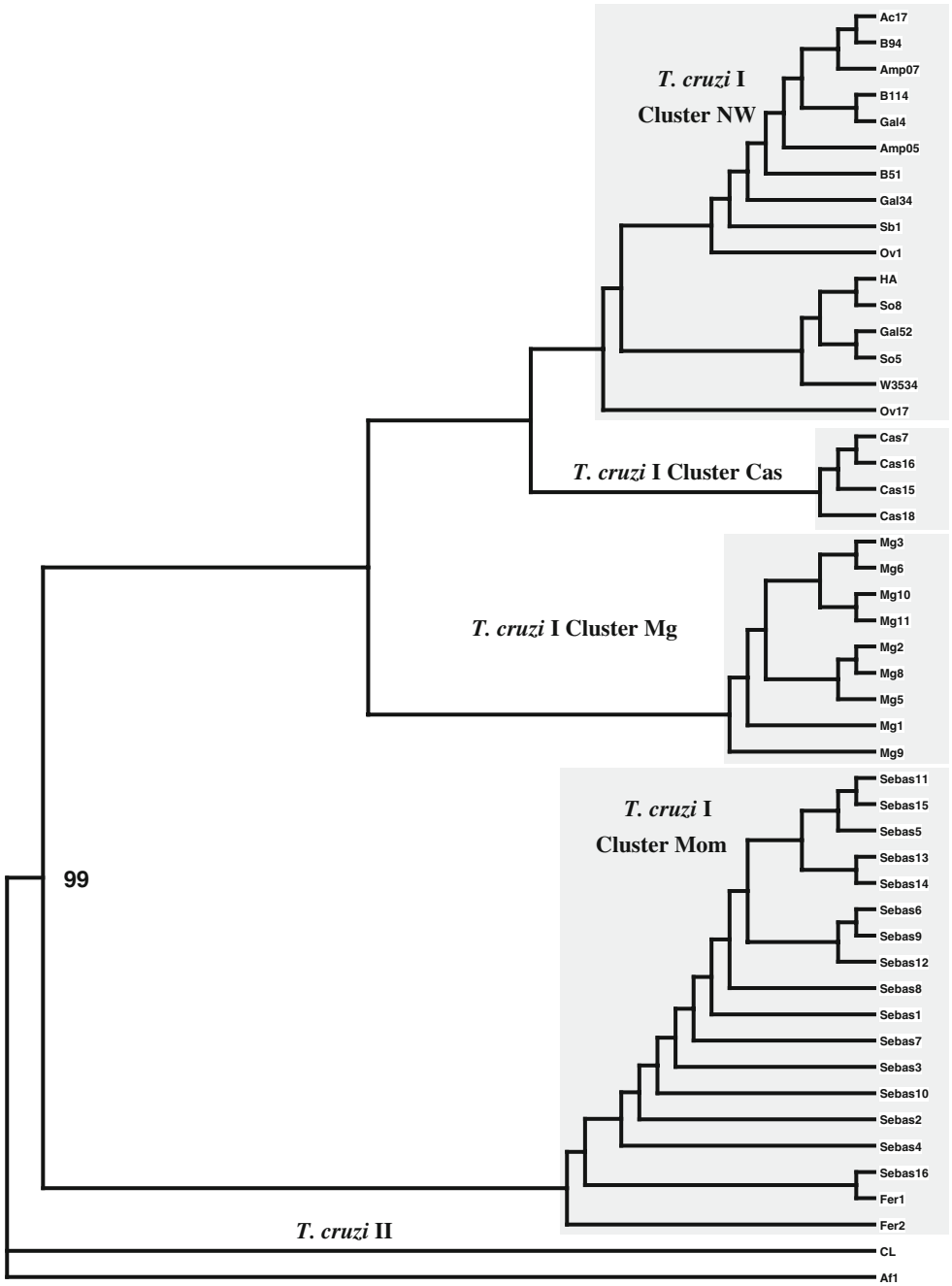

Fig. 5 Tree constructed by the NJ method from the genetic distances analyses of the SL-LSSP-PCR signatures of 47 Colombian *T. cruzi* I stocks, performed using PAUP 4.0. Only bootstrapping higher than 50% of 10,000 replications is shown. *NW* Northwestern region, *Cas* Casanare Department, *Mg* Magdalena Department, *Mom* Momposina Depression

country. Except for Mg10 stock (Haplotype 2) analysed by Herrera et al., which had been isolated from the Magdalena Department, the geographical origin of the other 11 stocks reported by them are different from those of our tested samples. We have recently sequenced the above-mentioned microsatellite motif of representative stocks obtained from the departments of Sucre (Cluster NW), Magdalena (Cluster Mg) and Casanare (Cluster Cas), and only haplotype 4 was identified (data not shown). Interestingly, at least for the Magdalena department, haplotypes 2 (Mg10) and 4 (Mg1) can be associated to different sylvatic triatomine species (Table 1). Further characterisation attempting to integrate genomic data from a higher number of *T. cruzi* I stocks, representing the different endemic regions and reservoir and vector species circulating in Colombia, will allow assessment of the epidemiological significance of these haplotypes, as well as to discover novel haplotypes within *T. cruzi* I.

Finally, SL-LSSP-PCR could be a useful tool to profile multiclonal isolates from Andean and central American countries where *T. cruzi* I prevails (Bosseno et al. 2002; Añez et al. 2004; Carrasco et al. 2005; Ruiz-Sanchez et al. 2005; Brenière et al. 2007; Samudio et al. 2007 Márquez et

Table 2 Polymorphic positions of a 211-bp sequence of the spliced-leader intergenic regions from 21 Colombian *T. cruzi* I stocks

T. cruzi strain	GB accession number	Cluster	Variable positions																			
			2	21	25	28	62	69	105	112	140	162	165	`183	186	188	198	200	202	203	208	211
Mg10C	AM259476	Mg	T	T	G	G	G	T	T	T	C	G	C	G	C	C	C	G	A	C	T	C
Amp05	AY646671	NW	A	.	.	.	.	.	.	C.	.	.	.	–	.	.	.	.	.	–	.	.
Ac17	AY646663	NW	.	.	.	.	.	.	.	–	.	.	.	C.	.	.	.	.	.	–	.	.
B51	AY646665	NW	C	.	.	.	.	A	.	–	.	.	.	–	.	.	.	.	.	–	.	.
B94	AY646657	NW	C	.	.	.	.	.	.	C.	.	.	.	–	.	.	.	.	.	–	.	.
B114	AY646659	NW	C	.	.	.	.	.	.	.C	.	.	.	–	T	.	.	.	.	A	.	.
Sb1	AY646658	NW	C	.	.	.	T	.	.	.C	.	.	.	–	T	.	.	.	.	–	.	.
Gal4	AY646672	NW	C	.	.	.	.	.	.	.C	.	.	.	–	.	.	.	.	.	–	.	A
Gal34	AY646662	NW	C	.	T	.	.	.	.	.C	.	.	.	–	.	.	.	.	.	–	.	.
Gal 52	AY646668	NW	.	.	.	.	.	.	.	–	.	.	.	.C	.	.	.	.	.	–	.	A
Ov1	AY646654	NW	C	.	.	.	T	.	C	.C	.	.	.	.C	.	T	.	.	.	–	.	.
Ov17	AY646675	NW	C	.	.	.	.	.	.	–	.	.	.	.C	.	.	.	.	.	–	.	.
So8	AY646667	NW	.	.	.	.	.	.	.	.C	.	.	.	.C	.	.	.	.	.	–	.	A
So5	AY646674	NW	.	.	.	.	.	.	.	–	.	.	.	.C	.	.	.	.	.	–	.	.
W3534	AY646664	NW	C	.	.	.	.	.	.	–	.	.	.	–	T	.	.	.	.	–	C	.
Mg1	AY646669	Mg	C	.	.	.	.	.	.	–	.	.	.	–	T	.	.	.	.	–	.	.
Mg3	AY646670	Mg	C	.	.	.	.	.	.	–	.	.	.	–	T	.	.	.		–	.	A
Cas7	AY646651	Cas	C	.	.	.	T	.	.	.C	.	.	.	.C	.	T	.	.	.	–	.	.
Cas15	AY646652	Cas	C	.	.	A	T	.	.	.C	T	C	.	–	.	T	.	.	.	–	.	.
Cas16	AY646653	Cas	C	.	.	.	T	.	.	.C	.	.	.	C	.	T	.	.	.	–	.	.
HA	AY646656	Cas	.	.	.	.	.	.	.	–	.	.	.	.C	.	.	.	.	.	–	.	.

al. 1998; Cuervo et al. 2002; Montilla et al. 2002; Triana et al. 1999, 2006; Salazar et al. 2006; Herrera et al. 2007) and also from the southern cone of southern America, where *T. cruzi* I has also been identified in different scenarios (Solari et al. 1998; Brenière et al. 2002; Burgos et al. 2007, 2008). This tool will allow performing studies about *T. cruzi* I host preferences, tissue tropism, clinical forms and susceptibility to drugs (Veloso et al. 2001; Camandaroba et al. 2003; Toledo et al. 2003). This remarkable genetic heterogeneity should also be taken into consideration when novel *T. cruzi* genome projects and new drugs trials are being initiated.

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