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Geographical structuring of *Trypanosoma cruzi* populations from Chilean *Triatoma infestans* triatomines and their genetic relationship with other Latino American counterparts

J. VENEGAS[*], T. ROJAS[*], F. DÍAZ[*], S. MIRANDA[*], M. I. JERCIC[†], C. GONZÁLEZ[†], W. COÑOEPÁN[*], S. PICHUANTES[*], J. RODRÍGUEZ[‡], M. GAJARDO[§] and G. SÁNCHEZ[¶]

[*]*Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Correo 70086, Santiago 7, Chile*
[†]*Laboratorio de Referencia en Parasitología, Instituto de Salud Pública, Casilla 48, Santiago, Chile*
[‡]*Departamento de Salud Pública, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago, Chile*
[§]*Departamento de Patología Facultad de Odontología, Universidad de Chile, Olivos 943, Comuna de Independencia, Santiago, Chile*
[¶]*Programa de Genética Humana, Instituto de Ciencias Biomédicas, Facultad de Medicina, Santiago, Chile*

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In order to obtain more information about the population structure of Chilean *Trypanosoma cruzi*, and their genetic relationship with other Latino American counterparts, we performed the study of *T. cruzi* samples detected in the midgut content of *Triatoma infestans* insects from three endemic regions of Chile. The genetic characteristics of these samples were analysed using microsatellite markers and PCR conditions that allow the detection of predominant *T. cruzi* clones directly in triatomine midgut content. Population genetic analyses using the Fisher's exact method, analysis of molecular variance (AMOVA) and the determination of F_{ST} showed that the northern *T. cruzi* population sample was genetically differentiated from the two southern population counterparts. Further analysis showed that the cause of this genetic differentiation was the asymmetrical distribution of TcIII *T. cruzi* predominant clones. Considering all triatomines from the three regions, the most frequent predominant lineages were TcIII (38%), followed by TcI (34%) and hybrid (8%). No TcII lineage was observed along the predominant *T. cruzi* clones. The best phylogenetic reconstruction using the shared allelic genetic distance was concordant with the population genetic analysis and tree topology previously described studying foreign samples. The correlation studies showed that the lineage TcIII from the III region was genetically differentiated from the other two, and this differentiation was correlated with geographical distance including Chilean and mainly Brazilian samples. It will be interesting to investigate whether this geographical structure may be related with different clinical manifestation of Chagas disease.

INTRODUCTION

Chagas disease is well recognized as the most serious human parasitic disease of the Americas in terms of its social and economic impact. *Trypanosoma cruzi* is the etiological agent of Chagas disease, transmitted by

Reprint requests to: J. Venegas, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Correo 70086, Santiago 7, Chile.
E-mail: jvenega@med.uchile.cl

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triatomine bugs; it is one of the most important vector-borne diseases in Latin America, infecting approximately 15 million people (WHO, 2007). Several reports have shown that *T. cruzi* is mainly diploid, with a clonal population structure with possible rare genetic exchange (Tibayrenc *et al.*, 1986; Brisse *et al.*, 2000; Machado and Ayala, 2001; Gaunt *et al.*, 2003; Tibayrenc, 2003; de Freitas *et al.*, 2006). *T. cruzi* has been classified into two major phylogenetic lineages, *T. cruzi* I (TCI) and *T. cruzi* II (TCII) (Souto *et al.*, 1996; Momen, 1999). Later, based on multilocus enzyme electrophoresis and random amplified polymorphic DNA, TCII was further subdivided into five discrete phylogenetic clusters (Brisse *et al.*, 2000). Today, the six *T. cruzi* lineages are named as: TcI, TcII, TcIII, TcIV, TcV and TcVI, respectively, which will be used in the present paper (Zingales *et al.*, 2009). Several lines of evidence support the idea that TcV and TcVI lineages are hybrid, originated by a relatively recent genetic fusion between TcII and TcIII (Machado and Ayala, 2001; Westenberger *et al.*, 2005). Later, using mitochondrial sequences and five microsatellite markers, it was shown that the two hybrids, TcV and TcVI lineages, are not separate clusters but rather make up a single hybrid lineage, which was confirmed recently by our group (de Freitas *et al.*, 2006; Venegas *et al.*, 2009a).

These phylogenetic analyses conducted in laboratory cultured *T. cruzi* strains and clones have provided some information related to the population structure of this parasite, such as the observation that the TcI lineage has been found in a vast geographical and ecological area of Latin America and has been associated both with sylvatic and domestic transmission cycles, as well as both with arboreal and terrestrial mammals (Brisse *et al.*, 2000; Tibayrenc, 2003; Miles *et al.*, 2009), whereas lineages TcII–TcVI exhibit a more restricted geographic and ecological distribution. Thus, the majority of *T. cruzi* clones of lineages TcV and TcVI were found in the southern cone of South America, while the TcIV lineage has been more frequently found in Venezuela and the Amazon Basin (Brisse *et al.*, 2000; Miles *et al.*, 2009). The TcIII lineage was more frequent in the Amazon Basin and in Paraguay and mainly associated with terrestrial mammals (Miles *et al.*, 1977, 2009; Fernandes *et al.*, 1998; Zingales *et al.*, 1998; Yeo *et al.*, 2005). Very interestingly, recently population genetic studies using a high-resolution microsatellite analysis, nuclear and mitochondrial genes have shown that the TcIII lineage is geographically differentiated and is strongly associated with a terrestrial ecotope (Marcili *et al.*, *et al.*, 2009; Llewellyn *et al.*, 2009 a, b). However, it is worth mentioning that since the majority of these studies have been conducted in laboratory cultured parasites, stocks or isolates, they may not represent the real population structure and ecological distribution of natural populations of *T. cruzi*, due to selective pressure that the laboratory culture and mouse passages exert over the clones and mixtures of lineages which the *T. cruzi* samples contain (Deane *et al.*, 1984; Macedo and Pena, 1998; Solari *et al.*, 2001; Coronado *et al.*, 2006).

Chagas disease is widespread in Chile, distributed in rural and peri-urban areas in the seven northern regions of the country. The principal and domiciliary vector of *T. cruzi* is *Triatoma infestans*, but the peridomestic and sylvatic triatomines *Mepraia spinolai* and *Mepraia gajardoi* also bear *T. cruzi*. The interruption of the domestic cycle of transmission of *T. cruzi* was completed by the Chilean Health Minister in 1999 (Lorca *et al.*, 2001). Nevertheless, a permanent entomological vigilance and serological study of people at risk must be conducted to avoid the re-colonisation of domestic niches by new triatomines.

The main aims of the present manuscript were to determine whether Chilean *T. cruzi* populations are geographically structured and whether this phenomenon may be due to genetic differences among or within lineages. In addition, the genetic

relationship of these natural *T. cruzi* populations with other Latino American counterparts, was investigated. We think that obtaining more insights about the organisation and the factors which originated the *T. cruzi* populations, is highly relevant since this information could be important in the knowledge about the epidemiology and pathogenesis of Chagas disease.

MATERIALS AND METHODS

Population of Triatomines

The triatomines were captured by the Program of Eradication of the Domiciliary Infestation of *Triatoma infestans* of the Ministry of Health during period 2005–2007. The sample size in the study corresponded to 15, 37 and 12 *T. infestans* from the Atacama (III) region (middle point among locations of Alto del Carmen and Tierra Amarilla, −27°27′S, −70°15′W), Valparaiso (V) region (mainly from Petorca location −32° 16′S, −70° 58′W) and Metropolitan (M) region (Calera de Tango location, −33°37′S, −70°47′W) regions of Chile (Table 1).

T. cruzi DNA Samples from *T. infestans*

Samples were obtained by the dissection of the complete digestive apparatus of each triatomine. These samples were boiled, centrifuged and the supernatants were used to obtain DNA for the PCR test, as described (Venegas *et al.*, 2010).

PCR Assay

PCR was used to detect the presence of DNA of *T. cruzi* and was performed in duplicate with the samples of triatomines, according to a described protocol (Dorn *et al.*, 1999). The kinetoplast primers used for the reaction of PCR were S35 and S36 and the nuclear primers were TcZ1 and TcZ2. The products of the PCR were electrophoresed in 2% agarose gel. Gels were stained with ethidium bromide (5 μg/ml) and photographed.

Analysis of Microsatellites

Three microsatellite loci were analysed (SCLE10, SCLE11 and MCLE01) with a modification of the technique described by Oliveira *et al.* (1998), using a second amplification with an aliquot of 1 μl from the first PCR and the same amplification conditions. The alleles were detected using primers stained with fluorescence (Oliveira *et al.*, 1998). The amplification products were sent to the Roy J. Carver Biotechnology Center, University of Illinois, USA, for analysis by capillary electrophoresis and fluorescence detection with an automatic sequencer and an appropriate software program. The number of base pairs (bps) of each allele was determined using cloned alleles from each marker as controls. In this analysis, the minimal detectable peak height was set to 80 arbitrary fluorescence units (FUs).

Genotyping of the Predominant *T. cruzi* Clone in Each DNA Sample

The determination of the predominant *T. cruzi* clone genotype in samples containing multiple alleles per locus was conducted taking into account the distance and the height in FU of the highest two peaks, the major and second peaks, directly in the electropherogram, as described (Venegas *et al.*, 2010). Briefly, when the height ratio between the second and the major peak was less than 0.33, it was concluded that the predominant clone genotype in this sample has only a single allele, and therefore, is homozygous. By contrast, if the height ratio between the second and the major peak was greater than or equal to 0.33, it was analysed further, locus by locus. For instance, Fig. 1a, shows the electropherogram of the sample 24 analysed by the SCLE10 microsatellite marker. Four alleles of 207, 217, 251 and 255 bp were detected. The major and the second peaks correspond to the alleles 207 and 251 with a height of 400 and

TABLE 1. *Genetic characteristics of* Trypanosoma cruzi *samples isolated from* Triatoma infestans *insects captured by the Chilean Vector Control Program of Health Ministry determined by three microsatellite markers*

Triatomine			Minimal number of clones[a]	Lineage of predominant *T. cruzi* clone[b]
Code	Region	Location		
	III			
1	"	Alto del Carmen (AC)	4	TcIII
9	"	AC	2	TcI
10	"	AC	2	ND
44	"	AC	2	ND
55	"	Tierra Amarilla (TA)	2	TcI
86	"	TA	3	TcIII
106	"	TA	3	ND
107	"	TA	4	TcIII
108	"	TA	5	TcIII
109	"	TA	6	ND
110		TA	6	TcIII
111	"	TA	4	TcIII
112	"	TA	5	TcIII
8–120	"	TA	7	TcI
51–163	"	Mallorca	4	TcI
	V			
23	"	Alto del Puerto	2	ND
101	"	Chincolco	3	TcIII
3	"	San Antonio	2	TcI
51	"	Pedernal (PED)	2	TcI
89	"	PED	2	TcIII
90	"	PED	4	TcIII
11	"	Petorca (PET)	2	ND
9	"	PET	2	TcI
24	"	PET	2	ND
25	"	PET	2	TcIII
27	"	PET	4	ND
43	"	PET	1	TcIII
56	"	PET	2	TcIII
59	"	PET	1	TcI
92	"	PET	3	Hybrid
102	"	PET	3	ND
103	"	PET	8	TcI
11–123	V	PET	1	ND
18–130	V	PET	7	TcI
31–143	V	PET	3	ND
58–170	V	PET	3	ND
60–172	V	PET	2	TcIII
61–173	V	PET	2	ND
63–175	V	PET	5	TcIII
64–176	V	PET	4	TcI
65–177	V	PET	2	TcIII
69–181	V	PET	4	Hybrid
72–184	V	PET	5	TcIII
75–187	V	PET	2	TcIII
76–188	V	PET	1	TcI
78–190	V	PET	1	TcIII
14	V	Sobrante (SO)	2	TcI
15	V	SO	2	TcI
16	V	SO	1	TcI
17	V	SO	2	TcI
64	V	SO	3	Hybrid
88–200	V	San Felipe	1	TcI
	M			
68	M	Calera de Tango (CT)	1	TcI
69	M		2	TcI
2–114	M	CT	3	TcIII
33–145	M	CT	9	TcI
34–146	M	CT	4	TcIII
35–147	M	CT	3	Hybrid
37–149	M	CT	3	TcI
39–151	M	CT	4	TcIII
45–157	M	CT	5	TcIII
49–161	M	CT	2	TcIII
50–162	M	CT	5	TcI
2	M	Sanatorio	5	Hybrid

[a]Based on the genotype of predominant *T. cruzi* clones and total number of alleles, as described (the section on 'Materials and methods').

[b]Lineage assignation was performed using three different methods, as described (the section on 'Materials and methods'). The nomenclature is according to Zingales *et al.* (2009): TcI (DTU I), TcII (DTU IIb), TcIII (DTU IIc), TcIV (DTU IIa), TcV (DTU IId) and TcVI (DTU IIe). Hybrid corresponds to TcV and TcVI (de Freitas *et al.*, 2006).

210 FU, respectively. The ratio of the heights of the second peak divided by the major one is 0.53, but the distance between them is 44 bp. This distance is beyond the 32 bp that separates two alleles of a heterozygous clone,

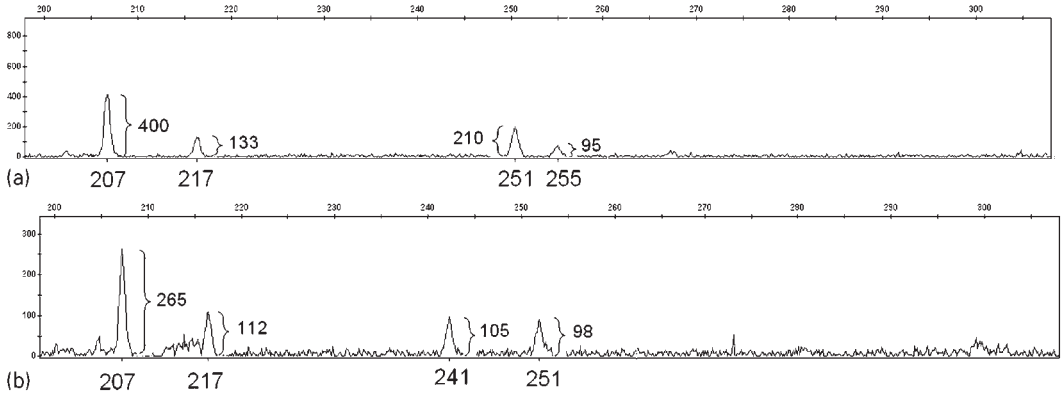

FIG. 1. Electropherogram of two midgut *Triatoma infestans* samples analysed by the SCLE10 microsatellite marker. The panels a and b correspond to the samples code 24 and 25 from the V region. The number below each peaks are the corresponding allele in base-pair length. The numbers beside each peak represent the height of each one in fluorescence units (FU). The genotype of predominant clones of both samples was estimated as 207/217 with two minimal clones (see the section on 'Materials and method').

according to the data previously found by de Freitas *et al.* (2006) and Venegas *et al.* (2009a). For this reason, when this happens, it is necessary to look for a third allele that is within the maximum range expected. In this sample, the allele 217 meets this requirement, whose heights ratio among the 217 and 207 alleles is 0.33, just the minimum ratio accepted for a heterozygous clone. The minimum ratio was decided based on those found in heterozygous clones previously described (de Freitas *et al.*, 2006; Venegas *et al.*, 2009a). So, the genotype of the predominant clone of this sample is 207/217, and the minimum number of clones detected in it is 2. In the triatomine sample 25, the major and second alleles are the 207 and 217, respectively, which are within the allowed distance for heterozygous *T. cruzi* clone (Fig. 1b). As well as the 24 sample, the minimal number of clones detected is 2.

A similar analysis was conducted using the SCLE11 and MCLE01 markers with the exception that the maximum distances of separation between the major and the second peaks were 16 and 20 bp, respectively. This approach was applied with corresponding SCLE10, SCLE11 and MCLE01 locus in each *T. cruzi* sample (Table 1).

The minimum number of *T. cruzi* clones in each triatomine sample was estimated based on the genotype of the predominant clone plus the total number of alleles found in each sample divided by 2. Thus, if the predominant clone is homozygous and the sample has three additional alleles (a total of four alleles), it is concluded that the MNC is 3, because an odd number of alleles is considered as another clone. By contrast, in the same sample, if the predominant clone is heterozygous, it is concluded that the MNC is 2, as occur for samples 24 and 25 (Fig. 1).

Determination of Genetic Variation within Each Population

After the genotype of each predominant *T. cruzi* clone was determined in the different midgut samples of triatomines, the genetic parameters within each population were estimated (Table 2). Allele number (a) was determined using the MSA software (Dieringer and Scholötterer, 2003); the observed heterozygosis (Ho), expected heterozygosis (He) and Hardy–Weinberg equilibrium (HWE) were determined using the Guo and Thompson (1992) method and the program Arlequin (Excoffier *et al.*, 2005).

Analysis of Genetic Differentiation

This was conducted by three approaches: locus by locus Fisher method, multilocus

TABLE 2. *Microsatellite diversity of three Chilean* T. cruzi *population samples found in* T. infestans *midgut from the III, V and Metropolitan regions*[a]

Population sample	Parameter	Locus		
		SCLE10	SCLE11	MCLE01
III	n	12	12	12
	a[b]	10	10	10
	Ho[c]	0.000	0.083	0.384
	He[c]	0.978	0.952	0.941
	HWE[c]	0.000*	0.000*	0.000*
V	N	35	32	33
	a	17	24	16
	Ho	0.241	0.414	0.393
	He	0.874	0.953	0.904
	HWE	0.000*	0.000*	0.000*
M	N	8	11	8
	a	7	15	11
	Ho	0.250	0.818	0.666
	He	0.766	0.9480	0.954
	HWE	0.000*	0.174	0.015*
Total number of different alleles observed		27	34	26

[a] III, V and M: groups of midgut samples from 15, 37 and 12 *Triatoma infestans* captured in the III, V and Metropolitan Chilean regions. Samples with information on at least two loci were considered.
[b]Using MSA program (Dieringer and Scholötterer, 2003).
[c]Using Arlequin program (Excoffier *et al.*, 2005).
n=individual number genotyped by locus; a=allele number; He=expected heterozygosis; Ho=observed heterozygosis; HWE=*P* value from the Hardy–Weinberg equilibrium test; asterisk=significant differences.

analysis by the Fisher method across three loci and analysis of molecular variance (AMOVA). The genetic markers used in the three approaches were SCLE10, SCLE11 and MCLE01, described previously (Oliveira *et al.*, 1998; de Freitas *et al.*, 2006). The genotypic differentiation by the first and the second approach were analysed with the Genepop program using the Markov algorithm (Raymond and Rousset, 1995a, *b*). The AMOVA studies were performed with the Arlequin software using the genotype data determined from the predominant *T. cruzi* clone in each sample with unknown gametic phase, inferring the haplotypes from the distance matrix, using F_{ST} as genetic distance and a significant *P* value of genetic differences among population samples of 0.05 (Weir and Cockerham, 1984; Excoffier *et al.*, 1992).

Lineage Determination of Predominant *T. cruzi* Clones

The nomenclature used in the present paper for the different lineages, according to the most recent agreement of several researchers, was: TcI (DTU I), TcII (DTU IIb), TcIII (DTU IIc), TcIV (DTU IIa), TcV (DTU IId) and TcVI (DTU IIe) (Brisse *et al.*, 2000; Zingales *et al.*, 2009). However, since that by several criteria, the lineages TcV and TcVI are hybrids, and by microsatellite markers, correspond to the same phylogenetic cluster, in the present paper, they were named as Hybrid (de Freitas *et al.*, 2006).

In order to have a robust assignation of the different *T. cruzi* lineages, three different methods were used simultaneously with the Geneclass program: the method based on microsatellite lengths described by Goldstein *et al.* (1995), the method based on allelic

frequencies described by Paetkau *et al.* (1995) and the Bayesian method described by Rannala and Mountain (1997). At least two coincidences in the same lineage with different methods were necessary to assign the final lineage of each predominant *T. cruzi* clone. Otherwise, the predominant clone was considered as not determined (ND, Table 1).

Correlation Analysis between Genetic and Geographical Distances

Correlation among genetic and geographical distances was estimated by the Mantel test using the Arlequin program and the linear genetic distance $F_{ST}/(1-F_{ST})$ (Mantel, 1967; Schneider *et al.*, 2000).

Phylogenetic Reconstruction

In order to determine the genetic relationships between the Chilean *T. cruzi* sample populations and its foreign counterparts, a phylogram tree was constructed by the program PHYLIP 3.5c (Felsenstein, 1989) using the allele frequencies obtained with the SCLE10, SCLE11 and MCLE01 microsatellite loci. In order to determine the best phylogenetic reconstruction according to the published tree topology obtained with microsatellite markers (de Freita *et al.*, 2006; Venegas *et al.*, 2009a), three measures of genetic distances were tested. Two measurements based on the infinite allelic model, the chord distance Dc of Cavalli-Sforza and Edwards (1967) and the shared allele distance of Bowcock *et al.* (1994). In addition, genetic distance based on the stepwise mutation model of Goldstein *et al.* (1995) was also used. The 'Neighbour-Joining' algorithm (Saitou and Nei, 1987) was employed for tree construction and 1000 bootstrap iterations were used to test the confidence of the nodes (Felsenstein, 1989).

Published Data of Laboratory Cultured *T. cruzi* Clones and Strains Genotyped with Different Microsatellite Markers

This was taken from the published data of de Freitas *et al.* (2006) and Venegas *et al.* (2009a). The several *T. cruzi* clones and strains were grouped based on their geographic origin (Table 3) in which the majority corresponded to regions of Brazil, such as Amazon (AM) (−4°30′S, −64°41′W), Espiritu Santos (ES), Minas Gerais (−19°42′S, −44°1′W), Para (PA), Rio de Janeiro (RJ), Sao Paulo (SP), Goias (GO) (−14°53′S, −49°34′W) and Piauí (PI).

RESULTS

Genetic Variation within Each *T. cruzi* Population

This study was performed in order to determine whether geographic origin has any impact on the genetic characteristics of *T. cruzi* populations. According to this goal, *T. cruzi* Chilean and foreign population samples were analysed. The Chilean population samples were from triatomine midguts collected in the III, V and Metropolitan regions (see the section on 'Materials and methods' and Table 1). The III region has a hyper-arid climate and is separated by several hundred kilometres of desert from the V and M regions. However, both the V and M regions have similar ecological conditions with a Mediterranean climate characterised by a moderate average temperature (20°C), occasional rain during the winter and close one from another. The sampling point of III region was located 560 and 707 km from the corresponding points in the V and M regions, respectively. In addition, the sampling points in these two last regions were located at 147 km.

The foreign *T. cruzi* population samples correspond to published data by de Freitas *et al.* (2006) and Venegas *et al.* (2009a). These published data are composed of *T. cruzi* clones and strains isolated and cultured in the laboratory, mainly from regions of Brazil. Most *T. cruzi* samples were from the Amazon (−4°30′S, −64°41′W), Goias (−14°53′S, −49°34′W) and Minas Gerais

TABLE 3. *Data previously published by* de Freitas *et al.* (2006) *and* Venegas *et al.* (2009a) *corresponding to* T. cruzi *clones and strains cultured in the laboratory and typed with five microsatellite markers. The groups analysed in the present manuscript are shown in the last column*

Lineages[a]	Strains	SCLE10	SCLE11	MCLE01	Origin[b]	Reference	Code: population samples studied here
	Rb1	255/259	139/139	128/136	AM/Brazil	de Freitas	TcI-Br-A
	1502	255/255	141/143	136/136 "	AM/	de Freitas	"
TcI	SE	287/297	143/151	136/140 "	AM/	de Freitas	"
(Z/Z)	Rb2	251/255	139/141	136/142 "	AM/	de Freitas	"
	1523	255/255	143/147	144/144 "	AM/	de Freitas	"
	Cutia cl1	255-255	143/143	132/132	ES/Brazil	Venegas	TcI-Br-B
	1004	253/255	139/139	130/130 "	MG/	de Freitas	"
	1006	253/255	139/141	130/130 "	MG/	de Freitas	"
	1001	255/255	143/143	130/130 "	MG/	de Freitas	"
	SilvioX10 cl1	235/275	153/153	130/130	PA/Brazil	de Freitas	"
	D7	245/253	143/143	134/142	RJ/Brazil	de Freitas	"
	Gamba cl1	253/255	143/143	130/130	SP/Brazil	de Freitas	"
	13379 cl7	239/251	139/143	132/132	Bolivia	Venegas	TcI-Br-C
	Colombiana	235/257	139/139	136/136	Colombia	de Freitas	"
	Col18/05	253/257	139/139	136/136	Colombia	de Freitas	"
	A83	253/253	141/141	134/138	FG	de Freitas	"
	A87	253/253	141/141	142/150	FG	de Freitas	"
TcII	GOCH	271/281	153/159	128/150	GO/Brazil	de Freitas	TcII-Br-G
(Y/Y)	580	279/279	149/149	130/134"	GO/	de Freitas	"
	183744	281/281	149/149	130/134"	GO/	de Freitas	"
	578	281/281	149/155	130/148"	GO/	de Freitas	"
	581	277/277	153/161	130/148"	GO/	de Freitas	"
	577	273/281	151/155	152/154"	GO/	de Freitas	"
	Esm cl3	289/289	151/159	126/138	MG/Brazil	Venegas	TcII-Br-M
	Mas 1 cl1	271/289	151/151	130/130 "	MG/	Venegas	"
	Tu 18 cl11	289/289	149/163	124/132 "	MG/	Venegas	"
	GMS	273/285	149/157	126/134 "	MG/	de Freitas	"
	84	267/271	153/155	126/134 "	MG/	de Freitas	"
	JSM	267/275	143/151	128/128 "	MG/	de Freitas	"
	Tu18 cl11	255/285	149/163	128/130 "	MG/	de Freitas	"
	1043	275/281	157/157	128/134 "	MG/	de Freitas	"
	169/1	273/275	149/149	128/138 "	MG/	de Freitas	"
	JAF	269/275	143/149	130/134 "	MG/	de Freitas	"
	1005	275/275	153/155	130/156 "	MG/	de Freitas	"
	209	269/275	153/155	132/132 "	MG/	de Freitas	"
	Ig539	281/281	151/153	132/150 "	MG/	de Freitas	"
	JG	273/275	145/149	134/136 "	MG/	de Freitas	"
	JHF	273/273	149/149	134/136 "	MG/	de Freitas	"
	207	245/251	151/155	134/142 "	MG/	de Freitas	"
	239	271/275	151/157	134/150 "	MG/	de Freitas	"
	84Ti	271/271	143/147	136/136 "	MG/	de Freitas	"
	200pm	277/277	153/155	136/136 "	MG/	de Freitas	"
	Be62	273/275	155/155	136/138 "	MG/	de Freitas	"
	Gil	271/271	155/157	136/144 "	MG/	de Freitas	"
	803	271/281	145/145	136/152 "	MG/	de Freitas	"
	MPD	267/271	143/153	138/138 "	MG/	de Freitas	"
	1931	275/275	141/153	140/140 "	MG/	de Freitas	"
	1014	269/275	149/149	140/142 "	MG/	de Freitas	"

TABLE 3. *Continued*

Lineages[a]	Strains	SCLE10	SCLE11	MCLE01	Origin[b]	Reference	Code: population samples studied here
	CPI95/94	277/277	149/155	124/132	PI/Brazil	de Freitas	TcII-Br-X
	OPS27/94	287/297	149/159	132/136	PI/	de Freitas	"
	GLT593	271/285	151/151	136/136	RJ/	de Freitas	"
	GLT564	275/275	151/151	136/136	RJ/	de Freitas	"
	Y	273/273	153/153	130/130	SP/	de Freitas	"
TcIII	M5631 cl5	245/245	155/155	134/134	PA/Brazil	Venegas	TcIII-Br
(X/X)	M6241 cl6	255/255	151/153	130/132	PA/	Venegas	"
	3663	245/245	155/155	128/132	AM/	de Freitas	"
	222	215/251	151/155	132/132	MG/	de Freitas	"
	115	245/251	153/153	132/132	MG/	de Freitas	"
	226	245/251	153/155	132/132	MG/	de Freitas	"
	3869	249/249	153/157	132/132	AM/	de Freitas	"
	231	245/251	151/155	134/134	MG/	de Freitas	"
Hybrid	167	237/275	153/153	130/130	MG/Brazil	de Freitas	H-Bra
TcV–TcVI	1022	233/275	153/153	130/134	MG/	de Freitas	"
(X/Y)	182	237/275	153/157	130/146	MG/	de Freitas	"
	CLBrener	237/275	153/159	132/148	RS/Brazil	Venegas	"
	MN cl2	255/289	149/149	130/132	IV/Chile	Venegas	H-Chi
	NR cl3	255/289	149/165	128/130	Salvador/Chile	Venegas	"
	Tula cl2	289/289	149/151	130/130	IV/Chile	de Freitas	"
	Tula 1954	237/273	153/159	132/132	IV/Chile	Venegas	"

[a]Each lineage is composed of *T. cruzi* clones and strains isolated and cultured in the laboratory using different microsatellite markers (de Freitas *et al.*, 2006; Venegas *et al.*, 2009a). The nomenclature used corresponds to the recent agreement: TcI (DTU I), TcII (DTU IIb), TcIII (DTU IIc), TcIV (DTU IIa), TcV (DTU IId) and TcVI (DTU IIe) (Brisse *et al.*, 2000; Zingales *et al.*, 2009). Hybrid corresponds to TcV and TcVI (de Freitas *et al.*, 2006).

[b]Brazil regions: Amazon (AM) (−4°30′S, −64°41′W), Espiritu Santos (ES), Minas Gerais (−19°42′S, −44°1′W), Para (PA), Rio de Janeiro (RJ), Sao Paulo (SP), Goias (GO) (−14°53′S, −49°34′W) and Piauí (PI). France Guyana (FG).

(−19°42′S, −44°1′W) (de Freitas *et al.*, 2006) (Table 3).

The intestinal contents of 15, 37 and 12 triatomines from the III, V and M regions positive by conventional PCR detection for *T. cruzi* DNA (Dorn *et al.*, 1999), were analysed with the SCLE10, SCLE11 and MCLE01 microsatellite markers (Oliveira *et al.*, 1998; de Freitas *et al.*, 2006; Venegas *et al.*, 2009a), as shown in Table 1. The genotype of the predominant *T. cruzi* clone was determined as described (Venegas *et al.*, 2010) and the genetic parameters within each population were estimated (Table 2). As shown, the total number of alleles observed in the Chilean population samples for SCLE10, SCLE11 and MCLE01 markers were 27, 34 and 26, respectively, which means that the total number of possible haplotypes was 23 868. In other words, in theory, the present methodology using these three microsatellite markers has the potential resolving power to differentiate 23 868 *T. cruzi* clones. In addition, as expected for clonal populations almost all loci were not found in Hardy–Weinberg equilibrium, as

TABLE 4. *Analysis of genotypic differentiation using the SCLE10, SCLE11 and MCLE01 loci for each Trypanosoma cruzi group pair*

Locus	Groups compared[a]		*P* value[b]	SE
SCLE10[c]	III	V	0.00025⋆	0.00010
	III	M	0.02763⋆	0.00113
	V	M	0.65085	0.00432
SCLE11	III	V	0.29116	0.00542
	III	M	0.32907	0.00327
	V	M	0.44513	0.00487
MCLE01	III	V	0.12652	0.00313
	III	M	0.07590	0.00132
	V	M	0.06904	0.00211
Across three loci (Fisher method)				
	III	V	0.000745⋆	
	III	M	0.023990⋆	
	V	M	0.251287	

[a]III, V and MR: groups of midgut samples from 15, 37 and 12 *Triatoma infestans* captured in the third, fifth and Metropolitan regions, respectively. Samples with information on at least two loci were considered.
[b]Markov chain parameters: dememorisation=10 000; Batches=100; iterations per batch=5000.
[c]Locus by locus using the exact G test (see the section on 'Materials and methods').
⋆$P<0.05$.

was evidenced by the significant differences among observed and expected heterozygosis. Interestingly, thought this is the first glance, these genetic characteristics were more similar among the V and M regions than among III and V, or III and M regions. For instance, a clear difference in heterozygosis between the III and V regions and between the III and M regions was observed.

Interpopulation Analysis

In order to determine whether the three *T. cruzi* Chilean population samples from triatomine midgut were genetically differentiated, three analytical methods were used: a locus by locus and multilocus analysis using the Fisher's exact test ((Raymond and Rousset, 1995a, *b*), and the AMOVA (Weir and Cockerham, 1984; Excoffier *et al.*, 1992). With the first approach and the SCLE10 locus, a significant genetic differentiation was detected both between III and V and between III and M *T. cruzi* population samples (Table 4).

With the second approach, the Fisher's exact method across those three loci, a significant genetic differentiation was detected between the III and V as well as between III and M (Table 4). The third approach confirmed the result obtained with the second one, although the contribution to the variance among the populations samples was low (4.12%), the F_{ST} of 0.0412 was statistically significant ($P=0.018$) (Table 5).

TABLE 5. *Multilocus AMOVA analysis of predominant* Trypanosoma cruzi *genotypes found in the midgut of* Triatoma infestans *from the III, V and Metropolitan regions*

Source of variation	df	Sum of squares	Variance components[b]	Percentage of variation
Among populations[a]	2	4.281	$0.03560V_a$	4.12
Within populations	125	103.586	$0.82869V_b$	95.88
Total	127	107.867	0.86429	
Fixation index	F_{ST}	0.04119		

[a]Populations: III, V and M: groups of midgut samples from 15, 37 and 12 *Triatoma infestans* captured in the III, V and Metropolitan regions. Samples with information on at least two loci were considered. Molecular distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').
[b]Significance tests (1023 permutations):
V_a and F_{ST}: P(rand. value>obs. value)=0.01760
P(rand. Value=obs. value)=0.00000
P(rand. value>=obs. value)=0.01760±0.00338.

TABLE 6. *Lineages of predominant* Trypanosoma cruzi *clones detected in the midgut of* T. infestans *triatomines from three Chilean regions (III, V and M)*

	No. (%) of *T. cruzi* clones found of each lineage[b]				
Sample groups[a]	TcI	TcII	TcIII	H	ND[d]
III (n^c=15)	4 (26.7)	0.0	7 (46.7)	0.0	4 (26.7)
V (n=37)	13 (35.1)	0.0	12 (32.4)	3 (8.1)	9 (24.3)
M (n=12)	5 (41.7)	0.0	5 (41.7)	2 (16.7)	0.0
Total (n=64)	22 (34.4)	0.0	24 (37.5)	5 (7.8)	13 (20.3)
Pair	*P* value[e]				
III/V	0.3478; 0.5	1	0.9326; 0.334	0.548	0.0312; 0.861
III/M	0.448	1	0.675; 0.795	0.188	0.106
V/M	0.683; 0.16	1	0.584	0.584	0.09

[a]III, V and M: groups of midgut samples from 15, 37 and 12 *Triatoma infestans* captured in the III, V and Metropolitan regions, respectively.
[b]Determined using the Geneclass program using simultaneously the methods of: Goldstein *et al.* (1995), Paetkau *et al.* (1995) and Rannala and Mountain (1997) (see the section on 'Materials and methods'). The lineage nomenclature used corresponds to the recent agreement (Zingales *et al.*, 2009).
[c] Triatomine number.
[d]ND=not determined.
[e]Significant (⋆P<0.05). Determined by Fischer's exact method (one value) or Chi-squared (two values).

Based on the previous results which showed that the *T. cruzi* population sample from the III region was genetically differentiated from its V and M counterparts, it was interesting to determine whether other biological characteristics could also be different among these *T. cruzi* population samples. Thus, we analysed the lineage

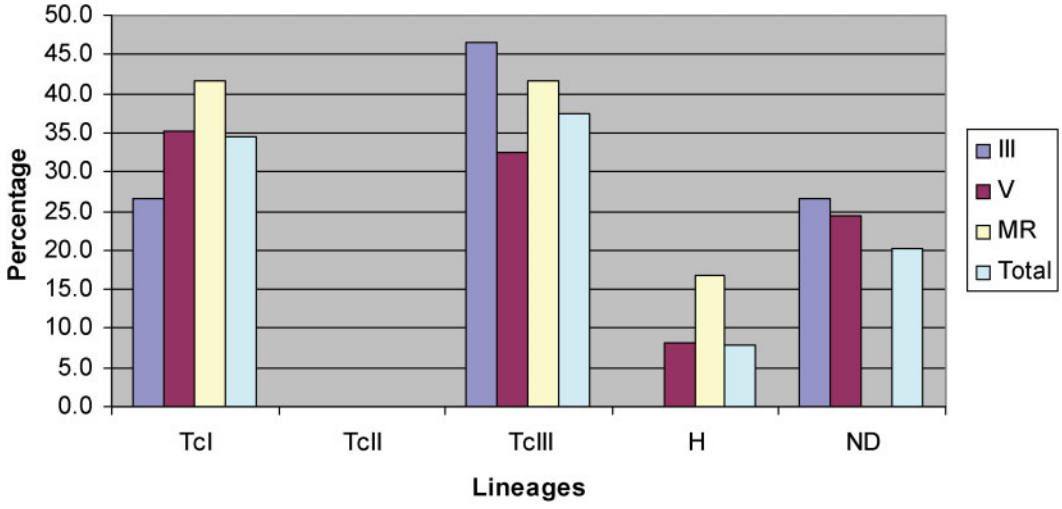

FIG. 2. Lineage frequency of predominant *T. cruzi* clones found in *T. infestans* midgut collected in the III, V and M regions. The lineage were determined using the microsatellite markers SCLE10, SCLE11 and MCLE01 using the Geneclass program by the methods of Goldstein *et al.* (1995), Paetkau *et al.* (1995) and Rannala and Mountain (1997) (see the section on 'Materials and methods'). The lineage nomenclature used corresponds to the recent agreement (Zingales *et al.*, 2009). Sample sizes were 15, 37 and 12 from III, V and M regions, respectively.

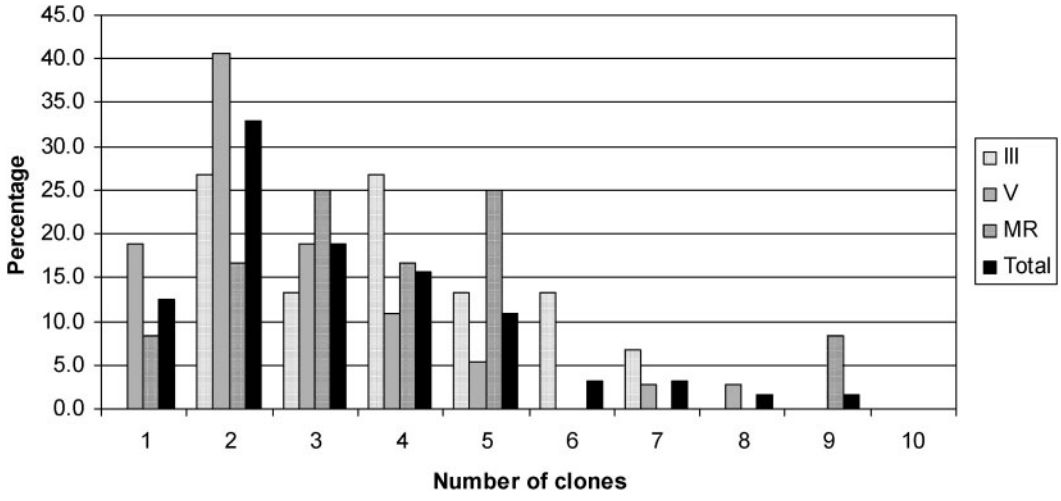

FIG. 3. Frequency of minimum number of *Trypanosoma cruzi* clones detected in *T. infestans* midguts by three microsatellite markers in population samples from the III, V and M regions. The markers used were SCLE10, SCLE11 and MCLE01, previously described (Oliveira *et al.*, 1998). The population samples included 15, 37 and 12 triatomine insects from the III, V and M regions, respectively (see the section on 'Materials and methods').

and the minimal number of *T. cruzi* clones (MNC). In order to have a robust assignation of the lineages, three dissimilar methods were used (see the section on 'Materials and methods'). The results showed that no significant differences were observed among the predominant *T. cruzi* lineages of the III, V and M regions (Table 6). Interestingly, the most frequent lineage in regions was TcIII, followed by TcI, while the TcII and Hybrid (TcV–TcVI) lineages were less frequent (Fig. 2). In addition, no significant statistical differences were detected among the minimal number of *T. cruzi* clones comparing the III, V and M regions (Fig. 3).

With the aim to conduct a deeper study to try to identify the possible source of the genetic differentiation among these three Chilean *T. cruzi* population samples, further analyses were performed with the AMOVA method and the determination of the F_{ST} index among all corresponding subpopulation pairs. Thus, the *T. cruzi* subpopulation

TABLE 7. *Multilocus AMOVA analysis of TcIII predominant* T. cruzi *genotypes found in the midgut of* T. infestans *from the III, V and Metropolitan regions*

Source of variation	df	Sum of squares	Variance components[b]	Percentage of variation
Among populations[a]	2	4.311	$0.09162V_a$	10.41
Within populations	45	35.501	$0.78892V_b$	89.59
Total	47	39.812	0.88054	
Fixation index	F_{ST}	0.10406		

[a]Populations III, V and M: subgroups of TcIII predominant *T. cruzi* clones found in the midgut of *T. infestans* captured in the III, V and Metropolitan regions; each subgroup have 7, 12 and 5 individuals, respectively. Samples with information on at least two loci were considered. Molecular distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').

Significance tests (1023 permutations):

V_a and F_{ST}: P(rand. value>obs. value)=0.01173

P(rand. value=obs. value)=0.00000

P(rand. value>=obs. value)=0.01173$\pm$0.00389.

TABLE 8. *Genetic distances* F_{ST} *and corresponding P values among TcIII* Trypanosoma cruzi *population samples from the III, V and M Chilean regions*

F_{ST} Regions[a]	III	V	MR
III	0.00000		
V	0.14036	0.00000	
MR	0.14829	0.02028	0.00000
F_{ST} *P* values[b]			
III	…		
V	0.00879⋆	…	
M	0.01074⋆	0.58008	…

[a]Population samples: III, V and M: predominant *T. cruzi* clones found in the midgut of 7, 12 and 5 *Triatoma infestans* captured in the third, fifth and Metropolitan Chilean regions, respectively. Molecular distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').
[b]Significant values with 1023 permutations (⋆$P<0.05$).

samples within the TcI and TcIII lineages were analysed. The result showed that there was no significant differentiation among the TcI subpopulation samples from the III, V and M regions. The percentage of variation among samples was 7.35%, but this was not statistically significant ($P=0.235$) (data not shown). This result was confirmed determining the corresponding F_{ST} among all subpopulation pairs, since all *P* values were superior to 0.05 (data not shown).

A significant differentiation was observed among TcIII subpopulations, as is observed by the statistically significant variation and F_{ST} among *T. cruzi* subpopulation samples, 10.41 and 0.104, respectively ($P=0.012$, Table 7). It is worth mentioning that only the F_{ST} values among the III and V and the III and M samples were statistically significant, among samples V and M F_{ST} was not significant (Table 8).

Genetic Relationship of Chilean *T. cruzi* Populations with Their Counterparts from Some Other Latin American Regions

In order to determine whether the Chilean *T. cruzi* population samples were genetically differentiated from their counterparts in other countries, AMOVA analyses and their corresponding F_{ST} distances among pairs of subpopulations were performed. To obtain this goal, the first step was to determine whether the *T. cruzi* population samples available from the literature could be genetically differentiated within each lineage. Therefore, *T. cruzi* clones and strains from the published data of de Freitas *et al.* (2006) and Venegas *et al.* (2009a) were separated by geographical origin. Three groups within the TcI (TcI-A, TcI-B and TcI-C), three groups within TcII (TcII-Br-G, TcII-Br-M and TcII-Br-X), one group of TcIII (TcIII-Br) and two groups of TcV–TcVI (H-Bra and H-Chi) were produced (Table 3). The results showed that within TcI as well as the TcV–TcVI lineages, *T. cruzi* subpopulation samples are genetically differentiated, as is evidenced by their

TABLE 9. *Multilocus AMOVA analysis of TcI* T. cruzi *subpopulations from Brazil*

Source of variation	df	Sum of squares	Variance components[b]	Percentage of variation
Among populations[a]	2	6.847	$0.21309V_a$	16.98
Within populations	31	32.300	$1.04194V_b$	83.02
Total	33	39.147	1.25503	
Fixation index	F_{ST}	0.16979		

[a]Populations: TcI Brazilian subpopulations TcI-Br-A, TcI-Br-B and TcI-Br-C (Table 3). Distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').
Significance tests (1023 permutations):
V_a and F_{ST}: *P*(rand. value>obs. value)=0.00196
P(rand. value=obs. value)=0.00000
P(rand. value>=obs. value)=0.00196±0.00136.

TABLE 10. *Multilocus AMOVA analysis of TcV–TcVI* T. cruzi *subpopulations from Brazil and Chile*

Source of variation	df	Sum of squares	Variance components[b]	Percentage of variation
Among populations[a]	1	2.938	$0.23772V_a$	18.67
Within populations	14	14.500	$1.03571V_b$	81.33
Total	15	17.438	1.27344	
Fixation index	F_{ST}	0.18668		

[a]Populations: TcV–TcVI *T. cruzi* subpopulations H-Bra and H-Chi (Table 3). Distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').
Significance tests (1023 permutations):
V_a and F_{ST}: P(rand. value>obs. value)=0.00000
P(rand. value=obs. value)=0.02444
P(rand. value>=obs. value)=0.02444±0.00495.

statistically significant F_{ST} P values ($P<0.05$) (Tables 9 and 10, respectively). In contrast, no significant differentiation was detected among the TcII *T. cruzi* subpopulation samples (data not shown).

AMOVA analysis and the corresponding F_{ST} determinations within the TcI lineage showed that the Chilean subpopulation from the III region is not differentiated from any Brazilian counterpart, since in all pairs of comparisons the P values were superior to 0.05 (data not shown). On the contrary, both the V and M Chilean *T. cruzi* subpopulation samples are differentiated from the Brazilian TcI-Br-B sample ($P<0.05$).

The AMOVA and F_{ST} determinations of the Chilean and Brazilian TcIII subpopulation samples showed that in all pairs of comparisons, the Chilean samples are significantly differentiated from their Brazilian counterparts, since the P values of F_{ST} for each subpopulation pair is much less than 0.05 (Table 11).

TABLE 11. *Genetic distances* F_{ST} *and corresponding* P *values among TcIII* Trypanosoma cruzi *population samples from three Chilean regions and one Brazilian region*

F_{ST}				
Regions[a]	III	V	M	Brazil
III	0.00000			
V	0.14036	0.00000		
M	0.14829	0.02028	0.00000	
Brazil	0.17918	0.17148	0.16412	0.00000
F_{ST} P values[b]				
III	…			
V	0.00684*	…		
M	0.01758*	0.60254	…	
Brazil	0.00195*	0.00000*	0.00195*	…

[a]Population samples: III, V and M: predominant *T. cruzi* clones found in 7, 12 and 5 *Triatoma infestans* captured in the III, V and Metropolitan Chilean regions. A fourth population sample of eight Brazilian *T. cruzi* clones mainly from the Minas Gerais region (MG) was included. Molecular distance: number of different alleles (F_{ST}) (see the section on 'Materials and methods').
[b]Significant values with 1023 permutations ($P<0.05$).

Phylogenetic Analysis of Chilean and Foreign *T. cruzi* Subpopulation Samples

To estimate the phylogenetic relationships among the Chilean and foreign *T. cruzi* subpopulation samples, phylogenetic trees were constructed based on four different genetic distances; the Cavalli-Sforza and Edwards (1967) chord distance, the Nei *et al.* (1983) distance, the Bowcock *et al.* (1994) distance based on the proportion of shared alleles and the Goldstein *et al.* (1995) distance based on the stepwise mutation model. According to the known *T. cruzi* phylogeny, the best result was obtained with the Bowcock method (Fig. 4), which is characterized by the main monophyletic clusters TcI and TcII located at the extremes of the tree. In addition, as indicated in the literature (Westenberger *et al.*, 2005; de Freitas *et al.*, 2006; Venegas *et al.*,

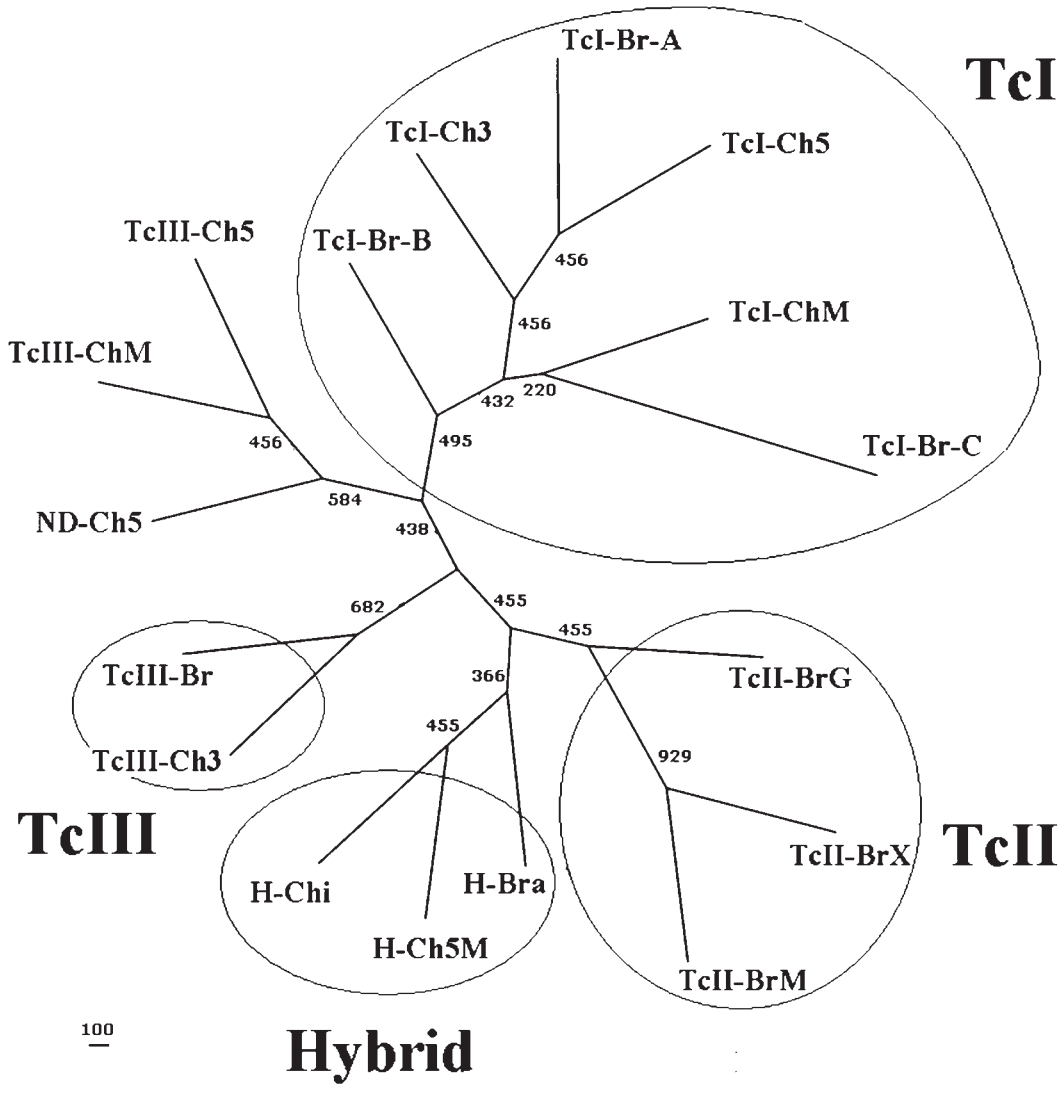

FIG. 4. Unrooted phylogenetic tree of the *T. cruzi* population samples from the midgut of triatomines from Chilean and Brazilian regions. The phylogenetic reconstruction was performed using the allele sharing distance method (Bowcock *et al.*, 1994) and the SCLE10, SCLE11 and MCLE01 microsatellite markers (Oliveira *et al.*, 1998). The *T. cruzi* population samples from triatomine midgut were directly genotyped and correspond to groups of insects collected in the III (TcI-Ch3 and TcIII-Ch3), V (TcI-Ch5, TcIII-Ch5 and ND-Ch5) and Metropolitan (TcI-ChM and TcIII-ChM) Chilean regions. The lineage nomenclature corresponds to the recent agreement (Zingales *et al.*, 2009); they were assigned based on three simultaneous methods and correspond to the following *T. cruzi* population samples: TcI lineage (TcI-Ch3, TcI-Ch5 and TcI-ChM) and TcIII lineage (TcIII-Ch3, TcIII-Ch5 and TcIII-ChM). H-Ch5M corresponds to a *T. cruzi* population sample of the TcV–TcVI lineage from triatomine midguts collected in the V and Metropolitan Chilean regions. The laboratory cultured *T. cruzi* population samples correspond to published data of de Freita *et al.* (2006) and Venegas *et al.* (2009a) and are the following: TcI from Brazil Amazon (TcI-Br-A), TcI mainly from Brazil-Minas Gerais (TcI-Br-B), TcI from Bolivia, Colombia and France Guyana (TcI-C), TcII from Brazil-Goias (TcII-Br-G), TcII from Brazil-Minas Gerais (TcII-Br-M), TcII from several Brazilian regions (TcII-Br-X), TcIII mainly from Minas Gerais (TcIII-Br), TcV–TcVI from Minas Gerais (H-Bra) and TcV–TcVI from Chile (H-Chi) (see Table 3 and the section on 'Materials and methods' for more details).

TABLE 12. *Matrix of Slatkin linearized* F_{ST} *genetic distance [*$F_{ST}/(1-F_{ST})$*] and geographic distances of TcIII* Trypanosoma cruzi *population samples from three Chilean regions and one Brazilian region*

$F_{ST}/(1-F_{ST})$[a]				
Regions[b]	III	V	M	Brazil
III	0.00000			
V	0.16328	0.00000		
M	0.17411	0.02070	0.00000	
Brazil	0.21829	0.20697	0.19635	0.00000
Geographic distance (km)[b]				
Regions				
III	0			
V	560	0		
M	707	147	0	
Brazil	4056	4616	4763	0

[a]Reference: Slatkin et al. (1995).
[b]Population samples: III, V and M: predominant *T. cruzi* clones found in 7, 12 and 5 *Triatoma infestans* captured in the III, V and Metropolitan Chilean regions, respectively. A fourth population sample of eight Brazilian *T. cruzi* clones mainly from the Minas Gerais region (MG) was included. The geographic distances were determined as the smallest distance between two regions with the Google heart online map (see the section on 'Materials and methods').

2009a), the hybrid lineage (TcV–TcVI) is located between the nodes of TcIII and TcII. In agreement with the results of AMOVA and F_{ST} determinations, the three TcI Chilean *T. cruzi* subpopulation samples are located in the TcI monophyletic node, while the TcIII Chilean subpopulation samples are located in two separate clusters: on the one hand, the TcIIICh3 sample forms an internal node with the single sample from Brazil TcIII-Br, whereas the TcIIICh5 and TcIIIChM Chilean samples form another cluster with the unknown ND-V sample. Interestingly, the hybrid sample including *T. cruzi* clones from the V and M regions (H-Ch5M) is grouped together with the Chilean laboratory cultured *T. cruzi* clones belonging to TcV–TcVI lineage (H-Chi).

Correlation among Chilean and Brazilian TcIII Subpopulations

In order to determine whether the geographic distances have played an important role in the differentiation of these *T. cruzi* subpopulation samples, we performed the Mantel test (Mantel, 1967). A correlation coefficient of 0.71 was obtained with a significant P value of 0.038.

Table 12 shows the matrices of lineal genetic distance and geographical distance of the Chilean and Brazilian TcIII *T. cruzi* subpulations.

DISCUSSION

The evidence presented in this study strongly suggests that the Chilean *T. cruzi* populations from the midgut of domestic *T. infestans* are geographically differentiated. The following evidences support this conclusion.

First, the significant differences detected with the SCLE10 marker among the *T. cruzi* populations from the III and V regions as well as between the III and Metropolitan regions. It is very interesting that using only a single locus, it is possible to detect significant genotypic differentiation, although methods based on a single locus have less power of resolution than those based on multiple loci. One possible explication that SCLE10 could detect this differentiation may be the high polymorphism we detected, as is illustrated

by its 27 total alleles. Considering that *T. cruzi* is meanly a diploid organism (Tibayrenc *et al.*, 1986; Oliveira *et al.*, 1998; Brisse *et al.*, 2000), this means that using this single SCLE10 marker, in theory, it is possible to distinguish $n(n-1)/2=27(27-1)/2=351$ different combinations or genotypes, which may explain why it was possible to detect subtle genetic differences between these two Chilean *T. cruzi* population samples.

Second, the significant genetic differences detected by the multilocus Fisher's exact method, using the microsatellite markers SCLE10, SCLE11 and MCLE01 between the III and V regions, as well as between the III and M regions of *T. cruzi* population samples. This result confirms the above evidence using a more powerful multilocus method. Considering that the total alleles observed with each microsatellite marker (27, 34 and 26 with SCLE10, SCLE11 and MCLE01, respectively), the total number of different haplotypes would be $27 \times 34 \times 26 = 23\,868$, in absence of linkage disequilibrium, which could explain the resolving power to detected genetic differences among these *T. cruzi* populations, apparently much higher than the resolving power of isoenzymes, traditionally used in the study of *T. cruzi* populations (Tibayrenc *et al.*, 1986; Tibayrenc *et al.*, 1990; Brisse *et al.*, 2000).

Third, the AMOVA of haplotype distribution among the *T. cruzi* population samples from the III and V regions, and from III and M regions. This result reinforces that the genetic differentiation between the III and the V and M regions most probably is real and not due to a fortuitous event or an experimental artefact, because the AMOVA and the Fisher's exact methods are based on dissimilar principles: the first on haplotype variance and the second on genotype frequences (Raymond and Rousset, 1995a, *b*).

Fourth, the determination of the genetic distance F_{ST} among the *T. cruzi* population samples from III, V and M regions. The fact that the F_{ST} values comparing III and V, and III and M *T. cruzi* population samples were statistically significant reinforced the first observations which showed that the *T. cruzi* population from the III region is genetically differentiated from its V and M counterparts. It is worth mentioning that important different geographic characteristics exist between the two triatomine population niches formed by the III region and V–M. They are separated by about 700 km and have dissimilar geographic, climatic and demographic conditions (Apt *et al.*, 1987). These different ecological niches also are associated with different peridomestic mammals as possible reservoirs (Rozas *et al.*, 2007). We think that these ecological and geographical factors of *T. cruzi* have favoured the conditions for population structuring, originating two dissimilar genetic *T. cruzi* subpopulations. On the other hand, the V and M regions are separated by only 150 km and they have similar climate and a high rate of human migration; for these reasons, it is very probable that these two regions may really constitute a single ecotope and in consequence, have a single *T. cruzi* 'deme' or subpopulation.

In order to determine whether the genetic differentiation detected between the *T. cruzi* population samples III and V or between III and M is manifested in another genetic characteristic, we studied the distribution of clones in these samples. Unexpectedly, no significant difference in distribution was observed. However, it is worth recalling the high percentage of multiclonal *T. cruzi* samples observed in all populations, illustrated by the presence of more than one *T. cruzi* clone in each triatomine midgut sample. This evidence is concordant with the previous results in showing that in the triatomine intestine the *T. cruzi* parasites have a more favourable environment for growth and development than in the highly selective environment of the mammalian host (Oliveira *et al.*, 1998; Coronado *et al.*, 2006; Venegas *et al.*, 2010).

At first glance, analysing simultaneously the distribution of the different *T. cruzi*

lineages among the III, V and M regions, no statistically significant dissimilarities were detected. However, it is important to mention that the lineage distributions compared here correspond to lineages of predominant *T. cruzi* clones; therefore, we cannot discard that the distribution of minor clones may have greater differences among these *T. cruzi* populations samples or that there may exist subpopulations within each lineages with asymmetric genetic distribution. To determine whether this latter possibility could be occurring in the populations studied here, we proceeded to perform AMOVA studies and determinations of F_{ST} between subgroups or subpopulations consisting of a single lineage. The results obtained showed that statistically significant dissimilar haplotype distributions and F_{ST} values were found comparing TcIII subpopulation samples either between the III and V regions or between the III and M regions. However, no significant differences were found comparing the same subpopulation pairs only containing TcI individuals. This result not only confirms again the fact that the III region *T. cruzi* population is genetically different from its V and M region counterparts, but also strongly suggests that the III population of *T. cruzi* is a discrete entity, physically separated from the other two southern regions, and that this genetic differentiation is only due to the asymmetrical distribution of TcIII *T. cruzi* clones. To our knowledge, this is the first time that in Chile, it has been shown by population genetics methods that *T. cruzi* populations really are structured, and not only between lineages; we also demonstrated differential distributions within the same lineage. It is true that in other previous studies, analysing kDNA with restriction enzymes or/and with minicycle probes, some dissimilar distributions of lineages among different localities were observed. Nevertheless, in none of these studies, statistical analyses were conducted to support their observations, and they were mainly based on the analysis of laboratory cultured parasites (Sanchez *et al.*, 1993; Solari *et al.*, 2001; Torres *et al.*, 2004). In this regard, there is a very interesting recent report from Brazil which used several microsatellite markers to show that between different regions, there is subtle but statistical significant genetic differentiation among TcIII *T. cruzi* populations and that this phylogeographic differentiation could have some impact in clinical manifestations of Chagas disease (Llewelyn *et al.*, 2009a, b).

The very low frequency of the hybrid lineage TcV–TcVI detected in all the population samples studied here (7.8%) was similar to that found in a previous study of our group analysing *T. cruzi* samples from chronic chagasic patients (Venegas *et al.*, 2010). However, this low percentage of the hybrid lineage is markedly different than published reports of *T. cruzi* stocks from triatomines, where 21–51% of stocks were characterized as TcIId, corresponding to the old Z2bol nomenclature and the current hybrid TcV lineage (Sanchez *et al.*, 1993; Venegas *et al.*, 1997; Torres *et al.*, 2004; Zingales *et al.*, 2009). Some of the causes which could explain this dissimilarity may be linked to the fact that here we are only typing the predominant *T. cruzi* clone found in each triatomine sample; therefore, minor *T. cruzi* clones such as the hybrid ones may not be detected. In addition, in the present paper, it was used as a direct method to type *T. cruzi* clones without previous isolating and culturing, as it was done in the cited literature (Sanchez *et al.*, 1993; Venegas *et al.*, 1997; Torres *et al.*, 2004). Another explanation to this dissimilarity could be related with the differences among the nature of molecular makers (Junqueira *et al.*, 2005; Coronado *et al.*, 2006; Tillerias *et al.*, 2006; Rozas *et al.*, 2007; Venegas *et al.*, 2009b).

The geographic distances separating these host insects may be a relevant factor involved in genetic differentiation of Chilean *T. cruzi* populations from triatomine midguts. The evidence which supports this suggestion is the correlation study using

the Mantel test (Mantel, 1967; Schneider *et al.*, 2000). Significant statistical correlation (correlation coefficient $r=0.71$ and $P=0.038$) was found between the Slatkin linearized F_{ST} genetic distance [$F_{ST}/(1-F_{ST})$] and the geographic distances of the III, V, M and Brazil TcIII subpopulation samples. This result is concordant with recent studies analysing *T. cruzi* TcIII isolates from the north and centre of Latin America with a large panel of 49 microsatellite markers and sequences of the glucose-6-phophate isomerase locus (Llewellyn *et al.*, 2009*a*). In this study, a significant statistical correlation was found between genetic and geographical distances among *T. cruzi* isolates from four localities of Latin America made up of population samples from the north (Brazil, Venezuela and Colombia), north of Bolivia, south of Bolivia and Paraguay.

With the aim to further obtain more information about the genetic relationship among the studied Chilean and foreign population samples, we conducted phylogenetic studies using different genetic distances. The best result, based on the expected topology and the high polymorphism of the three microsatellite markers used, was found using the infinite allelic model as genetic distance which corresponded to the shared genetic distance (Dc) and Neighbour-Joining method (Cavalli-Sforza and Edwards, 1967; Saitou and Nei, 1987). The tree topology was very similar to the described topology using five microsatellite markers, characterized by the most divergent TcI and TcII lineages at the each end of tree and the hybrid lineage node surrounded by the nodes of TCII and TCIII (de Freitas *et al.*, 2006; Venegas *et al.*, 2009a). The tree also reflects, shown by population genetics studies, the more homogenous nature of the TcI lineages from the III, V and M regions, because all these subpopulation samples were grouped in the same node. However, as expected, the TcIII from the III region was grouped in a distinct node in relation to the node in which the subpopulations in V and M regions were clustered. This phylogenetic construction strongly supports and validates the above population genetic results, giving more evidence that these results are probably not due to a biased sample or experimental error.

CONCLUSIONS

Taken together, the evidence shown in the present paper strongly supports the idea that the *T. cruzi* populations really are geographically structured in Chile and in other localities of Latin America. Second, in Chile, this geographical differentiation would be due to a dissimilar distribution of TcIII *T. cruzi* clones, at least in some regions. In addition, it was found that the genetic distance of TcIII populations is correlated with geographical distances among Chilean and foreign populations. The fact that the *T. cruzi* populations are geographical structured not only among lineages but even within one lineage could have relevant impact on the epidemiological and pathogenic characteristic of Chagas disease. These interesting aspects should be further investigated.

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