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Microsatellite loci-based distribution of *Trypanosoma cruzi* genotypes from Chilean chronic Chagas disease patients and *Triatoma infestans* is concordant with a specific host-parasite association hypothesis

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Abstract

The objective of this study was to investigate if there is specific host-parasite association in Chilean populations of *Trypanosoma cruzi*. For this purpose, two groups of parasites were analyzed, one from chronic chagasic patients, and the other from *Triatoma infestans* triatomines in three regions of the country. The first group consisted of four types of samples: parasites from peripheral blood of non-cardiopathic *T. cruzi* infected patients (NB); parasites from their corresponding xenodiagnosis (NX); parasites from peripheral blood of *T. cruzi* infected cardiopathic patients (CB) and parasites from their xenodiagnostics (CX). The *T. infestans* sample in turn was from three regions: III, V and M (Metropolitan). The genetic differentiation by the Fisher exact method, the lineage distribution of the samples, the molecular phylogeny and the frequency of multiclonality were analysed. The results show that not only are the groups of *T. cruzi* clones from Chagas disease patients and vectors genetically differentiated, but also all the sub-groups (NB, NX, CB and CX) from the III, V and M regions. The analysis of lineage distribution was concordant with the above results, because significant differences among the percentages of TcI, TcIII and hybrids (TcV or TcVI) were observed. The phylogenetic reconstruction with these Chilean *T. cruzi* samples was coherent with the above results because the four chagasic samples clustered together in a node with high bootstrap support, whereas the three triatomine samples (III, V and M) were located apart from that node. The topology of the tree including published *T. cruzi* clones and isolates was concordant with the known topology, which confirmed that the results presented here are correct and are not biased by experimental error. Taken together the results presented here are concordant with a specific host-parasite association between some Chilean *T. cruzi* populations.

Keywords

Chagas disease, *Trypanosoma cruzi*, *Triatoma infestans*, population genetics, phylogeny, host-parasite interaction

Introduction

The protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease, a parasitic zoonosis transmitted by triatomine insects which are commonly known as kissing bugs. In Chile, the triatomines *Triatoma infestans* and *Mepraia spinolai* are associated with the cycles of domestic and wild transmission of the disease, respectively. Despite major advances in the control of vector transmission and transfusion of its causative agent, the protozoan *Trypanosoma cruzi*, this is still the etiologic agent that causes the greatest amount of morbidity and mortality in the Americas (WHO 2000, 2002). *T. cruzi* has

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been divided 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 (RAPD), TCII was further subdivided into five discrete phylogenetic clusters (Brisse *et al.* 2000). Thus, *T. cruzi* was subdivided into six discrete typing units (DTUs) which were named as DTU1, DTU2a-DTU2e and later, TCI, TCIIa – TCIIe (Marcili *et al.* 2009). Today, the six *T. cruzi* lineages are called TcI, TcIV, TcII, TcIII, TcV and TcVI, respectively, which will be used in the present paper (Zingales *et al.* 2009; Zingales *et al.* 2012).

In patients, *T. cruzi* clones circulating in the blood may not be a representative sample of the infecting clones contained in the midgut of the triatomine bugs, because certain clones may be eliminated or their numbers reduced by the host immune response, or conversely, some clones could be favoured by the host conditions or tissue tropism (Machado *et al*. 2001; Burgos *et al*. 2007). In general, it is expected and observed that the midgut conditions of triatomines are more favourable to the proliferation of *T. cruzi* clones, since this environment has several rich nutrients for the parasites and is less selective (Schmidt *et al*. 1998; Devera *et al.* 2003). This same situation could influence the biological, pathological and genetic characteristics of *T. cruzi* present in the triatomine midgut (Schmidt *et al*. 1998; Devera *et al.* 2003).

There is currently no consensus as to what extent there is an association between specific lineages and host species, nor as to whether there is an association between lineages and geographic regions (Brisse *et al.* 2000; De Freitas *et al.* 2006; Marcili *et al.* 2009; Miles *et al.* 2009; Ramos-Ligonio *et al.* 2012). It is true that there has been a continuing tendency that certain lineages are more frequent in certain cycles (domestic or wild) or in certain host ecotypes (terrestrial or arboreal), but even so there is no absolute segregation, i.e. a particular lineage that appears only associated with a given host species or is found only in a particular geographic region (Brisse *et al.* 2000; De Freitas *et al.* 2006; Marcili *et al.* 2009; Rodrigues and Borges-Pereira 2010). Thus, for instance the TcI lineage, which was initially thought to be only associated with the wild cycle (Apt *et al.* 1987), has now been found spread over a wide range of hosts and vectors of both wild and domestic cycles and both terrestrial and arboreal host ecotypes (Marcili *et al.* 2009; Ramos-Ligonio *et al.* 2012). A similar situation occurs with the TcIII lineage, poorly studied until recently. Until a few years ago, this lineage was detected almost exclusively in mammalian hosts and insect vectors of the terrestrial host ecotype, in which armadillos were one of the paradigmatic examples (De Freitas *et al.* 2006; Marcili *et al.* 2009). Since the TcII and TcV lineages (formerly called TcIIb and TcIId, respectively) were also found in this host, it was postulated that this mammal is an example of a natural reservoir which would have favoured the hybridization of the TcII and TcIII lineages to generate the hybrid lineage TcV (Gaunt *et al.* 2003; Yeo *et al.* 2005; De Freitas *et al.* 2006; Miles *et al.* 2009; Rodrigues and Borges-Pereira 2010).

As in the case of lineage-host association, it is still unclear if there are associations between lineages and geographic regions (Brisse *et al.* 2000; De Freitas *et al.* 2006; Marcili *et al.* 2009, Zingales *et al.* 2012). Some tendencies have been found with some lineages, for instance the lineage TcII which is detected frequently in Brazilian chagasic patients but almost never in Chilean or Bolivian people, and the TcV linage which is very frequent in Bolivian and Chilean patients but very infrequent in Brazilians, which suggests that there is an association between certain lineages and geographic regions (Sanchez *et al.* 1993; Torres *et al.* 2004; De Freitas *et al.* 2006; Marcili *et al.* 2009). However, the majority of these reports come from data obtained from *T. cruzi* parasites cultured in laboratory media. It is important to mention that there are reports which show that laboratory culture changes the constitution of *T. cruzi* samples, increasing the concentration of some clones and reducing (even eliminating) others (Deane *et al.* 1984; Tibayrenc 2003). Therefore, the results obtained with samples cultured in the laboratory (isolates) do not necessarily reflect the distribution of clones and lineages found in nature.

In order to obtain more information on host – parasite lineage associations and geography - parasite lineage associations, in this study we analyzed a group of *T. cruzi* samples from the midgut of *Triatoma infestans* and a group of *T. cruzi* samples from infected humans. The triatomine samples came from three geographical regions of Chile. The chagasic samples came from non-cardiopathic and cardiopathic *T. cruzi* infected patients. The analysis of samples was performed by population genetics and molecular phylogenetic methods, using three microsatellite markers able to detect directly the lineages as well as the genotype of the majority clone both present in the insect midgut and in peripheral blood of *T. cruzi* infected patients.

Materials and Methods

Samples of triatomines

The triatomines were captured by the Program of Eradication of the Domiciliary Infestation of *Triatoma infestans* of the Chilean Ministry of Health in the period 2005–2007. Sample sizes in the study were 15, 37 and 12 *T. infestans* from the Atacama (III) Region (midpoint between Alto del Carmen and Tierra Amarilla 27°27´S, 70°15´W), Valparaiso (V) Region (mainly from Petorca, 32°16´S, 70°58´W) and the Metropolitan (M) Region (Calera de Tango, 33°37´S, 70°47´W), respectively (Table S1).

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 PCR, as described (Venegas *et al.* 2010).

T. cruzi DNA samples from chronic Chagas disease patients

Peripheral blood samples were obtained from Chagas disease Chilean patients classified as non-cardiopathic (NB, 29 individuals) and cardiopathic (CB, 24 individuals), according to criteria described before (Venegas *et al.* 2010). The patients were inhabitants from the IV region (between parallels 29°10´and 32°10´).

In addition, all individuals were studied by xenodiagnosis. The triatomine faeces from each xenodiagnostic assay obtained from the non-cardiopathic (NX, 35 individuals) and cardiopathic (CX, 26 individuals) groups were used to isolate *T. cruzi* DNA.

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 gels. Gels were stained with ethidium bromide (5ug/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 marked by fluorophores (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 (bp) of each allele was determined using cloned alleles from each marker as controls. In this analysis the minimum detectable peak height was set to 80 arbitrary fluorescence units (FU).

Genotyping of the predominant T. cruzi clone in each DNA sample

The determination of the genotype of the predominant *T. cruzi* clone in samples containing multiple alleles per locus was conducted taking into account the distance and the height in FU of the two highest peaks, the major and secondary peaks directly in the electropherogram, as described (Venegas *et al.* 2011).

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 two. Thus, if the predominant clone was homozygous and the sample had three additional alleles (a total of four alleles), it was concluded that the minimum number of clones (MNC) is 3, because an odd number of alleles was considered as another clone. By contrast, in the same sample if the predominant clone was heterozygous, it was concluded that the MNC is 2, as described (Venegas *et al.* 2011).

Analysis of genetic differentiation

This was conducted by the Fisher method across the loci SCLE10, SCLE11 and MCLE01, described previously (Oliveira *et al.* 1998; De Freitas *et al.* 2006; Venegas *et al.* 2011). The genotypic differentiation was analysed with the Genepop program using the Markov algorithm (Raymond and Rousset 1995a; 1995b).

Lineage determination of predominant Trypanosoma cruzi clones

The nomenclature used in this study 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 by several criteria lineages TcV and TcVI are hybrids, and by microsatellite markers correspond to the same phylogenetic cluster, here they were called 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 required to assign the final lineage of each predominant *T. cruzi* clone. Otherwise, the predominant clone was considered as not determined (ND, Table S1). To determine whether or not differences between the percentages of lineages were significant, a study was conducted by the Chi-square and Fisher methods, using a significant P-value of less than 0.05).

Phylogenetic reconstruction

To determine the genetic relationships between the Chilean *T. cruzi* population samples and their foreign counterparts, a phylogram tree was constructed with the program PHYLIP 3.5c (Felsenstein 1989) using the allele frequencies obtained with the SCLE10, SCLE11 and MCLE01 microsatellite loci. The phylogenetic reconstruction was based on the infinite allelic model (IAM) using the shared allele distance of Bowcock *et al.* (1994). 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).

Data from literature

Information was used from the published data of De Freitas *et al.* (2006), Venegas *et al.* (2009a) and Venegas *et al.* (2011). The several *T. cruzi* clones and strains were grouped based on their geographic origin, of which the majority corresponded to regions of Brazil: 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°34W) and Piauí (PI).

Results

We analyzed the intestinal contents of 64 triatomines from the regions of Atacama (III), Valparaiso (V) and Metropolitan (M), using previously described microsatellite markers SCLE10, SCLE11 and MCLE01 (Oliveira *et al.* 1998). These three population samples will be called the "triatomine group" (G1). The second group will be called "Chagas disease group" (G2), is composed of samples from non-cardiopathic and cardiopathic *T. cruzi* infected patients, data obtained earlier by our laboratory with the same three microsatellite markers (Venegas *et al.* 2010).

The analysis of genotypic differentiation using the Fisher exact method with these three microsatellite markers showed that the triatomine population samples from the III, V and M regions (G1) were different from the populations found in the Chagas disease group (G2), both in blood (NB and CB) and xenodiagnosis samples (NX and CX) (Table I). As expected from the previous analysis (Venegas *et al.* 2011), the results showed that the sample from the III Region was differentiated either from the V or the M samples.

Analysis of the predominant lineages from each triatomine and Chagas disease population showed that there were significant differences among the triatomine (G1) and Chagas disease patient group (G2), with the exception of TcII linage (Table II). These differences are also evident when comparing samples from each group against either total G1 or G2 groups. Thus for example, the lineages TcI and H from the V region differs significantly from the group of *T. cruzi* infected patients (V/G2). Another example, the lineages TcI and TcIII from the triatomine group (G1) differs significantly from the NB sample (G1/NB). As expected, according to previously published analyzes (Venegas *et al*. 2010), no significant differences were detected when comparing non-cardiopathic patients with cardiopathic patients both in blood as xenodiagnosis samples (NB / CB and NX / CX).

Moreover, differences in lineage percentage were detected within each group of triatomine and chagasic samples, for instance in the triatomine group the M sample showed the highest percentage of TcII (16.7%), nearly tripling the percentage found in the other two regions. A similar observation can be made with respect to the H lineage, in which the V sample (18.4%) doubled the percentage detected in the other two regions. Within the *T. cruzi* infected patients group, the percentage of the TcII lineage for the CB sample (25%), was more than double of the percentages of the other three samples (Table II). The same was observed in the TcIII lineage, in which the percentage of the CX sample (23.1%) was twice as large as the other three. However, the differences within each group were not significant, as is shown by comparison of the NB/CB and NX/CX samples (P-value between 0.194–1 > 0.05).

Analysing the minimum number of *T. cruzi* clones in each sample, in general the triatomine group had a more wide-

Table I. Analysis of population differentiation using the Fisher method with the loci SCLE10, SCLE11 and MCLE01 for each *Trypanosoma cruzi* group pair

Samples compared[a]		Probability[b]
III	V	0.002989
III	M	0.025153
V	M	0.411318*
III	NB	Highly sign[c].
III	CB	Highly sign.
III	NX	Highly sign.
III	CX	Highly sign.
V	NB	Highly sign.
V	CB	0.000000
V	NX	Highly sign.
V	CX	Highly sign.
M	NB	Highly sign.
M	CB	0.000002
M	NX	0.000000
M	CX	0.000000
Triatomine group (G1)	Chagasic group (G2)	Highly sign

[a] Triatomine group (G1): *T. cruzi* samples from *Triatoma infestans* midgut from insects captured in the third (sample III, 15 insects), fifth (sample V, 37 insects) and Metropolitan (sample M, 12 insects) regions, respectively. Chagasic group (G2): *T. cruzi* samples from chronic *T. cruzi* infected patients. NB and CB correspond to blood samples from non-cardiopathic and cardiopathic *T. cruzi* infected patients, respectively. NX and CX correspond to dejections of triatomines fed on non-cardiopathic and cardiopathic *T. cruzi* infected patients, respectively. The numbers of patients in groups NB, CB, NX and CX were: 29, 24, 35 and 26, respectively. Samples with information on at least two loci were considered.
[b] An unbiased estimate of the P-value of a log-likelihood ratio (G) based exact test is performed for all pair of sample for all loci (Fisher method) (Raymond M., Rousset F. 1995a). Markov chain parameters: Dememorisation = 10000; Batches = 100; Iterations per batch = 5000.
[c] Highly sign = is reported when at least one of the individual tests being combined yielded a zero P-value estimate.
[*]not significant (P > 0.05).

spread distribution, ranging from 1–9 clones (Table III), while in the *T. cruzi* infected patients group we found only 1–4 clones.

The results of the phylogenetic analysis are concordant with the previous observations. For example, the four samples of the *T. cruzi* infected patients group clustered together in the same node nearest to the TcI group (Fig. 1), as expected from the high percentage of TcI lineages shown in Table II. By contrast, the samples from the triatomine group were distributed between the TcI node and the TcIII samples, as expected from the mixing of the total percentage of TcI (42%) and TcIII (23.1%). Also, as expected from previous

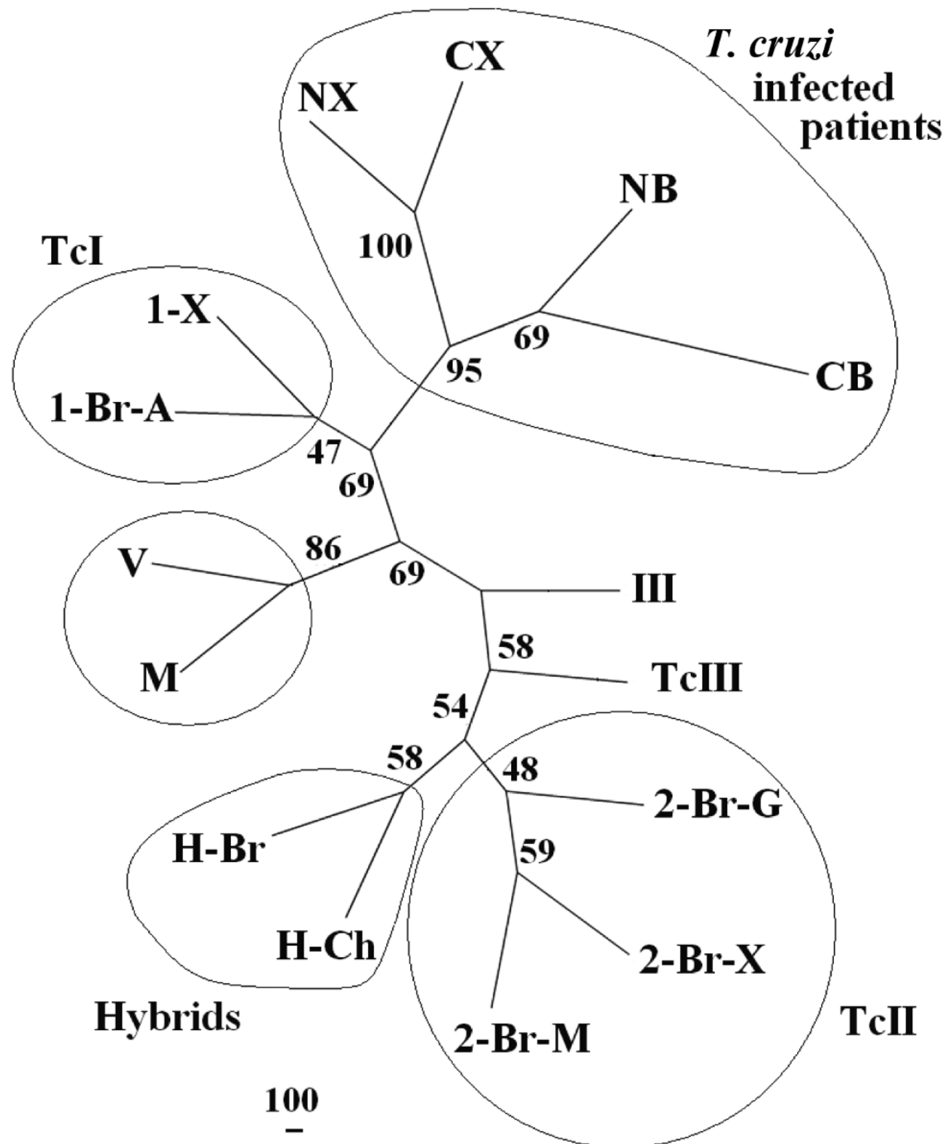

Fig. 1. Unrooted phylogenetic tree of *Trypanosoma cruzi* population samples detected in the midgut of *Triatoma infestans* triatomines from three Chilean regions, Chilean *T. cruzi* infected patients and Brazilian regions. The phylogenetic reconstruction was performed using the allele sharing distance method (Bowcock *et al.* 1994) and the SCLE10, SCLE11 and MCLE01 microsatellite markers (Oliveira *et al.* 1998). The *T. cruzi* population samples from triatomine midguts were directly genotyped and correspond to insects collected in the III, V and Metropolitan (M) regions of Chile (Group 1). The chagasic patient samples correspond to: blood samples from non-cardiopathic (NB, 29 individuals) and cardiopathic (CB, 24 individuals) and their respective xenodiagnostic samples (NX, 35 individuals) and (CX, 26 individuals) (Group 2). Samples with information on at least two loci were considered (see Material and Methods). The lineage nomenclature corresponds to the recent agreement (Zingales *et al.* 2009); they were assigned based on three simultaneous methods as described (Venegas *et al*. 2011). The laboratory cultured *T. cruzi* population samples correspond to published data of De Freitas *et al*. (2006) and Venegas *et al*. (2009a) and are the following: TcI from Brazil Amazon (1-Br-A) and TcI from Bolivia, Colombia and French Guyana (1-X);.TcII Brazilian samples from Goias (2-Br-G), Minas Gerais (2-Br-M), and other regions (2-Br-X). A TcIII sample mainly from Minas Gerais (TcIII). TcV-TcVI from Minas Gerais (H-Br) and TcV-TcVI from Chile (H-Ch). Percentage of bootstrap support is near each node (1000 replicas)

Table II. Lineages of predominant *Trypanosoma cruzi* clones detected in the midgut of *Triatoma infestans* triatomines and *T. cruzi* infected patients

Population samples[a]	Number (%) of *T. cruzi* clones found for each lineage[b]				
	TcI	TcII	TcIII	H[c]	ND[d]
Triatomine Group (G1):					
III(n[c]=15)	5 (33.3)	1(6.7)	4(26.7)	1(6.7)	4(26.7)
V(n=38)	18(47.4)	2(5.3)	8(21.1)	7(18.4)	3(7.9)
M (n=12)	4(33.3)	2(16.7)	3(25)	1(8.3)	2(16.7)
Total (n=65)	27(42.0)	5(8.0)	15(23.1)	9(13.8)	9(13.8)
Chagasic Group (G2):					
NB (n=29)	24(82.8)	3(10.3)	1(3.4)	0	1(3.4)
CB (n=24)	17(70.8)	6(25.0)	1(4.2)	0	0
NX (n=35)	27(77.1)	1(2.9)	4(11.4)	0	3(8.6)
CX (n=26)	18(69.2)	1(3.8)	6(23.1)	1(3.8)	0
Total (n= 114)	86(75.4)	11(9.6)	12(10.5)	1(0.9)	4(3.5)
Pair:	P-value[e]				
NB/CB	0.194	0.271	1		1
NX/CX	0.563	1	0.3	0.426	0.254
G1 / G2	0.0001*	0.659	0.024*	0.0001*	0.031*
III/G2	0.006*	1	0.093	0.22	0.034*
V/G2	0.0001*	0.519	0.096	0.0001*	0.367
M/G2	0.004*	0.358	0.155	0.182	0.101
G1 /NB	0.0001*	0.699	0.019*	0.053	0.266
G1/CB	0.02*	0.062	0.059	0.106	0.102
G1/NX	0.0001*	0.662	0.157	0.025*	0.743
G1/CX	0.024*	0.67	1	0.271	0.099
G1/NB+CB	0.0001*	0.121	0.003*	0.004*	0.04*
G1/NX+CX	0.0001*	0.441	0.347	0.017*	0.142

[a] Group 1: Population samples from *Triatoma infestans* captured in: III (15 insects), V (38 insects) and M (12 insects) regions. Group 2: Blood samples from non-cardiopathic (NB, 29 individuals) and cardiopathic (CB, N = 24) individuals, and their respective xenodiagnostic samples (NX, 35 individuals) and (CX, 26 individuals). Samples with information on at least two loci were considered (see Materials and Methods).
[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 Materials and Methods). The lineage nomenclature used corresponds to the recent agreement (Zingales *et al*. 2009).
[c]H = hybrid lineages TcIV and/or TcV.
[d]ND = not determined.
[e] Comparison of the sample populations and groups listed in the first column by the Fisher or Chi-squared (shading cells) methods. Asterisks correspond to significant differences (P-value < 0.05).

Table III. Minimum number of *Trypanosoma cruzi* clones detected in the midgut of *T. infestans* triatomines (Group 1) and *T. cruzi* infected patients (Group 2)

Samples[a]	Number of clones (%)								
G1	1	2	3	4	5	6	7	8	9
III (15)	0	4(26.7)	2(13.3)	4(26.7)	2(13.3)	2(13.3)	1(6.7)	0	0
V (37)	7(18.9)	16(43.2)	6(16.2)	4(10.8)	2(5.4)	0	1(2.7)	1(2.7)	0
MR (12)	1(8.3)	2(16.7)	3(25.0)	2(16.7)	3(25.0)	0	0	0	1(8.3)
Subtotal (64):	8(12.5)	22(34.4)	11(17.2)	10(15.6)	7(10.9)	2(3.1)	2(3.1)	1(1.6)	1(1.6)
G2									
NB (29)	12(41.4)	9(31.0)	7(24.1)	1(3.4)	0	0	0	0	0
CB (24)	15(62.5)	3(12.5)	6(25.0)	0	0	0	0	0	0
NX (35)	20(57.1)	11(31.4)	4(11.4)	0	0	0	0	0	0
CX (26)	14(53.8)	9(34.6)	3(11.5)	0	0	0	0	0	0
Subtotal (114):	61(53.5)	32(28.1)	20(17.5)	1(0.9)	0	0	0	0	0

[a] Group 1: Population samples from *Triatoma infestans* captured in: III (15 insects), V (38 insects) and M (12 insects) regions. Group 2: Blood samples from non-cardiopathic (NB, 29 individuals) and cardiopathic (CB, N = 24) individuals, and their respective xenodiagnostic samples (NX, 35 individuals) and (CX, 26 individuals). Samples with information on at least two loci were considered (see Materials and Methods).

results in which the genetic characteristics of the same kind of sample were more similar to each other than to the type of patient (Venegas *et al*. 2010), the NX and CX clustered together, as well as NB and CB. Finally, the topology of the tree produced from the foreign *T. cruzi* clones is concordant with the previous published topology (De Freitas *et al.* 2006); the hybrid clones clustered together in a node located between the TcII and TcIII lineages and the TcI lineage was furthest from TcII.

Discussion

The key question analyzed in the present study is: Is there specific host-parasite association between the *T. cruzi* populations that are transported by the insect vector *T. infestans* and the *T. cruzi* populations detected in chagasic patients? If the answer to this question is affirmative, then the *T. cruzi* populations circulating in the insects from the III, V and Metropolitan regions, should be different from the *T. cruzi* populations detected in patients either in peripheral blood or xenodiagnosis specimens. Precisely, the results were concordant with this prediction. It was observed that all genetic comparisons performed between the *T. cruzi* samples from triatomine (G1 group) and *T. cruzi* infected patients samples (G2 group) were genetically differentiated. This was observed not only when comparing the entire G1 group against the entire G2 group, but also when comparing any samples from one group against any samples from the another one (Table I). The causes of these genetic differentiation could be several: differential selection processes conducted by triatomines and mammalian hosts, specific parasite-host interaction phenomena, differential tissue tropism, different geographical origin of the parasites, as several authors have suggested previously (Brisse *et al*. 2000; Tibayrenc 2003; de Freitas *et al*. 2006; Marcili *et al.* 2009; Rodrigues and Borges-Pereira 2010). However, we cannot rule out combinations of these causes in which the differences between these populations could be due to infective parasites with a strong tissue tropism, low replication rate and parasitic load in peripheral blood, and even we cannot ruled out geographical effect due *T. cruzi* infected patients were inhabitants from another Chilean region (IV).

The analysis of lineage distribution among the present population samples was also consistent with a specific host-parasite association hypothesis, because statistical significant differences were observed among the percentages of some lineages comparing the triatomine group (G1) and the *T. cruzi* infected patients group (G2), as was found on TcI, TcIII and H (hybrids TcV-TcVI) distribution. Interestingly, in both groups TcI was the most frequent lineage and TcIII was present in a significant percentage. There are reports in the literature which support that there are some specific host-parasite interactions (Brisse *et al*. 2000; De Freitas *et al*. 2006; Marcili *et al*. 2009). However, this specificity is not absolute, because no lineage has been associated exclusively with a particular host or vector species. Thus, for example TcI, initially associated with the wild cycle, has now been found in a wide range of different mammal species and also in different species of triatomines in Latin America (Tibayrenc and Ayala 1988; Sanchez *et al.* 1993; Breniere *et al*. 1998; Brisse *et al.* 2000; De Freitas *et al.* 2006; Marcili *et al.* 2009; Del Puerto *et al.* 2010; Ramos-Ligonio *et al.* 2012). The same is true for the other lineages; for example TcII in Brazil has been found very frequently associated with chagasic patients, but also in the same country it has been found in *Triatoma infestans* and *Panstrongylus megistus* (De Freitas *et al.* 2006; Miles *et al.* 2009). Moreover, the less studied TcIII, initially found associated with the terrestrial armadillo, now has been detected in different transmission cycles and hosts, even in the domestic cycle in humans and corresponding vectors (De Freitas *et al.* 2006; Marcili *et al.* 2009; Miles *et al.* 2009). For these reasons, today in *T. cruzi* there is not a clear picture about the degree of specificity of this host-parasite interaction and the factors that determine this phenomenon. The evidence which emerges from the studies of *T. cruzi* samples from patients and their corresponding xenodiagnosis is very interesting (Coronado *et al.* 2006; Venegas *et al*. 2010), because in this case the genetic differences found between the blood and xenodiagnosis samples of *T. cruzi* should be due to differential selection of parasites produced by the human and xenodiagnostic triatomines, because here the geographic effect can be ruled out, since the xenodiagnosis samples come from the same patient groups.

Comparing the distribution of lineages among *T. cruzi* infected patients (NB and CB) and their corresponding xenodiagnosis (NX and CX) samples, the results presented here are also concordant with the specific host-parasite interaction hypothesis, because significant differences were observed among the percentages of the lineages TcI, TcIII and H (hybrids TcV-TcVI). Other reports on this same topic studying Chilean chronic *T. cruzi* infected patients have been published (Coronado *et al.* 2006; Venegas *et al.* 2009b; 2010), however, there are some differences between our results and previous observations. For instance, although TcI was the most frequent lineage found in the peripheral blood of *T. cruzi* infected patients, with a frequency of 0.70 similar to the present study (0.71–0.82), the frequency of the other lineages was different (Coronado *et al.* 2006). For example, the frequency of TcII in blood samples was 0.54, whereas in the present study was 0.10 or 0.25 in non-cardiopathic (NB) or cardiopathic (CB) patients blood samples, respectively. There are even greater differences comparing the prevalence of lineage TcV between these studies; in Coronado *et al.* (2006) the frequency of this lineage in bood samples was 0.33, whereas in our study this lineage was not detected in blood. In the xenodiagnosis samples there were also important differences with Coronado *et al.* (2006). The TcV lineage had the highest frequency (0.65), while in this study we found a frequency of only 0.04 which could be TcV or TcVI. Also, there are important differences between the present results and the evidence obtained previously by our

group using minicircle kDNA probes (Venegas *et al.* 2009b). It is important to mention that both previous reports used probes derived from kDNA, compared to the microsatellite nuclear marker used here. Although there are some reports in which data obtained with kDNA markers show good lineage association with nuclear markers in this parasite (Veas *et al.* 1990; Breniere *et al.* 1992), strong molecular evidence showed that in each kinetoplastid genome there are thousands of different minicircle sequences, which could not reconstruct phylogeny and lineages obtained with nuclear markers (Telleria *et al.* 2006). The difference in the nature of these molecular markers may explain the dissimilar results observed in the lineage distribution between the present study and previous studies (Coronado *et al.* 2006, Venegas *et al.* 2009b); nevertheless, other causes cannot be not ruled out. For example the fact that in this study only typifies the predominant clone, not considering other minority lineages that are present both in triatomines and patients which can be detected by other methods. It is also possible that *T. cruzi* populations analyzed here are different from those previously published due to distinct geographical origin of the samples, or ecosystem changes produced by surveillance and control programs of Chagas disease applied in our country.

Another important point is whether the differences observed between frequencies of lineages in different geographic regions of Latin America are due to specific host-parasite interactions or only due to geographic factors such as distance and/or geological barriers. It is clear that geography has an important impact on natural *T. cruzi* populations, as was shown recently by other authors and our group for the same lineage (TcIII), in which significant correlations between genetic and geographic distances have been observed (Llewellyn *et al*. 2009; Venegas *et al.* 2011). The geographic distances could favour the genetic differentiation of *T. cruzi* populations by the restriction of gene flow among different allopatric cycles and host ecotypes. Although an asymmetrical geographic distribution of *T. cruzi* clones and lineages was observed very early and in several previous studies (Tibayrenc and Ayala 1988; Sanchez *et al.* 1993; Brisse *et al.* 2000; Torres *et al.* 2004; De Freitas *et al.* 2006; Marcili *et al.* 2009), the fact that the majority of this evidence came from laboratory cultured parasites introduces some doubt about the representativeness of the *T. cruzi* clones and lineage distributions with respect to what actually exists in nature. For this reason, our group has made great efforts to develop an effective direct method that allows us to identify and quantify the clones and lineages actually circulating in the different cycles and habitats. Although in this study we used only three microsatellite markers which can directly distinguish the predominant clones found in each sample, previously published results showed that this method not only allows distinguishing between different lineages, but can also differentiate clones within the same lineage (Venegas *et al.* 2011). This fact gives us some confidence that the results presented here reflect the distribution of lineages that are present both in triatomine samples from the three regions (III, V and M) and the distribution of lineages that were detected in chagasic patients. Moreover, the topology of the phylogenetic tree obtained with this method is consistent with the genetic differentiation shown by the Fisher exact method and the lineage distribution described here. For instance, the four samples *T. cruzi* infected patients (NB, CB, NX and CX) clustered together with a high bootstrap support (95%), apart from the node of the V-M samples and the branch of the III sample, confirming that the samples of *T. cruzi* infected patients and triatomines are genetically distinct, which in turn is consistent with the hypothesis of a specific host-parasite association. In addition, the fact that the topology of the present tree obtained with published data of the *T. cruzi* clones and isolates was concordant with the classical topology described with different nuclear markers and even with several microsatellite markers previously described (Brisse *et al.* 2000, De Freitas *et al.* 2006), supports the idea that the method employed here allows reliable results on the genetic characteristics of *T. cruzi* populations analyzed in the present study.

As expected from published information (Brisse *et al.* 2000, Coronado *et al.* 2006, Venegas *et al.* 2010), a greater number of clones was found in the triatomine samples than in samples from *T. cruzi* infected patients (Table III). This is expected and is in agreement with the hypothesis that triatomine midgut favours the growth of a wider range of parasite lineages and clones, since it is known that better conditions for the proliferation of different *T. cruzi* lineages and clones exist in the midgut of triatomines than in the mammalian host conditions, in which the parasites are subject to attack by the host immune response (Deane *et al.* 1984, Brisse *et al.* 2000, Solari *et al.* 2001, Coronado *et al*. 2006). This is also consistent with the hypothesis that certain clones or strains of the parasite have different specificity for both vector and mammalian hosts, which would correspond to parasites circulating in different ecotopes (Brisse *et al.* 2000, De Freitas *et al.* 2006, Marceli *et al.* 2009).

Conclusions

The results presented in this article support the hypothesis that in Chilean *T. cruzi* populations there are specific host-parasite associations. However, to strengthen this hypothesis, it is necessary to perform further studies with larger sample sizes from each host and vector species from the same region of precedence, to rule out geographical or ecological causes. Our group is devoting its best effort to follow up on this important issue.

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