Genetic structure of *Trypanosoma cruzi* in American continents: special emphasis on sexual reproduction in Central America

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SUMMARY

Isozyme analysis (12 enzymes:14 loci) was conducted on 99 isolates of *Trypanosoma cruzi*: 77 from Guatemala, 5 from Mexico and 17 from South American countries. Analyses of 4 population-genetic indices were undertaken to assess the possibility of genetic exchange occurring among Guatemalan isolates. The results provide evidence for a degree of genetic exchange occurring among isolates from this relatively small geographical area. Previous studies of population genetics on *T. cruzi* might have failed to detect this phenomenon because they tended to use isolates originating far from one another, rendering gene exchange unlikely for geographical reasons. Phylogenetic data, presented here, show considerable differences in genetic structure between Central and South American isolates, suggesting that different biological and clinical properties might be expected. For example, there are differences in clinical syndromes between Central and South America, a situation discussed further here.

Key words: *Trypanosoma cruzi*, genetic recombination, Chagas' disease, isozyme, phylogeny, pathogenicity.

INTRODUCTION

Chagas' disease, caused by *Trypanosoma cruzi*, is a serious public health problem in Latin America, affecting between 16 and 18 million people (Dias, 1992). However, many biological questions of epidemiological significance remain unanswered. For example, little is known about the reproductive biology of the species. If populations are mainly clonal, which has been shown by some researchers (Tibayrenc & Ayala, 1988; Tibayrenc, Kjellberg & Ayala, 1990; Ayala, 1993; Tibayrenc, 1995), then specific genetic and epidemiological attributes will arise and be maintained independently in different lineages. If sexual reproduction can occur, a possibility recently claimed by some researchers (Lewicka *et al.* 1995; Bogliolo, Lauria-Pires & Gibson, 1996; Carrasco *et al.* 1996), even to a limited extent, then those attributes can be recombined to co-occur in the same individual organism. From the point of view of strain-specific PCR diagnosis and chemotherapy, this is a more worrying situation.

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Previous studies investigating reproductive biology in *T. cruzi* have often examined isolates originating very far from one another. Thus, it is not surprising that they have failed to find evidence for gene exchange. In our previous isozyme study (Higo *et al.* 1997), we examined the degree of genetic exchange among a small number of isolates from Guatemala by using 2 population-genetic indices (1) deviation from Hardy–Weinberg equilibrium and (2) the differences between observed and expected heterozygosity. The former index suggested clonal reproduction. However, the latter did not. In the present study, we repeated the previous analyses and added a further 2 indices in an investigation of a far larger collection (75) of isolates from Guatemala. Being a small country, where local movements of infected people or reservoir hosts could bring different strains of *T. cruzi* into sympatry, Guatemala provided a very suitable location for this work. Examination of numerous sympatric (or nearly so) isolates should detect gene exchange if this is happening with at least moderate frequency. We also constructed a phylogenetic tree to determine the relationships among our isolates. This indicated that 2 Guatemalan isolates were extremely different genetically from the majority of other Guatemalan isolates, so they were excluded from the population

Table 1. Geographical and host origin, and zymodemes of isolates of *Trypanosoma cruzi*

Isolate	No. of isolates	Zymodeme	Host	Locality
H6–8, H10, HN4004, SM53, SM249, SM392	8	Z-1	Human	Santa Rosa, Guatemala
H18, H31	2	Z-1	Human	Zacapa, Guatemala
TM1–4, TM6–7, TM9–13, TM15–16, TM18–21, TM23, TM25–39, 171	34	Z-1	*Triatoma dimidiata*	Santa Rosa, Guatemala
MAR5, MAR6	2	Z-1	*Triatoma dimidiata*	Guatemala, Guatemala
VER1, VER3–6	5	Z-1	Opossum	Mexico
VC	1	Z-1	*Triatoma nitida*	Guatemala, Guatemala
RM1, RM5	2	Z-1	*Rhodonius prolixus*	Zacapa, Guatemala
MTM1	1	Z-1	Wild rat	Santa Rosa, Guatemala
G	1	Z-1	Unknown	Guatemala
H23	1	Z-2	Human	Zacapa, Guatemala
TM8, TM14, 172	3	Z-2	*Triatoma dimidiata*	Santa Rosa, Guatemala
H13.2, H14–15, H21, H25, H28–30	8	Z-3	Human	Zacapa, Guatemala
TM17	1	Z-3	*Triatoma dimidiata*	Santa Rosa, Guatemala
RM3, RM4	2	Z-3	*Rhodonius prolixus*	Zacapa, Guatemala
MTM2	1	Z-3	Squirrel	Guatemala, Guatemala
TM22, TM24	2	Z-4	*Triatoma dimidiata*	Santa Rosa, Guatemala
H12	1	Z-5	Human	Santa Rosa, Guatemala
H27	1	Z-5	Human	Zacapa, Guatemala
H17	1	Z-6	Human	Santa Rosa, Guatemala
H22, H26	2	Z-7	Human	Zacapa, Guatemala
H24	1	Z-8	Human	Santa Rosa, Guatemala
HN274	1	Z-9	Human	Santa Rosa, Guatemala
H20	1	Z-9	Human	Zacapa, Guatemala
PH1, PH2	2	Z-9	Human	Peru
Colombia	1	Z-10	Human	Colombia
CL	1	Z-10	Insect vector	Brazil
PTM1–4	4	Z-11	*Triatoma infestans*	Peru
119	1	Z-12	Opossum	Ecuador
SX10/4, SX10/7	2	Z-13	Human	Brazil
ESM3	1	Z-14	Human	Brazil
Y	1	Z-15	Human	Brazil
GS, LO, RF	3	Z-15	Human	Paraguay
Tul-L	1	Z-15	Unknown	Chile

genetic analysis. All Guatemalan isolates used for the analysis of genetic exchange therefore belonged to a single phylogenetic Group (Group 1) identified in our previous study (Higo *et al.* 1997).

MATERIALS AND METHODS

Parasites

A total of 99 isolates of *T. cruzi* was used in this study, 82 of these from Guatemala and Mexico, and 17 from South America (see Table 1 for details). Isolation of parasites from humans and insects, and sample preparation for isozyme analysis were done according to the methods of Higo *et al.* (1997).

Sample preparation and isozyme analysis

Buffer systems, electrode conditions and the enzymatic staining of the gel slices for isozymes followed the modified methods of Agatsuma & Habe (1986) and Shaw & Prasad (1970). Twelve enzymes, AK, CK, DIA, GAPD, GPI, IDH, MDH, ME, MPI, PEP, 6PGD, PGM, were investigated (Higo *et al.* 1997).

Calculation of genetic distances and construction of a dendrogram

As *T. cruzi* is considered to be at least partially clonal in reproduction (Tibayrenc & Ayala, 1988), it is not possible to calculate the genetic distances among the populations by assuming random mating. So we assigned all the isolates into zymodemes according to the pattern of zymograms. Nei's genetic distances (Nei, 1987) for each pair of zymodemes were calculated by GENDIST program in PHYLIP package version 3.572 (Felsenstein, 1993). A dendrogram was drawn from the matrix of genetic distances by the unweighted pair-group method

Table 2. Zymodemes of *Trypanosoma cruzi* generated from isozyme analyses

Zymodeme	AK1	AK2	CK	DIA	GAPD	GPI	IDH	MDH	ME2	MPI	PEP1	PEP2	6PGD	PGM
Z-1	1/1	1/1	1/1	1/1	1/1	4/4	3/3	1/1	2/2	1/3	3/3	3/3	1/1	1/1
Z-2	1/1	1/1	1/1	1/1	3/3	4/4	3/3	1/1	2/3	3/3	3/3	3/3	1/1	1/1
Z-3	1/1	1/1	1/1	1/1	2/2	4/4	3/3	1/1	2/3	3/3	3/3	3/3	1/1	1/1
Z-4	1/1	1/1	1/1	1/1	3/3	4/4	3/3	1/1	2/2	3/3	3/3	3/3	1/1	1/1
Z-5	1/1	1/1	1/1	1/1	3/3	4/4	3/3	1/1	2/4	1/3	3/3	3/3	1/1	1/1
Z-6	1/1	1/1	1/1	1/1	2/2	4/4	3/3	1/1	2/4	1/3	3/3	3/3	1/1	1/1
Z-7	1/1	1/1	1/1	1/1	3/3	4/4	3/3	1/1	2/3	1/3	3/3	3/3	1/1	1/1
Z-8	1/1	1/1	1/1	1/1	2/2	4/4	3/3	1/1	2/3	1/3	3/3	3/3	1/1	1/1
Z-9	1/1	1/1	0/0	3/3	6/6	3/3	1/1	1/1	1/1	4/4	1/1	1/2	0/0	2/2
Z-10	1/1	1/1	1/1	1/1	3/3	4/4	3/3	1/1	2/2	1/3	3/3	3/3	1/1	1/1
Z-11	1/1	1/1	1/1	1/1	6/6	4/4	3/3	1/1	2/2	3/3	3/4	3/3	1/1	1/1
Z-12	1/1	1/1	1/1	1/1	3/3	4/4	2/2	1/1	2/2	1/1	3/3	6/6	1/1	1/1
Z-13	1/1	1/1	1/1	1/1	4/5	4/4	3/3	1/1	2/2	3/3	2/2	4/4	1/1	1/1
Z-14	1/1	1/1	0/0	2/2	6/6	1/1	1/3	1/1	4/4	2/2	4/4	5/5	0/0	2/2
Z-15	1/1	1/1	0/0	1/1	6/6	2/2	1/3	1/2	4/4	2/2	4/4	7/7	0/0	2/2

with arithmetic mean (UPGMA) (Nei, 1987) by using NEIGHBOR and DRAWGRAM program in PHYLIP. We performed 1000 bootstraps to estimate the significance of branching in the tree by SEQBOOT program in PHYLIP.

Criteria for inferring clonality versus genetic exchange

All 75 *T. cruzi* isolates from Guatemala belonging to Group 1 (see dendrogram below) were used in analyses to estimate the degree of genetic exchange occurring. As criteria of clonality, the following 4 indices were employed (1) the deviation of observed distribution of genotypes from the expected one based on the Hardy–Weinberg equilibrium with 3 loci, GAPD, ME2, MPI which were polymorphic among the Guatemalan population, (2) the difference between the expected value of heterozygosity (He) and the observed one (Ho), (3) normalized coefficient of linkage disequilibrium which is expressed by the ratio of the coefficient of linkage disequilibrium to the corresponding maximum value in pairs of 3 loci, GAPD, ME2, MPI, and (4) the degree of association between loci examined (IA) which can be a generalized measure of linkage disequilibrium. Indices (1) and (2) were calculated by the same methods as Higo *et al*. (1997), indices (3) according to Ayala (1993). For indices (4) we followed Brown, Feldman & Nevo (1980), Maynard Smith *et al*. (1993) and Stevens & Tibayrenc (1996).

RESULTS

Of 12 enzymes examined, AK and PEP had 2 loci, indicated by the suffix 1 (faster electromorph) and 2 (slower electromorph) in Table 2. All of the other enzymes were encoded by 1 locus, so that in total 14 loci were identified. AK showed no variation among all the isolates. Five loci, GAPD, ME2, MPI, PEP1

and PEP2 varied among the isolates of Group 1, and all the other loci except AK varied between major phylogenetic groups.

Fifteen zymodemes were identified by isozyme analysis of the 99 isolates of *T. cruzi*: zymodemes 1–9 from Guatemala and Mexico, and zymodemes 10–15 from South America (Table 1). Table 2 shows the genotype of each zymodeme which was determined by the zymogram patterns. The dendrogram of relationships among isolates (Fig. 1) revealed 3 main lineages. The majority of Guatemalan, Mexican and some South American isolates formed Group 1. Two human isolates from Guatemala and 2 from Peru formed Group 2, and isolates obtained only from South America fell into Group 3.

No significant difference was shown with the locus ME2 when comparison was made between observed and expected gene frequencies. However, a significant deviation from the Hardy–Weinberg equilibrium was found for GAPD and MPI (Table 3). The expected value of heterozygosity (He = 0·087) was not different from the observed one (Ho = 0·075). The ratio of linkage disequilibrium of combinations for 3 loci, GAPD, ME2 and MPI, were small for GAPD/ME2 and GAPD/MPI, being 0·171 and 0·45 respectively, but high (0·8) for the other combination ME2/MPI (Table 4). The indices of association (IA) were $2·016 \pm 0·830$ for 75 isolates in Group 1 from Guatemala and $-0·401 \pm 0·593$ for 8 electrophoretic types (ETs) of zymodeme 1–8, indicating the possibility of genetic recombination by the value that is not significantly different from 0 for ETs (Maynard Smith *et al*. 1993). Of these results, the genetic exchange, possibly partially occurring, seems to be suggested by (1) the lack of deviation from the Hardy–Weinberg equilibrium at the locus ME2, (2) lack of difference between expected and observed heterozygosity, (3) deviation of the linkage disequilibrium coefficient (D′) from a value of 1 for the locus pairs GAPD/ME2 and
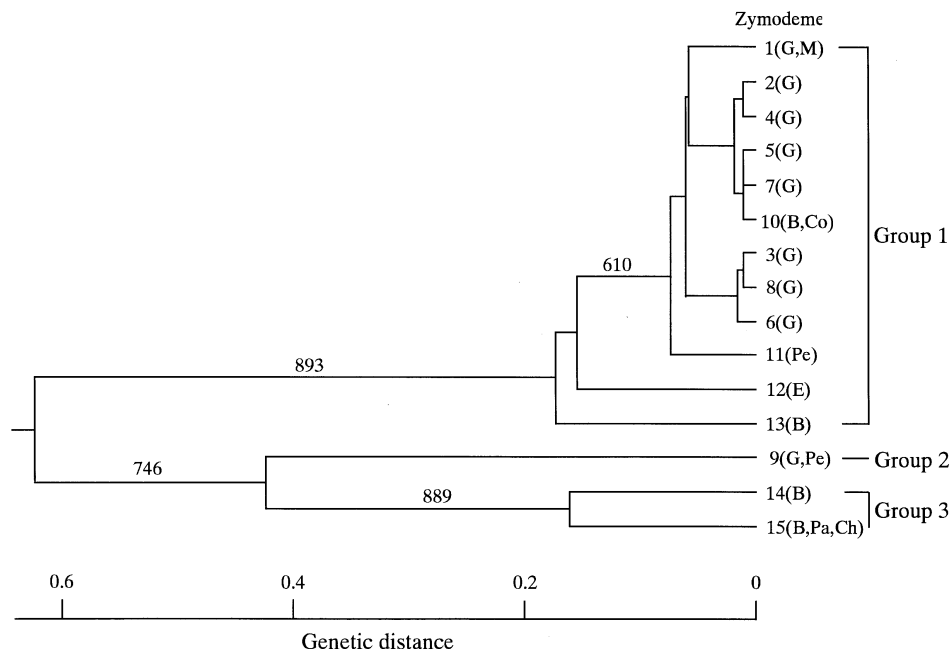
Fig. 1. Dendrogram drawn based on the matrix of genetic distances among 15 zymodemes of *Trypanosoma cruzi* using UPGMA. The figures on branches indicate the number of times that the branch was observed in 1000 bootstraps. Bootstrap values below 600 are not given. Abbreviations: B, Brazil; Ch, Chile; Co, Colombia; E, Ecuador; G, Guatemala; M, Mexico; Pa, Paraguay; Pe, Peru.

Table 3. Expected and observed distribution of genotype of *Trypanosoma cruzi* population from Guatemala

(The differences between observed and expected distributions were significant in GAPD and MPI ($P < 0.01$), not in ME2 ($P > 0.05$) by χ^2 test. χ^2 tests were calculated between 4 groups (1/1, 2/2+3/3+2/3, 1/2, 1/3) for GAPD, 3 groups (2/2, 3/3+4/4+2/4+3/4, 2/3) for ME2 to prevent expected values from being less than 5.)

Enzyme	Genotype	Frequency	
		Observed	Expected
GAPD	1/1	51	34·68
	2/2	14	2·623
	3/3	10	1·327
	1/2	0	19·074
	1/3	0	13·566
	2/3	0	3·73
ME2	2/2	53	54·571
	3/3	0	1·21
	4/4	0	0·03
	2/3	19	15·25
	2/4	3	2·559
	3/4	0	0·381
MPI	1/1	0	10·83
	3/3	18	28·83
	1/3	57	35·34

GAPD/MPI, and (4) the value of IA for ETs not significantly different from 0. However, the remaining results, deviations from the Hardy–Weinberg equilibrium at GAPD and MPI, the D′ value of nearly 1 for the pair of ME2/MPI, are as expected for clonal organisms.

Table 4. Linkage disequilibrium in *Trypanosoma cruzi* population from Guatemala

(D(obs) is the disequilibrium observed; D(max) is the maximum value possible for the observed allelic frequencies; D′ indicates D(obs)/D(max). D′ values of GAPD × ME2 and GAPD × MPI are much lower than 1.)

Compared loci	D(obs)	D(max)	D′
GAPD × ME2	0·029	0·172	0·171
GAPD × MPI	0·031	0·073	0·425
ME2 × MPI	0·04	0·05	0·8

DISCUSSION

Substantially clonal reproduction of *T. cruzi* has been shown in the natural population by isozyme and DNA analyses (Ayala, 1993; Tibayrenc, 1995; Tibayrenc & Ayala, 1988; Tibayrenc *et al.* 1990). However, recent studies (Lewicka *et al.* 1995; Bogliolo *et al.* 1996; Carrasco *et al.* 1996), suggested the possibility of genetic exchange in *T. cruzi* using data from isozyme and DNA analyses. In the present study population genetic analyses did not show necessarily clonal reproduction in Guatemala, and suggest that genetic exchange may occur to some extent in this small geographical area. However, the divergences from Hardy–Weinberg equilibrium at loci for GAPD and MPI and the homozygous patterns of some polymorphic loci, especially GAPD, are indicative of clonality, suggesting that genetic exchange, if present, is at a low level. Even a low level of genetic exchange will permit recom-

bination of any biological attributes and genetic markers such as sensitivity to drugs and PCR patterns for strain-specific diagnosis. Further population genetic studies of *T. cruzi* are necessary to assess the degree of clonality using many more populations.

To identify the genetic structure of Central American isolates and also to confirm the validity of Guatemalan isolates for the population genetic analyses of gene exchange, we constructed a dendrogram to show relationships among all available isolates. Many such studies on *T. cruzi* have been carried out by isozyme and DNA analyses in South America (Miles *et al.* 1984; Tibayrenc & Ayala, 1988; DeLucad'oro *et al.* 1993; Lewicka *et al.* 1995). However, there have been many fewer studies on isolates from North and Central America. DeLeon *et al.* (1998) and Lopez-Olmos *et al.* (1998) reported many lineages of *T. cruzi* in Guatemala and Mexico by DNA and isozyme analyses, respectively. In the present study, a dendrogram based on isozyme analysis of 99 isolates showed almost the same features as that of Higo *et al.* (1997), except for an increase in the number of zymodemes. Group 1 consists of the majority of isolates (from humans, insects and reservoir hosts) from Guatemala, all the Mexican ones and those from northern countries of South America. Only 4 isolates were placed in Group 2 (2 patients from Peru and 2 from Guatemala). The isolates of Group 3 were found in humans, insect vectors and reservoirs from the southern regions of South America only. Group 1, distributed mainly in the northern part of Latin America, is quite distinct from the South American unique strain of Group 3.

Isozyme studies have indicated 3 main phylogenetic groups (Z1, Z2, Z3) on *T. cruzi* in South America (Carrasco *et al.* 1996; Miles *et al.* 1984; Tibayrenc & Miles, 1983), and also recently the presence of 2 main lineages has been reported based on isozyme, RAPD and other DNA analyses (Tibayrenc, 1995; Oury *et al.* 1997; Gomes *et al.* 1998; Oliveira *et al.* 1998). Comparing the data of previous studies and ours with reference strains SX10 and ESM3, Groups 1 and 3 in the present study seem to correspond to Z1 and Z2 in the study of Miles *et al.* (1984) and Tibayrenc & Miles (1983). Further data and analyses are also required to determine the correct correspondence between these 2 or 3 lineages of *T. cruzi* shown by various studies.

Luquetti *et al.* (1986) reported that 12 isolates from chronic patients in Central Brazil belonged to only Z2, possibly Group 3 in our study, and more than 80 % of them were from patients with intestinal disorder. This finding appears to indicate the relation of Z2 strain to mega syndrome. However, the following studies mapping clinical syndromes on phylogenetic trees of South American isolates failed to find any phylogenetic correlation (Apt *et al.* 1987;

Breniere *et al.* 1989; DeLucad'oro *et al.* 1993; Henriksson *et al.* 1993; Lauria-Pires & Teixeira, 1996; Montamat *et al.* 1996). If genetic recombination had occurred among these isolates, this might explain the lack of correlation. On a larger geographical scale, we have detected such a correlation: the Central American isolates forming our Group 1 cause only the cardiac form of the disease whereas both the intestinal and cardiac manifestations occur in South America where 2 major strains, Groups 1 and 3, exist. Geographical separation between Central and South America could possibly have prevented genetic recombination across the Isthmus of Panama. Certainly, one very distinct lineage (Group 1) occurs primarily in Central America and should be the focus of further work.

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