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An isoenzyme study of naturally occurring clones of *Trypanosoma cruzi* isolated from both sides of the West Andes highland

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Abstract

Seventy-two stocks of *Trypanosoma cruzi* isolated from both sides of the West Andes highland (Bolivia, Chile and Peru) were analysed by isoenzyme electrophoresis at 12 loci. The data, which were interpreted in terms of population and evolutionary genetics, corroborated the hypothesis of *T. cruzi* clonal population structure previously proposed, and indicated extensive genetic variability within the taxon *T. cruzi*. Fifteen different clones (or zymodemes) were identified, which could be grouped into 3 different clusters. Several clones from 2 of these clusters were isolated both in Chile and Bolivia, suggesting a significant circulation of invertebrate and/or vertebrate hosts of *T. cruzi* between these 2 countries. Low clonal variability in Peru suggested the occurrence of a 'founder effect' in this country. The potential usefulness of a cladistic approach in epidemiology is discussed.

Introduction

Isoenzyme electrophoresis has been widely used for the characterization of *Trypanosoma cruzi* populations (TOYÉ, 1974; MILES *et al.*, 1977, 1980) and extensive population genetical analysis has demonstrated a basically clonal structure in natural populations (TIBAYRENC *et al.*, 1986). The zymodemes described can be equated to natural clones of the parasite, as they can be identified by isozyme patterns. To date, 43 different natural clones have been described from an extensive sample of stocks isolated from different geographical areas and from various hosts. The distribution of these clones is noteworthy in two respects: (i) radically dissimilar clones are frequently sampled in close geographical proximity, in the same house or from the same individual host, either triatomine bug or human (BRENIÈRE *et al.*, 1985b; TIBAYRENC *et al.*, 1985); and (ii), while most of the clones are rarely recorded, a limited number appear to be widely distributed: for example, clone 19 was recorded in Brazil, Bolivia, Columbia and Venezuela (TIBAYRENC & AYALA, 1988). These predominant clones, the medical importance of which might be considerable, were termed 'major clones' (TIBAYRENC & BRENIÈRE, 1988).

The Bolivian domestic cycle is now well known: it is characterized by a large number of different clones (13) with a heterogeneous distribution throughout the whole endemic area, and by the presence of 3 predominant clones (19, 20 and 39), the last being radically dissimilar from the other two (large genetic distances). These 3 clones are found in sympatric circulation in most of the areas studied (TIBAYRENC *et al.*, 1986). In Chile, isoenzyme studies of *T. cruzi* stocks suggest the existence of 2 separate transmission cycles (domestic and sylvatic) in northern Chile with possible overlap between the 2 cycles (APT *et al.*, 1987). In southern Peru, BRENIÈRE *et al.* (1985a) reported the existence of a prevalence of stocks related to zymodeme 1 of READY & MILES (1980) and exhibiting a low variability. The purpose of the present study was to elucidate the distribution of natural clones of *T. cruzi* on either side of the Andean highlands, to check whether the Andes represent an efficient geographical barrier for *T. cruzi*, and to elucidate the phylogenetic relationships between clones of *T. cruzi*.

Material and Methods

Parasites. Table 1 gives all data about the origin of the 72 stocks studied.

Isoenzyme analysis. Eleven enzyme systems, as previously listed, corresponding to 12 genetic loci, were assayed (BRENIÈRE *et al.*, 1989). Electrophoresis was performed on cellulose acetate plates as described by TIBAYRENC *et al.* (1985), with slight modifications. Genetic interpretation and clone (or 'zymodeme') numbering was according to TIBAYRENC & AYALA (1988). The following stocks, already fully characterized (TIBAYRENC & AYALA, 1988), were used as references for allelic identification: SC43, cl.2, C8 cl.1, Mil3, Tulahuen, Tehuentepec, A107, Can III cl.1 (Z3f), Esmeraldo cl.3 (Z2) and 27R27. Isoenzymic analysis of the Bolivian and Peruvian stocks was done soon after isolation (one month), while the Chilean stocks were analysed after a longer period in culture.

Taxonomic clustering. Phylogenetic relationships among the clones were evaluated by using two different methods. (i) Based on the standard genetic distance matrix of NEI (1972), a dendogram was built by using the KITSCH program of hierarchized agglomeration (LANCE & WILLIAMS, 1966; LEGENDRE & LEGENDRE, 1979). (ii) After transforming the allelic frequency matrix into an allelic presence/absence (1/0) matrix, a cladistic network was built by using both the MIX program of Wagner algorithm (FARRIS, 1970), based on the parsimony principle (FELSENSTEIN, 1978, 1982) and the CLIQUE program, based on the compatibility principle (LE QUESNE, 1974; ECK & DAYHOFF, 1966). Several runs of the MIX program

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were carried out, by shuffling clone presentation order, to obtain the most parsimonious tree and check clustering stability. All programs pertain to the PHYLIP package communicated by J. Felsenstein.

Results

Further genetic variability in T. cruzi

Fifteen different clones, with the genotypes detailed in Table 2, were identified (see Table 1). Among them, 5 have already been reported, namely clones 2, 19, 20, 33 and 39 (TIBAYRENC & AYALA, 1988). The others were closely related to them, since they presented at most 6 allelic differences out of 24 possible ones, by comparison with the previously identified clones. We did not number any of the new clones, since more complete genotype characterization is required for fuller understanding of their phylogenetic relationships with the previously described clones.

For the locus *Pep 1*, a putative three-banded heterozygous aspect of a dimeric enzyme (the central band being more intensely stained than the two extreme ones) was observed for the first time in *T. cruzi*.

Stocks presenting isoenzyme patterns typical of two mixed clones

Some stocks from Bolivia (Potosi, 5 stocks) and Peru (Majes valley, 1 stock) exhibited patterns typical (TIBAYRENC *et al.*, 1985) of a mixture of two different clones (Table 1). We did not observe such patterns in the other localities under study. The patterns have been extensively described previously (BRENIÈRE *et al.*, 1985b; TIBAYRENC *et al.*, 1985). The fact that they are due to the presence of mixtures of various genotypes has been ascertained either by cloning experiments (TIBAYRENC & MILES, 1982) or by preparing artificial mixtures of cloned stocks (our unpublished data).

Phylogenetic relationships and geographical distribution of the clones

Standard genetic distances (NEI, 1972) are shown in

Table 1. Geographical and hostal origin and genotypes of the 72 *Trypanosoma cruzi* stocks studied[a]

Number of stocks	Designation	Country	Place	Host	Genotype
4	TPA 1–4	Bolivia	Trinidad Pampa Yungas (1600 m)	*Triatoma infestans*	cl.20
2	TPC 1,2	Bolivia	Coripata Yungas (1800 m)	*Triatoma infestans*	cl.20
3	TPL 3,4,7	Bolivia	Chillamani Yungas (1800 m)	*Triatoma infestans*	cl.20
2	TPN 12,13	Bolivia	Parrarani Yungas (1600 m)	*Triatoma infestans*	cl.20
1	SO 34	Bolivia	Toropalca Potosio (2700 m)	*Triatoma infestans*	cl.20
2	SO 44, 51	Bolivia	Vitichi Potosio (3000 m)	*Triatoma infestans*	cl.20
1	SO 18	Bolivia	Miculpaya Potosio (3000 m)	*Triatoma infestans*	cl.19
1	SP 104	Chile	IVa Region	*Triatoma spinolai*	cl.19
2	SO 16,28	Bolivia	Miculpava Potosi (3000 m)	*Triatoma infestans*	NP3
1	SO 30	Bolivia	Calcha Potosio (2700 m)	*Triatoma infestans*	NP3
1	SO 40	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	NP3
1	LGN	Chile	IVa Region	Man (chronic case)	NP4
1	SPAII	Chile	IVa Region	*Triatoma spinolai*	NP5
1	SP 31	Chile	IVa Region	*Triatoma spinolai*	NP6
1	LQ	Chile	IVa Region	Man (chronic case)	NP7
1	A 97	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	cl.2
1	A 105	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP9
1	A 82	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP10
4	A 1,4,6,7	Peru	Victor valley (2000 m)	*Triatoma infestans*	NP11
5	A 34, 38, 70, 76, 87	Peru	Sihuas valley (1500 m)	*Triatoma infestans*	NP11
5	A 136,138,141,144,146	Peru	Majes valley (600 m)	*Triatoma infestans*	NP11
2	SO19, 23	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	cl.39
1	SO 31	Bolivia	Calcha Potosi (2700 m)	*Triatoma infestans*	cl.39
1	SO 33	Bolivia	Toropalca Potosi (2700 m)	*Triatoma infestans*	cl.39
2	SO 35, 38	Bolivia	Huatina Potosi	*Triatoma infestans*	cl.39
2	SO 45, 48	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	cl.39
3	SO 3–5	Bolivia	Otavi Potosi (3400 m)	*Triatoma infestans*	cl.39
1	TPK 1	Bolivia	Khala-khala Yungas (1700 m)	*Triatoma infestans*	cl.39
3	TPN 1,21,22	Bolivia	Parrarani Yungas (1600 m)	*Triatoma infestans*	cl.39
2	MN, RMS	Chile	IVa Region	Man (chronic case)	cl.39
1	NR	Chile	IIIa Region	Man (chronic case)	cl.39
1	V2X	Chile	IVa Region	*Triatoma infestans*	cl.39
2	PCM, MCC	Chile	IVa Region	Man (chronic case)	NP13
1	GR	Chile	IIIa Region	Man (chronic case)	NP13
1	CBB	Chile	IVa Region	Man (chronic case)	cl.33
1	SO 21	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	NP15
1	SO 50	Bolivia	Vitichi Potosi (3000 m)	*Triatoma infestans*	NP15
2	SO 22, 25	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	NP3+NP15
2	SO 36,37	Bolivia	Huatina Potosi	*Triatoma infestans*	cl.39+NP3
1	SO 15	Bolivia	Miculpaya Potosi (3000 m)	*Triatoma infestans*	cl.39+NP15
1	A 141	Peru	Majes valley (600 m)	*Triatoma infestans*	NP11+cl.39
Total=72					

[a]Fifteen different genotyes were identified; clone numbers (cl.) indicate genotypes described by TIBAYRENC & AYALA (1988), NP indicates provisional numbers (see footnote to Table 2). For discussion of relationship with zymodemes defined by READY & MILES (1980), see TIBAYRENC *et al.* (1986) and text.

Table 2. Genotypes of *Trypanosoma cruzi* clones identified in Bolivia, Chile and Peru[a]

	Genotypes[b]										
	G6pd	*Gpi*	*Gd1*	*Gd2*	*Idh*	*Me-1*	*Me-2*	*Pep-1*	*Pep-2*	*Pgm*	*6Pgd*
cl. 20	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	2/4
cl. 19	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	4/4
NP3	5/5	5/5	3/3	2/2	1/1	2/2	4/4	3/3	1*/1*	3/3	4/4
NP4	5/5	5/5	3/3	2/2	1/1	2/2	4/4	1/4	1*/1*	3/3	4/4
NP5	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1	3/3	4/4
NP6	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	4/4
NP7	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1*	1*/1*	3/3	4/4
cl. 2	6/6	5/5	3/3	2/2	1/1	2/2	4/4	3/3	1 /1	3/3	4/4
NP9	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	3 /3	8/10	4/4
NP10	6/6	5/5	3/3	2/2	1/1	2/2	4/4	4/4	1*/1*	3/3	2/4
NP11	6/6	5/5	3/3	2/2	1/1	2/2	4/4	1/1	1*/1*	3/3	2/4
cl. 39	4/4	2/4	1/1	1/1	2/2	1/1	5/5	5/5	1 /1	6/10	1/4
NP13	4/4	2/4	1/1	1/1	2/2	1/1	6/6	5/5	1 /1	6/10	1/4
cl. 33	2/2	3/3	2/2	5/5	2/2	2/2	6/6	4/4	1 /1	10/12	1/1
NP15	2/2	3/3	2/2	5/5	2/2	2/2	5/5	5/5	1 /1	10/12	1/1

[a]For each locus, allele 1 codes for the fastest electromorph. The malate dehydrogenase enzyme system is not listed as it was monomorphic in this sample (*Mdh*=2/2). Each electromorph was determined according to TIBAYRENC & AYALA (1988) using appropriate reference clones. Clone numbering (cl.) refers to TIBAYRENC & AYALA (1988), the other genotypes (NP) are newly recorded. For better understanding, provisional numbering is given to the new genotypes recorded here; nevertheless, they need additional characterization to confirm their phylogenetic position (see text).
[b]The asterisks (*) indicate new allelic positions, slightly faster than the fastest position previously recorded (=1).

Genotypes	cl.20	cl.19	NP3	NP4	NP5	NP6	NP7	cl.2	NP9	NP10	NP11	cl.39	NP13	cl.33	NP15
cl.20	-	1	3	2	5	3	5	7	8	6	5	26	26	24	24
cl.19	0.02	-	2	1	4	2	4	6	7	5	4	25	25	23	23
NP3	0.11	0.09	-	3	6	4	6	8	9	7	6	27	27	25	25
NP4	0.40	0.02	0.09	-	5	3	5	7	8	6	5	26	26	24	24
NP5	0.21	0.18	0.29	0.21	-	2	4	2	5	5	4	21	21	19	19
NP6	0.11	0.09	0.18	0.11	0.09	-	2	4	5	3	2	23	23	21	21
NP7	0.21	0.18	0.18	0.16	0.18	0.09	-	6	7	3	2	25	25	23	23
cl.2	0.32	0.29	0.16	0.27	0.09	0.18	0.18	-	7	7	6	21	21	19	19
NP9	0.03	0.27	0.58	0.30	0.16	0.16	0.27	0.27	-	8	7	26	26	24	24
NP10	0.19	0.21	0.21	0.14	0.21	0.16	0.11	0.21	0.30	-	1	26	26	24	24
NP11	0.09	0.11	0.21	0.14	0.11	0.02	0.11	0.21	0.19	0.09	-	25	25	23	25
cl.39	2.17	2.01	2.01	1.20	1.50	2.01	2.01	1.50	1.84	2.17	2.17	-	2	10	10
NP13	2.17	2.01	2.01	1.20	1.50	2.01	2.01	1.50	1.84	2.17	2.17	0.10	-	10	10
cl.33	1.75	1.77	1.77	1.57	1.36	1.77	1.77	1.36	1.63	1.34	1.75	1.07	0.42	-	4
NP15	1.75	1.77	1.77	1.75	1.36	1.77	1.77	1.36	1.63	1.75	1.75	0.65	0.04	0.19	-

Fig. 1. Matrix of patristic or evolutionary distances (above the diagonal: see Fig. 3) and Nei's standard genetic distances (below the diagonal) between the 15 genotypes identified by 12 isoenzyme loci in *Trypanosoma cruzi* stocks isolated from Bolivia, Chile and Peru. Clone numbering (cl.) refers to TIBAYRENC & AYALA (1988); the other genotypes (NP) are newly recorded (see Table 2).

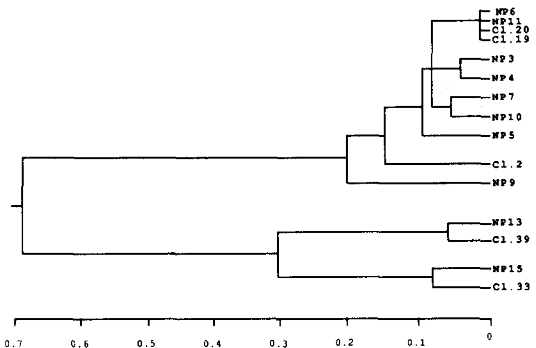

Fig. 2. Dendogram obtained from the genetic distance matrix of NEI (1972) for the different clones of *Trypanosoma cruzi*, using the KITSCH program (package PHYLIP). The P parameter was fixed by (=0) in order to obtain a treatment similar to UPGMA.

Fig. 1. As previously noted (TIBAYRENC *et al.*, 1986), some pair-wise comparisons exhibited very high values (up to 2·17) while others showed values as low as 0·02.

Three clusters can be discerned (Figs 2, 3). (i) A cluster including 10 different multilocus genotypes (clones) which share 14 alleles out of 24 possible allelic positions, with allelic differences among the clones ranging from 1 to 6. Within this group, 3 clones have been recorded previously, namely clones 2, 19 and 20 (TIBAYRENC & AYALA, 1988). Stocks pertaining to this cluster have been isolated from all three countries studied. The Peruvian stocks were all included in this cluster, except one stock which exhibited a weak pattern for glucose phosphate isomerase, suggesting a mixture with clone 39, which is radically different (see below). (ii) A second cluster included 2 clones (one corresponding to the formerly described clone 39, and the other closely related to it). These clones differed from any clone of the first cluster by 19 to 21 allelic differences. In the present sample, clone 39 (identified as a 'major clone': TIBAYRENC & BRENIÈRE, 1988) was frequently found in Bolivia as well as in Chile, and was possibly recorded in Peru (see Table 1). (iii) The third cluster included two different clones, the previously recorded clone 33 and another closely related to it. These two clones differed from any clone of the two other clusters in at least 17 allelic positions. Relationships between these various genotypes and the previously described zymodemes I, II and III (READY & MILES, 1980) have been extensively discussed by TIBAYRENC *et al.* (1986) and TIBAYRENC & AYALA (1988). Briefly, zymodemes II and III are clearly distinct from any of the stocks

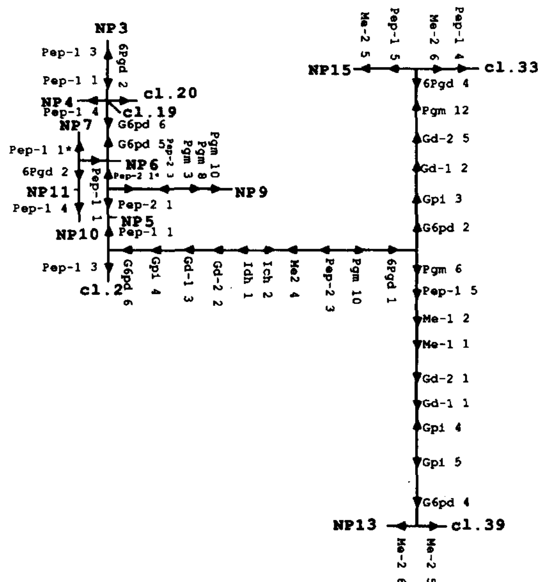

Fig. 3. Phylogenetic network showing the evolutionary relationships between the *Trypanosoma cruzi* clones using the MIX program; CLIQUE program yielded a quite similar pattern (see text). The matrix was built from the presence/absence criterion for 38 alleles. The cl. and NP numbers refer to the genotypes identified in this study (see Table 2). The arrows represent the different evolutionary steps between the clones, either by gain or loss of an allele.

surveyed here (minimum genetic distance 0·87; maximum genetic distance 1·79). Zymodeme I appears to be related to the stocks from the first cluster (see above) only, although the minimum genetic distance (between clone 2 and zymodeme I) was not negligible (0·28).

Discussion

Several stocks of *T. cruzi* from Bolivia, Chile and Peru presented new electrophoretic patterns which differed at one to three loci from clones previously described by TIBAYRENC & AYALA (1988). These results are consistent with the clonal structure of *T. cruzi* populations proposed by TIBAYRENC *et al.* (1986): when stock sampling increases and genetic labelling improves, the number of clones identified is expected to become higher. Nevertheless, the main results indicating clonal structure are fully confirmed by the present study, that is to say fixed heterozygosity of some genotypes (see clone 39) and strong linkage disequilibrium; if the alleles recorded here combined randomly, the total number of possible genotypes would be considerable, so that frequent isolation of the same multilocus genotype, as in this work, would be extremely improbable.

Some clones were identified on both sides of the Andes, in Chile and Bolivia (clones 19 and 39), which suggests the existence of communication between the two countries for the invertebrate and/or vertebrate hosts of *T. cruzi*: this could be explained by the important commercial contacts between the two countries. Nevertheless, although stock sampling is still limited, some clones appear to be specific for each of the two countries (see Table 1). This suggests that communication between the two countries is not sufficient to allow complete mixing of the clone populations between them. In Peru, the low genetic variability existing among the clones (maximum genetic distance=0·30) and the presence of a predominant clone (defined by genotype NP11) circulating in the three Peruvian valleys studied suggest the occurrence of founder effects in this area (see below).

The rate of mixed stocks recorded is similar to those in previous results—about 10% (BRENIÈRE *et al.*, 1985a; TIBAYRENC *et al.*, 1985). Due to technical limitations, this is probably an underestimation of the real rate. This is particularly true for the present sample, where Chilean stocks were maintained in culture for a long time, which probably led to the elimination of some clones in mixtures.

Three clusters of clones were identified. This clustering pattern, and the relative configuration of the clones, seem to be 'robust', since they remained unchanged when studied by the different methods used, namely the hierarchized agglomeration technique, the MIX program (with several runs) based on the parsimony principle, and the CLIQUE program based on the compatibility principle: this program rejects incompatible characters (9 out of 38 in the present case) implicating reversion, convergence or an error of interpretation.

The first cluster is rather heterogeneous, and exhibits a complex topology. Within this cluster, Peruvian clones (see Table 1) cannot be equated with a monophyletic group which could be distinguished from the other clones belonging to this first cluster. Although founder effects can be inferred to explain the present genetical variability of *T. cruzi* in Peru, it is probable that the Peruvian stocks do not derive from a unique common ancestor which had differentiated locally: the divergence among NP11/NP10, NP9 and clone 2 seems to have occurred before their introduction in Peru. To date we have no indication about the possible geographical origin of these *T. cruzi* populations from Peru. A larger sample of stocks isolated from *Triatoma spinolai* and human hosts in Chile is needed to understand better the overlap between wild and domestic cycles in this country.

Stock characterization based upon the use of a sufficient range of loci, and on the working hypothesis of clonality, provides an efficient means of studying spatial and temporal distribution of *T. cruzi* populations. Moreover, cladistic analysis makes it possible to establish firmly the phylogenetic relationships among the clones, and to infer working hypotheses concerning their patterns of geographical divergence. Work is in hand to complete the present study by increasing the number of stocks and of isoenzyme loci studied, and by comparing the results with those obtained using other genetic markers, such as kinetoplast deoxyribonucleic acid fragment polymorphism (VEAS *et al.*, 1990).

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