Am. J. Trop. Med. Hyg., 61(6), 1999, pp. 986–993

GENETIC STRUCTURE AND PHYLOGENETIC RELATIONSHIPS OF COLOMBIAN *TRYPANOSOMA CRUZI* POPULATIONS AS DETERMINED BY SCHIZODEME AND ISOENZYME MARKERS

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Abstract. Twenty-four *Trypanosoma cruzi* stocks isolated from vectors and from human and *Didelphis marsupialis* hosts from highly separated sylvatic localities in Colombia were characterized by isoenzyme and schizodeme analyses. The stocks were collected primarily from sylvatic ecotopes representing areas of low, moderate, and high endemicity for Chagas' diseases in Colombia. Parasites were characterized mainly by schizodeme analysis with the restriction enzyme *Eco* RI and the isoenzyme analysis was performed at 10 genetic loci. These analyses demonstrated an agreement between the classifications based on the isoenzyme analysis and on the restriction fragment length polymorphism patterns obtained with the Colombian stocks. There is clear evidence of demic subdivision between the eastern (E) and western (W) stocks separated by the Andean Mountains and Magdalena River, which is likely due to the geographic isolation generated by these topographic features. Heterozygosity estimates indicate that the E group could be more ancient than the W group. As was postulated in a previous study, these results are also compatible with the existence of a clonal population structure in Colombian sylvatic *T. cruzi*. Evidence presented here failed to demonstrate a correlation between the degree of endemy and genetic clustering. Finally, schizodeme and isoenzymatic analyses comparing Colombian *T. cruzi* stocks with others from Chile confirm that Colombian isolates are genetically related to zymodeme 1 and distant from zymodeme 2.

Trypanosoma cruzi is a parasitic protozoan of biologic and medical importance. It is the etiologic agent of Chagas' disease, which affects approximately 20 million people in Central and South America.[1] The information available concerning the epidemiology of Chagas' disease from different geographic areas in Latin America[2] is highly variable, but Colombia is one of those countries in which epidemiological data on Chagas' disease is now accumulating. Clinical signs from chronic cases throughout Latin America are mainly cardiac and intestinal, but these clinical forms are not equally distributed in this region. It has been proposed that different *T. cruzi* strains, as identified by different genetic markers, could explain this fact. It is interesting that only cardiac cases are seen in the nearby country of Venezuela, which has led us to suggest a possible link between infective zymodemes and clinical forms of Chagas'disease.[3] A similar situation exist in Colombia, where only cardiac cases have been reported.[4,5]

Isoenzyme and schizodeme profiles have been widely used to characterize *T. cruzi* stocks from different countries. North of the Amazon basin, only the isoenzyme profiles zymodeme 1 and zymodeme 3 have been found. While the first one is ubiquitous, the second is rare, with a sporadic distribution.[3] In contrast, zymodemes Z2Bra and Z2Bol circulate only in the southern portion of South America.[6] Previous studies of the isoenzyme profiles of *T. cruzi* stocks from the Cundinamarca and Meta Departments in Colombia revealed an extensive polymorphism when studied with 13 enzyme systems.[7] Only zymodemes Z1 and Z3-like were found in that sample, although the isolates from the sylvatic and domiciliary transmission cycles diverged extensively.

In a previous study,[8] we evaluated isoenzyme variability in 75 wild stocks of *T. cruzi* obtained from differing hosts from 5 geographic regions within the endemic area in Colombia. The results supported a scenario of long-term clonal evolution in Colombian sylvatic *T. cruzi* populations. In this paper, distinct endemic areas of Colombia were chosen to further characterize *T. cruzi* isolates from different sylvatic environments. The geographic localities considered are the Antioquia, Sucre, Arauca, Casanare, Caquetá, Meta, and Tolima Departments, which are geographically representative of areas of low, moderate, and high endemicity. The degree of endemicity was defined according to the prevalence of Chagas' disease in 3 different regions of Colombia, as previously reported.[5] Here, we report the isoenzyme and schizodeme profiles of 24 *T. cruzi* stocks obtained from *Rhodnius prolixus* and *R. pallescens* in these areas. Analyses on genetic structure, phylogenetic inferences, and their concordance with geographic distribution are presented. Such information should eventually permit the elucidation of the normal mating system of *T. cruzi*, and development of phylogenetic scenarios, which will be important for the diagnosis, treatment, and prevention of Chagas' disease.

MATERIALS AND METHODS

***Trypanosoma cruzi* stocks.** Figure 1 shows the geographic area where the Colombian *T. cruzi* stocks were sampled. Table 1 provides information on the geographic area and host origin of the stocks. The *T. cruzi* stocks Ev13, Ev63, Ep222 and Daza from the Meta and Casanare Departments were previously studied at the isoenzyme level.[7] Similarly, the Colombiana and vinC7 isolates were previously characterized as zymodeme 1.[9,10] The Brazilian zymodeme Z1 Sylvio X10 clone 1 (SX10 cl 1) and the Chilean zymodemes Z2 Bra (IVV stock), Z2 Bol (GTP), and Z1 (CHI22 and spAI) were included as references for clustering.

Schizodeme analysis. Restriction fragment length polymorphism (RFLP) was performed with the restriction enzyme *Eco* RI. The digestion products were electrophoresed on a 4.5–10% polyacrylamide gradient gel and stained with silver nitrate.[11] The quantification of common bands was per-

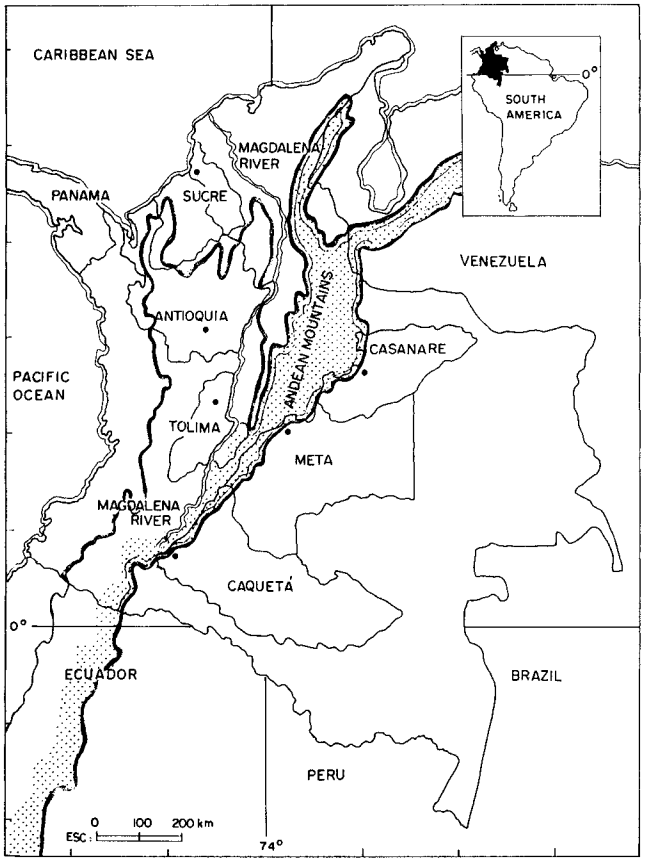

FIGURE 1. Map of Colombia showing the Departments where the *Trypanosoma cruzi* stocks were isolated. The Magdalena River and the Andean Mountains, which separate the eastern and western portions of the country, are indicated.

formed with the formula C/A + B + C, where C is the number of bands common to the two stocks being compared, and B and A correspond to bands present only on the first and second stocks, respectively.

Extract preparation and isoenzyme electrophoresis. Parasites were grown in liver infusion tryptose medium at 28°C, harvested by centrifugation at 3,000 × *g* at 4°C for 10 min and stored at −70°C until analyzed. For isoenzyme electrophoresis, parasites were lysed by suspension in a hyposmotic solution containing 2 mM EDTA, 2 mM dithiothreitol, and 2 mM ϵ-aminocaproic acid. The lysed solution obtained was centrifuged at 15,000 × *g* at 4°C for 30 min and the soluble fraction was used for electrophoresis. Ten enzymatic systems were used: glucose phosphate isomerase (E.C. 5.3.1.9, GPI), peptidase D (E.C. 3.4.11.1, PEP), mannose phosphate isomerase (E.C. 5.3.1.8, MPI), phosphoglucomutase (E.C. 2.7.5.1, PGM), aconitate hydratase (E.C. 4.2.1.3, ACON), 6-phosphogluconic dehydrogenase (E.C. 1.1.1.44, 6PGD), malate dehydrogenase NAD (E.C. 1.1.1.37, MDH), pyruvate kinase (E.C. 2.7.1.40, PK), glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PD), and phosphoglycerate kinase (E.C. 2.7.2.3, PGK). They were examined by thin-layer starch gel electrophoresis as previously described,[7,12] with minor modifications.

Evolutionary scenarios. Three hypothetical evolutionary scenarios were considered as explanations for the genetic structure and clustering of the *T. cruzi* stocks identified. In evolutionary scenario I (ESI), all stocks were analyzed assuming their existence as demes in nature. In evolutionary scenario II (ESII), the stocks were clustered into 4 subpopulations according to geographic origin (Antioquia, Sucre, Tolima, and a fourth subpopulation comprised of stocks from the Arauca, Meta, Casanare and Caquetá Departments, designated Eastern Plains). In evolutionary Scenario III (ESIII), the stocks were grouped into 2 subpopulations, based upon the geographic subdivision created by the eastern Andean Mountain Range and the Magdalena River. The western group (W) included stocks from Sucre, Antioquia, and Tolima and the eastern (E) group included samples from Arauca, Meta, Casanare, and Caquetá.

Data analysis. Multilocus enzyme genotypes of each stock were determined by numbering isoenzyme bands from 1 to n in decreasing electrophoretic mobility order. The unbiased genetic distances of Nei[13] and their respective standard deviations were used to compare gene frequencies between *T. cruzi* stocks according to each evolutionary scenario. The matrix of genetic distances was used to generate phenetic trees via unweighted pair-group method with the arithmetic mean (UPGMA) and neighbor-joining (NJ) analyses. This approach was chosen to detect departures from the molecular clock theory.[14] We also performed bootstrap test with 1,000 replications to determine the significance of individual branches within a tree. The criterion was that if a particular branching pattern is observed 70% of the time, this branching pattern is said to have 70% bootstrap support. The exact statistical interpretation of bootstrap results is still

TABLE 1
Biologic and geographic origins of Colombian *Trypanosoma cruzi* stocks

Stock	Host*	Locality, Department, Country
J7, SC-1, SC-6, SC-13	*R. pallescens*	San Carlos, Antioquia, Colombia
SC-2	*D. marsupialis*	San Carlos, Antioquia, Colombia
VINC-7	*R. prolixus*	Puerto Ele, Arauca, Colombia
COLOMBIANA	Human	Florencia, Caquetá, Colombia
DAZA	Human	Monterrey, Casanare, Colombia
EP-222	*R. prolixus*	Cravo Sur, Casanare, Colombia
EV-13, EV-63	*R. prolixus*	Calaguala, Meta, Colombia
FX-18	*D. marsupialis*	Galeras, Sucre, Colombia
GAL-1, GAL-8, GAL-9, SU-4, SU-5	*R. pallescens*	Galeras, Sucre, Colombia
RTD	*D. marsupialis*	Coyaima, Tolima, Colombia
STP-2.2, STP-3.1, STP-3.4, STP-3.5, STP-3.6, Susunaga	*R. prolixus*	Coyaima, Tolima, Colombia

* *R. = Rhodnius*; *D. = Didelphis.*

TABLE 2
Genotypes of Colombian *Trypanosoma cruzi* stocks for 10 isoenzymes*

Stock	GPI	G6PD	PEP1	MPI	ACON	PGM	MDH	PK	6PGD	PGK
J7	5/5	2/2	2/2	2/2	3/4	2/2	1/1	1/1	1/1	1/1
SC1	5/5	3/3	4/4	2/2	3/4	3/3	1/1	1/1	1/1	1/1
SC2	5/5	3/3	4/4	1/1	3/4	3/3	1/1	1/1	1/1	1/1
SC6	5/5	3/3	4/4	1/1	3/4	4/4	1/1	2/2	1/1	1/1
SC13	5/5	3/3	4/4	1/1	3/4	3/3	1/1	1/1	1/1	1/1
Daza	5/5	2/2	3/3	2/2	3/4	3/3	1/1	1/1	1/1	1/1
EP222	4/7	3/3	2/6	4/6	4/5	3/3	1/1	1/1	1/1	1/1
EV13	8/8	3/3	2/7	3/6	4/5	3/3	1/1	1/1	1/1	1/1
EV63	5/5	3/3	2/8	6/6	4/5	4/4	1/1	1/1	1/1	1/1
FX18	6/6	2/2	3/3	4/4	1/2	1/1	1/1	2/2	1/1	1/1
GAL1	5/5	3/3	1/2	2/2	2/2	3/3	1/1	1/1	1/1	1/1
GAL8	5/5	3/3	1/2	2/2	2/2	3/3	1/1	1/1	1/1	1/1
GAL9	5/5	3/3	1/2	2/2	2/2	3/3	1/1	1/1	1/1	1/1
SU4	5/5	3/3	1/2	1/1	3/4	3/3	1/1	1/1	1/1	1/1
SU5	5/5	3/3	1/2	1/1	3/4	3/3	1/1	1/1	1/1	1/1
RTD	5/5	3/3	4/4	2/3	3/4	4/4	1/1	2/2	1/1	1/1
STP2.2	5/5	3/3	1/2	1/3	4/5	4/4	1/1	2/2	1/1	1/1
STP3.1	6/6	2/2	3/3	2/2	1/2	1/1	1/1	1/1	1/1	1/1
STP3.4	6/6	3/3	5/5	1/1	3/4	2/2	1/1	1/1	1/1	1/1
STP3.5	5/5	3/3	3/3	1/1	4/5	4/4	1/1	2/2	1/1	1/1
STP3.6	5/5	3/3	3/3	1/1	4/5	4/4	1/1	2/2	1/1	1/1
Susunaga	5/5	3/3	4/4	3/3	3/4	4/4	1/1	2/2	1/1	1/1

* For definitions of isoenzymes, see Materials and Methods.

an active subject of study, but the rule of thumb is that internal tree branches that have 70% bootstrap support are likely to be corrected at the 95% level.[15] All analyses were computed using the programs GNKDST and TREVIEW obtained from Tatsuya Ota (Pennsylvania State University, University Park, PA). In all 3 evolutionary scenarios, population subdivision was quantified by calculating Wright's *F* statistics according to the methods of Weir and Cockerham[16] for the 3 separate levels. Bootstrap tests involving 1,000 replications were performed to determine significant differences from 0 with 95% confidence intervals using the Genetic Data Analysis (GDA) computer program.[17] Tests for clonal structure of *T. cruzi* demes were performed as described by Tibayrenc and others,[18] with deviations from expected Hardy-Weinberg genotype proportions assessed by Fisher exact tests. Linkage disequilibrium analyses were used to test for the absence of recombination among all pair-wise combinations of loci by performing a shuffling test, which randomly permuted the multilocus enzyme genotypes, again using the GDA software.

RESULTS

Genotypes obtained for each isoenzyme are shown in Table 2. The 6PGD and PGK enzymes were the only ones totally monomorphic, while MDH was monomorphic in all stocks with the exception of FX18. All remaining enzymes were polymorphic. Figure 2A and B shows the UPGMA and NJ trees, respectively. Comparable clustering was demonstrated by each method and significant branch length departures were not observed in the NJ tree. Bootstrap results with the UPGMA tree were significant (95% confidence interval) primarily in the terminal nodes of branching. If one considers these terminal nodes, it is possible to define at least 5 groups: groups SU4-SU5 and GAL1-GAL8-GAL9 from Sucre, group EP22-EV13 from the Eastern Plains, group RTD-SUSUNAGA from Tolima, and group STP3.5-STP3.6 from Tolima. Figure 2A shows that mainly Antioquia and Sucre populations cluster together. Some stocks from Sucre and Antioquia show clustering with the Tolima stocks (SC6-FX18). Stocks from the Eastern Plains showed an important clustering in the middle area of the tree, with the DAZA stock grouped with Antioquian stocks. If one considers ESIII, the W and E groups are well discriminated by the main root node (a node with a 70% bootstrap support). Results of the population subdivision F statistics analysis are shown in Table 3. If one considers ESIII, non-significant departures from 0 were found in the estimation of F_{st}, suggesting no populational subdivision between groups E and W. For all evolutionary scenarios, highly significant deviations from Hardy-Weinberg expected proportions were found in most loci (70%), with some loci (40%) lacking heterozygous genotypes and others lacking homozygous individuals. Both fixed heterozygosity and fixed homozygosity were detected. Moreover, the absence of segregating genotypes was observed in all loci. The genotype-shuffling test revealed significant linkage disequilibrium between loci ($P < 0.05$), especially when ESIII was examined.

The kinetoplast DNA (kDNA) RFLP patterns did not produce an interpretable grouping of the *T. cruzi* stocks based on their RFLP similarities (Figures 3–5). Figure 3 shows the results of the schizodeme analysis of *T. cruzi* stocks obtained from the Antioquia Department. This method differentiated among the isolates of Antioquia, with the kDNA pattern quite similar in all of them except stock J7 (Figure 3A). The average quantification of common bands within the stocks SC2, SC6, and SC13 of the Antioquia Department was 0.85, and the average between any one of these 3 stocks and the J7 stock was only 0.05. On the other hand, the samples from the Sucre Department were highly homogeneous, with similar or identical patterns demonstrated for all the isolates from this geographic region (Figure 3B). Figure 3 also in-

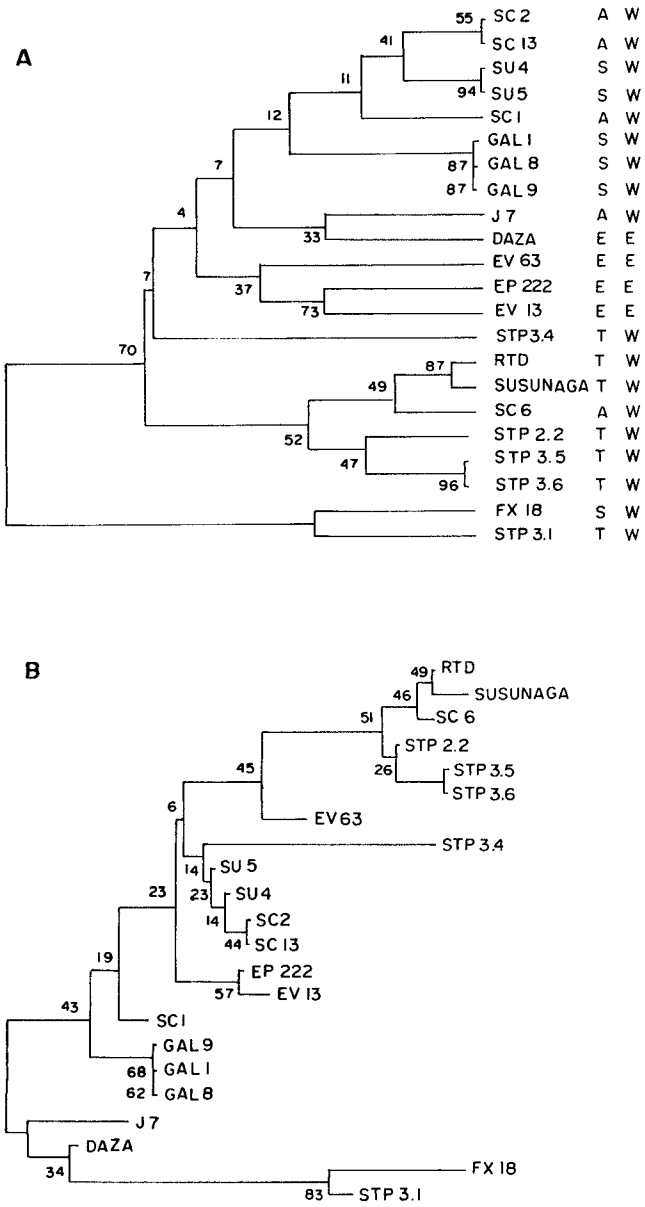

FIGURE 2. Unweighted pair-group method with the arithmetic mean (**A**) and neighbor-joining (**B**) trees of Colombian *Trypanosoma cruzi* stocks based on isoenzyme phenotypes. Values of bootstrap support are given on each node. A = Antioquia; S = Sucre; T = Tolima; W = Western Plains; E = Eastern Plains.

cludes the Ev13 and Ep222 stocks from the Meta and Casanare Departments, which show different profiles. The average quantification of common bands within stocks SU4, SU5, Gal 1, Gal 8, and Gal 9 of the Sucre Department is 0.53. However, this figure decreases to 0.07 and 0.11 when the stocks from the Sucre Department are compared with the EV13 and Ep222 stocks, respectively. Figure 4 compares the results obtained from the *T. cruzi* stocks from the Meta, Canasare, Arauca, and Caquetá Departments. It was also possible to detect different kDNA RFLPs on each *T. cruzi* sample, as shown in Figure 4A. The average quantification of common bands within the stocks EV13, Ep222, Daza and EV63 of the Meta and Casanare Departments was 0.31. Finally, kDNA patterns of the *T. cruzi* stocks from the Tolima Department displayed similar profiles with only minor differences detected among them (Figure 4B). The average quantification of common bands within all the stocks from the Tolima Department (with the exception of STP 3.4) was 0.91, but the similarity index was 1.0 between the STP3.5 and STP3.6 stocks. The only stock with a slightly different RFLP pattern was STP3.4, which yielded an average similarity index of 0.48 when compared with the others stocks from the same Department. One *T. cruzi* stock from Antioquia (the SC2 isolate) was included for comparative purposes. The similarity index with the Susunaga stock was 0.12.

A selected sample of Chilean zymodemes was included in this study for comparative purposes. Figure 5 show the results of kDNA RFLP analyses of the Colombiana stock from the Caquetá Department and the Chilean stock marker of zymodemes: Z2Bra (IVV), Z2Bol (GTP), and Z1 (CHI22 and SpAI), and the Brazilian Z1 (SX10 cl 1). Whereas the Chilean zymodeme 2 produced the characteristic patterns, the Colombian stocks resembled the general profile of the Chilean and Brazilian zymodeme 1. Similar isoenzyme results comparing Colombian *T. cruzi* stocks from *R. prolixus* with the Brazilian SX10 cl1 stock have also been documented for 5 enzymatic systems.[10] Schizodeme analyses were also performed with *Msp* I, but the resulting patterns were less discriminative than the ones obtained with *Eco* RI.

DISCUSSION

A large number of *T. cruzi* stocks from different geographic regions of Colombia were studied, using 2 independent methods. The *T. cruzi* stocks analyzed come from a highly endemic area (Coyaima municipality in the Tolima Department) in the central part of the country, an area of moderate endemicity in the eastern region (Meta and Casanare Departments) and an area of low endemicity in the

TABLE 3

Wright's *F* statistics for population subdivision according to the non-biased estimators of Weir and Cockerham[16] (f = F_{is}, F = F_{it}, and θ = F_{st}).

	Evolutionary scenario I 22 subpopulations (individual stocks)*			Evolutionary scenario II four subpopulations			Evolutionary scenario III two subpopulations		
Wright's F statistics	f	F	θ	f	F	θ	f	F	θ
Upper bound	********	********	0.960967	0.958389	0.962341	0.229908	0.960893	0.963249	0.139224
Lower bound	********	********	0.303483	0.200217	0.327050	0.036626	0.286866	0.336463	−0.015213
Nominal confidence interval†	95.0			95.0			95.0		

* Asterisks indicate that f and F values cannot be estimated for evolutionary scenario I.
† Bootstrapping over loci. Number of replicates = 1,000.

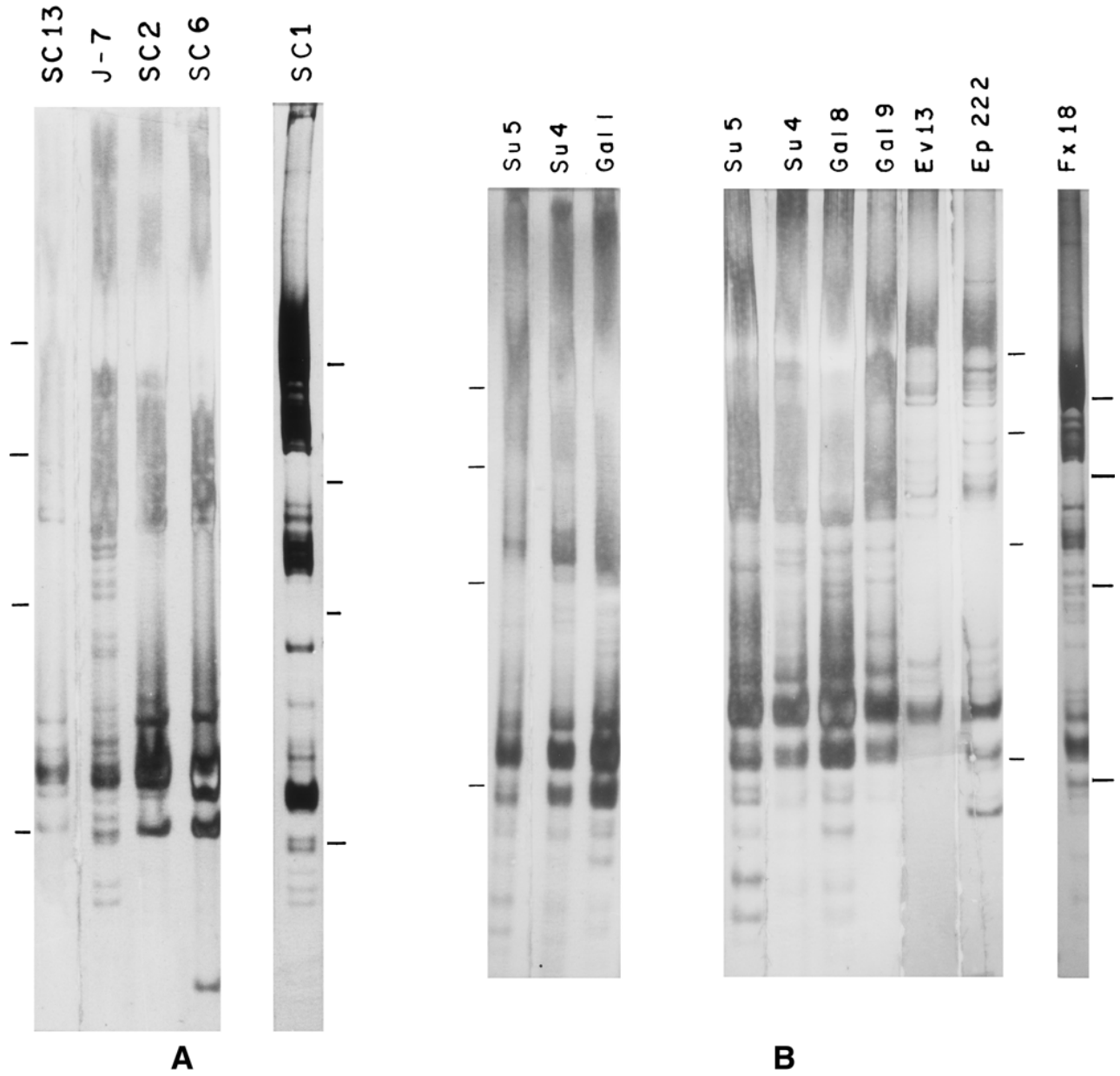

FIGURE 3. Schizodeme profiles of *Trypanosoma cruzi* stocks from Antioquia (**A**) and Sucre (**B**) Departments based upon polyacrylamide gradient gel electrophoresis and staining with silver nitrate. Molecular weight markers (**dashes**) are (from top to bottom) 1,353, 872, 603, and 310 basepairs.

northwestern portion of Colombia (Sucre and Antioquia Departments).

Two main insect vectors have been implicated in the parasite propagation of the sylvatic and domestic transmission cycles in this country (*R. pallescens* and *R. prolixus*, respectively). *Rhodnius prolixus* is the most important vector in the northeastern and central departments, which transmit different *T. cruzi* genotypes than those described from the sylvatic and domestic host from the Piedmont and Eastern Plains.[7] *Rhodnius pallescens* is frequently found on the northwest side of the Magdalena valley and on the Panamanian border. *Rhodnius prolixus* and rarely *R. pallescens* colonize houses from sylvatic ecotopes; thus, some overlapping of *T. cruzi* stocks from both transmission cycles should be expected.

Three *T. cruzi* stocks from the sylvatic reservoir *Didelphis marsupialis* were studied. Two of them (RTD and SC 2) were similar to others from the same reservoir, but one of them (FX 18) displayed a different isoenzymatic profile, suggesting that some *T. cruzi* stocks circulating within *D. marsupialis* could be unique.

A great variety of different schizodemes and isoenzyme profiles were documented in this survey with evidence of geographic structuring in this variation. For example *T. cruzi* stocks from the Antioquia, Sucre and Tolima Departments were more similar genetically to the ones from the Eastern

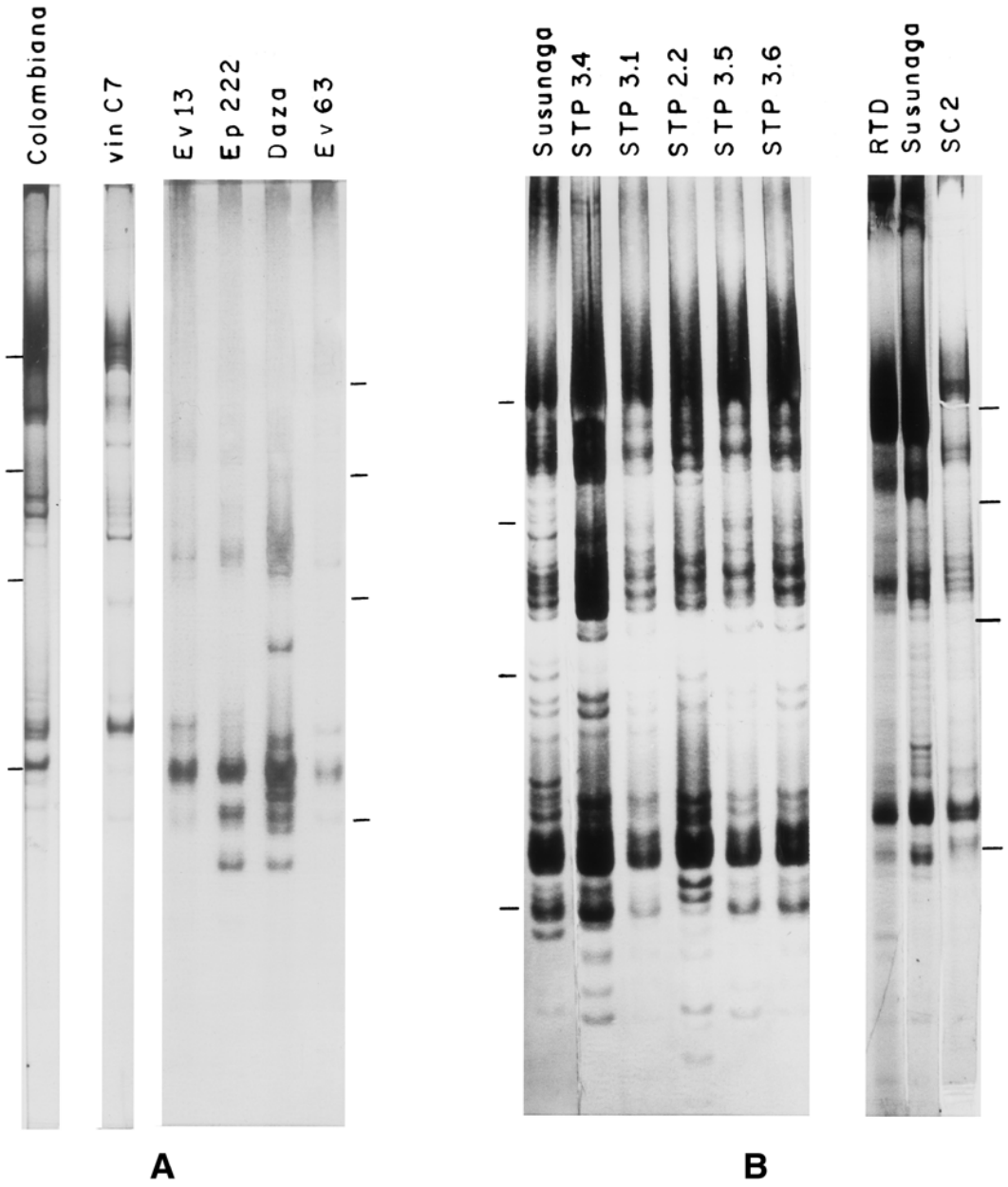

FIGURE 4. Schizodeme profiles of *Trypanosoma cruzi* stocks from the Meta, Arauca, Caquetá, and Casanare departments (**A**) and the Tolima Department (**B**). Conditions of polyacrylamide gradient gel electrophoresis and staining were the same as in Figure 3.

Plains (Meta and Casanare Departments). Interestingly, *T. cruzi* stocks from the Antioquia and Sucre Departments are transmitted by *R. pallescens*, while the other stocks circulate via *R. prolixus*, which colonizes human dwellings in the most endemic areas. This observation could have epidemiologic relevance for Chagas' disease in Colombia, since these 2 major parasite groups have different biologic effects on humans.

Our characterization of *T. cruzi* isolates from Colombia has extended our knowledge of the distribution of *T. cruzi* populations in this part of South America. This is the second schizodeme characterization study of *T. cruzi* for this country,[19] so documenting such high heterogeneity among the stocks was not entirely surprising. A previous study of *T. cruzi* enzyme profiles also described a great genetic heterogeneity in parasites from the sylvatic transmission cycle,[7] which the enzyme and molecular karyotype analyses in the present study have corroborated.[20]

The geographic and ecologic diversity of northern Colombia and the occurrence of a variety of insect vectors in this region make it an area of special interest. Parasite samples comprised mainly *T. cruzi* from sylvatic areas exhibited greater heterogeneity than that shown by parasites from only domestic areas, as described before.[7] This also has been documented elsewhere,[10] and could be explained by the preferential adaptation of some *T. cruzi* clones to human hosts from the mixed populations circulating in nature. One major *T. cruzi* genetic lineage was detected in this work. This par-

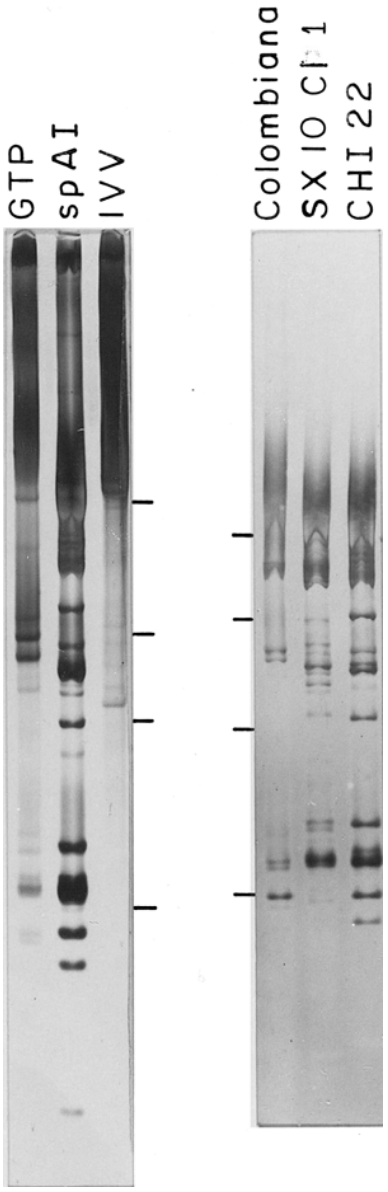

FIGURE 5. Schizodeme profiles of the Colombiana *Trypanosoma cruzi* stock from Colombia and *T. cruzi* stocks from Brazil **(left)** and Chile **(right)**. Conditions of polyacrylamide gradient gel electrophoresis and staining were the same as in Figure 3.

asite type corresponded to zymodeme 1, which is a very heterogeneous group in terms of its schizodeme profiles.

In general, there was agreement between the classifications based on analyses of isoenzyme and RFLP data from the Colombian stocks, despite the complex schizodeme patterns that are sometimes difficult to quantify accurately between 2 or more isolates. There is clear evidence that those groups originating from the western side of Andean Mountains and Magdalena River region represent an homogeneous genetic group differentiated from those originating from the eastern side of the Andean Mountains and Magdalena River valley (those distributed in the Eastern Plains). This implies that these topographic features represent geographic barriers that have exerted a significant influence in the *T. cruzi* micro-evolutionary process. Heterozygosity estimates were higher in the E group than in the W group, suggesting that the group from the Eastern Plains could be ancestral to the W group.

As we argued in a previous study,[8] the results presented in this paper are compatible with the clonal population structure in Colombian sylvatic *T. cruzi.* Evidence supporting this interpretation comes from several sources. 1) The null hypotheses that genotype proportions do not depart from Hardy-Weinberg expectations and that alleles at different loci assort independently were rejected. 2) The existence of both fixed heterozygosity and fixed homozygosity in stocks was shown. 3) *Trypanosoma cruzi* stocks with different isoenzyme patterns also exhibited different and characteristic schizodeme profiles. This result suggests that independent genetic markers in *T. cruzi*[18] subpopulations covary, which reinforces an interpretation that reproduction is clonal.

Further microgeographic studies are required to determine the biologic properties of *T. cruzi* stocks from areas of low endemicity (Antioquia and Sucre Departments), as well as to compare stocks from areas of moderate and high endemicity (Meta, Cundinamarca, and Tolima Departments). The evidence presented here does not predict a significant correlation between the degree of endemy and genetic clustering within a given region, but does show a correlation due to the long geographic isolation among subpopulations located on opposite sides of the Andeans Mountains and Magdalena River.

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