Am. J. Trop. Med. Hyg., 66(4), 2002, pp. 394–400

ISOENZYME CLUSTERING OF *TRYPANOSOMATIDAE* COLOMBIAN POPULATIONS

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Abstract. Thirty-six *Trypanosomatidae* stocks isolated from various hosts and geographical areas in Colombia and 7 others from Bolivia, Chile, Honduras and Panama have been surveyed by Multilocus Enzyme Electrophoresis (MLEE). Part of the Colombian stocks were previously characterized by morphology and biological behavior as belonging to *Trypanosoma cruzi* and *Trypanosoma rangeli* taxa, others were unknown species. The genetic variability observed at 13 different loci was considerable, since 38 zymodemes could be distinguished and 2 upper branches were observed. The first branch corresponded to *T. cruzi* and was divided in the two major phylogenetic subdivision of *T. cruzi* (*T. cruzi* I, *T. cruzi* II). The majority of the Colombian *T. cruzi* stocks (92%) felt into *T. cruzi* I. Only two stocks, isolated from sylvatic mammals, belonged to *T. cruzi* II. Among *T. cruzi* I, we did not observed any additional phylogenetic subdivision and host-dependent genotype specificity. The second branch was genetically very heterogeneous and included all *T. rangeli* stocks, the stocks isolated from bats and one stock isolated from a sylvatic *R. prolixus* vector. The stocks belonging to *T. rangeli* presented only one locus instead of two for the malic enzyme system. Since, the upper level of resolution of the isoenzyme method was exceeded, the current clustering study failed to draw a clear distinction between such a diverse set of *Trypanosomatidae* species.

INTRODUCTION

Chagas disease is a widespread Trypanosomiasis in Latin America, however, its manifestations and epidemiological characteristics are highly variable. *Trypanosoma cruzi,* the agent of the disease, circulates between many species of triatomine vectors, human and mammal reservoirs. In Colombia, *Rhodnius prolixus* is the main vector and the areas at risk has been related with the distribution of this vector.[1] The highest transmission rates have been found in the Magdalena River valley, the Catatumbo River basin and in the department of Norte de Santander where 9% of chagasic patients presented electrocardiographical alterations.[2] *R. prolixus* is also present in sylvatic cycles as well as several other species. Sylvatic cycles constitute a source of human infections when man penetrates the forest, although many triatomine species are infected by *T. cruzi* and trend toward domesticity.[2,3,4]

T. cruzi is composed of natural clones, which present a high genetic diversity and some of them are dominant clones.[5] These clonal genotypes have been distributed into two major lineages recently named *T. cruzi* I and *T. cruzi* II.[6,7,8] Each group remained very heterogeneous and only *T. cruzi* II should be divided into 5 phylogenetic subgroups as proposed using Random Amplified Polymorphic DNA analysis (RAPD).[9,10] A first work of isoenzyme analysis have related *T. cruzi* Colombian stocks to zymodeme I which belong to *T. cruzi* I.[11,12] Other study have found separate genotype groups in domestic and sylvatic ecotopes.[13] *Trypanosoma rangeli*, unlike *T. cruzi,* is a no pathogenic agent largely associated with vectors of the genus Rhodnius and it is abundant in.[14] The two parasites were distinguishable by their morphology and biological behavior but few specific genetic markers have been proposed.[15,16,17,18] Moreover, mammals can harbor several species of the subgenus *Schizotrypanum* and genetic characterization should help to determine their importance as a reservoir for *T. cruzi.*

In the current work, isoenzyme variability of new Colombian *T. cruzi, T. rangeli* and unknown trypanosomes (35 stocks) derived from sylvatic and domestic cycles was explored at 13 polymorphic loci. Isoenzyme patterns were compared with those of South American stocks and numerical and cladistic cluster analysis were carried out.

MATERIALS AND METHODS

Origins of stocks. Table 1 summarized the place and host origin of the 43 stocks (36 from Colombia, 5 from Bolivia, Chile, Honduras and Panama and 2 reference clones) under study. The reference clones SO34 cl4 (*T. cruzi* I) and MN cl2 (*T. cruzi* II) belonged to the 2 different lineages that composed *T. cruzi* taxon.[6] Colombian *T. cruzi* (10 stocks) and *T. rangeli* (6 stocks) were previously characterized by morphology after Giemsa staining of mice blood smear, and biological behavior in experimental mice infections (low virulence of *T. rangeli* stocks). Moreover, unlike *T. cruzi, T. rangeli* is transmitted by salivary inoculation during feeding and the present *T. rangeli* stocks were all isolated from salivary glands. The specific salivary gland localization of *T. rangeli* stocks was confirmed by experimental infections of bugs. The stocks P-19, Choachi, Perijá, Durán were maintained in laboratory through culture, vector and mice. *T. rangeli* stock C23 was also previously characterized by gene mini-exon PCR.[19]

Parasite extracts. The stocks were isolated in NNN and REI medium with 2% of fetal calf serum and were amplified in biphasic Tobie medium (*T. rangeli*) and Maekelt medium (*T. cruzi*).[20] Parasites were then harvested by centrifugation. The enzymes were extracted from the pellets with enzyme stabilizer buffer.[21,22] The extracts were divided in 20 μl aliquots and stored at –70°C or used immediately for further MLEE.

MLEE protocols. The electrophoretic study was carried out on cellulose acetate plates with slight modifications of migration times for G6PD and GPI (25 min), IDH (30 min) and MDH (25 min) systems.[23] Twelve different enzyme systems were surveyed (13 enzymatic loci): glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), glucose-6-phosphate

TABLE 1

Origin of 43 *Trypanosoma cruzi, Trypanosoma rangeli* and *Trypanosoma* sp. stocks examined

Stocks	Host	Geographical origin	Region	Cycle	Zymodeme	Putative classification
T. cruzi stocks						
MHOM/CO/86/M. Rangel	Human	Chiriguaná, Cesar (Colombia)	Atlantic coast	Domestic	31	*T. cruzi* I
MHOM/CO/86/O. González	Human	Simití, Bolivar (Colombia)	Atlantic coast	Domestic	23	*T. cruzi* I
IRHO/CO/92/Sta Marta	*R. prolixus*	Sierra Nevada, Magdalena (Colombia)	Atlantic coast	Domestic	29	*T. cruzi* I
MHOM/CO/92/F. Chaparro	Human	Tibú, Norte Santander, (Colombia)	Catatumbo River basin	Sylvatic	25	*T. cruzi* I
IRHO/CO/86/G. Castillo	*R. prolixus*	El Zulia, Norte Santander (Colombia)	Catatumbo River basin	Domestic	19	*T. cruzi* I
MHOM/CO/94/E. Acosta	Human	Lago Mure, Vaupés (Colombia)	Oriental part of the Amazonian forest	Domestic	28	*T. cruzi* I
MHOM/CO/87/B.M. Lopez	Human	Paratebueno, Cundinamarca (Colombia)	Magdalena River valley	Domestic	21	*T. cruzi* I
IRHO/CO/86/Choachi	*R. prolixus*	Choachi, Cundinamarca (Colombia)	Magdalena River valley	Domestic	17	*T. cruzi* I
IRHO/CO/82/Molino	*R. prolixus*	Molino, Cundinamarca (Colombia)	Magdalena River valley	Domestic	23	*T. cruzi* I
ITRI/BO/89/Sta Cruz	*Triatoma* sp.	Santa Cruz (Bolivia)			35	*T. cruzi* II
ITRI/BO/86/SO34 cl4	*T. infestans*	Toropalca, Potosi (Bolivia)		Domestic	30	*T. cruzi* I
MHOM/CO/90/N. Guevara	Human	Bogotá (Colombia)		Domestic	24	*T. cruzi* I
MHOM/CL/00/MN cl2	Human	IVa region, (Chile)		Domestic	36	*T. cruzi* II
ITRI/CHI/82/Tulahuén	*Triatoma* sp.	(Chile)			38	*T. cruzi* II
ITRI/CL/82/clon Tulahuén	*Triatoma* sp.	(Chile)			37	*T. cruzi* II
MHOM/PA/84/Y. Sousa	Human	(Panama)			34	*T. cruzi* II
T. rangeli stocks						
MAOT/CO/86/C-23	*Aotus* sp.	San Marcos, Sucre (Colombia)	Atlantic coast	Sylvatic	9	*T. rangeli*
IRHO/CO/84/Perijá	*R. prolixus*	Perijá, Guajira (Colombia)	Atlantic coast	Sylvatic	7	*T. rangeli*
IRHO/CO/87/Durán	*R. prolixus*	Tibú, Norte Santander, (Colombia)	Catatumbo River basin	Sylvatic	6	*T. rangeli*
IRHO/CO/85/P-19	*R. prolixus*	Tibú, Norte Santander, (Colombia)	Catatumbo River basin	Sylvatic	3	*T. rangeli*
MHOM/CO/85/S. Agustin	Human	San Agustín, Cundinamarca (Colombia)	Magdalena River valley	Domestic	4	*T. rangeli*
IRHO/CO/82/Choachi	*R. prolixus*	Choachi, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	5	*T. rangeli*
MHOM/HN/92/H-9	Human	(Honduras)			2	*T. rangeli*
Trypanosoma sp. stocks						
M/CO/92/333	Bat sp.	Antioquia (Colombia)	Atlantic coast	Sylvatic	13	Neither *T. cruzi* nor *T. rangeli*
MDID/CO/86/216	*D. masupialis*	Arboledas, Norte Santander (Colombia)	Catatumbo River basin	Sylvatic	26	*T. cruzi* I
MDID/CO/86/531	*D. masupialis*	Durania, Norte Santander (Colombia)	Catatumbo River basin	Sylvatic	25	*T. cruzi* I
MDID/CO/86/No. 3	*D. masupialis*	El Zulia, Norte Santander (Colombia)	Catatumbo River basin	Sylvatic	15	*T. cruzi* I
MRAT/CO/86/223	*Rattus rattus*	Arboledas, Norte Santander (Colombia)	Catatumbo River basin	Sylvatic	20	*T. cruzi* I
M/CO/92/111	Bat sp.	Antioquia (Colombia)	Magdalena River valley	Sylvatic	11	Neither *T. cruzi* nor *T. rangeli*
M/CO/92/114	Bat sp.	Antioquia (Colombia)	Magdalena River valley	Sylvatic	12	Neither *T. cruzi* nor *T. rangeli*
M/CO/92/121	Bat sp.	Antioquia (Colombia)	Magdalena River valley	Sylvatic	8	*T. rangeli*?
MCAL/CO/93/*C. Lanatus*	*Caluromys lanatus*	Ricaurte, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	27	*T. cruzi* I
MDID/CO/91/006	*D. masupialis*	La Vega, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	14	*T. cruzi* I
MDID/CO/88/No. 69	*D. masupialis*	Mariquita, Tolima (Colombia)	Magdalena River valley	Sylvatic	25	*T. cruzi* I
MDID/CO/93/791	*D. masupialis*	Ricaurte, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	22	*T. cruzi* I
MDID/CO/89/R-12	*D. masupialis*	Ricaurte, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	16	*T. cruzi* I
MDID/CO/88/R-46	*D. masupialis*	Ricaurte, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	25	*T. cruzi* I
MDID/CO/82/Ubaque 52	*R. Prolixus*	Ubaque, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	15	*T. cruzi* I
MDID/CO/82/Ubaque 67	*R. Prolixus*	Ubaque, Cundinamarca (Colombia)	Magdalena River valley	Sylvatic	10	Neither *T. cruzi* nor *T. rangeli*
IPAN/CO/94/Cepa 1	*P. geniculatus*	Antioquia (Colombia)	Magdalena River valley	Sylvatic	32	*T. cruzi* II
IPAN/CO/94/Cepa 6	*P. geniculatus*	Antioquia (Colombia)	Magdalena River valley	Sylvatic	33	*T. cruzi* II
IRHO/CO/86/Palo Gordo	*R. Prolixus*	Palo Gordo, Santander (Colombia)	Magdalena River valley	Domestic	18	*T. cruzi* I
Crithidia sp. stock						
MHOM/CO/00/M-Mayorga	Human	Charalá, Santander (Colombia)				

dehydrogenase (G6PD, EC 1.1.1.49), glucose phosphate isomerase (GPI, EC 5.3.1.9), glutamate dehydrogenase NAD+ (GDH NAD+, EC 1.4.1.2), glutamate dehydrogenase NADP+ (GDH NADP+, EC 1.4.1.4), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), peptidases (substrates: L-leucyl-leucine-leucine and L-leucyl-L-alanine) (PEP 1 and PEP 2, EC 3.4.11 or 13.*), 6-phospho-gluconate dehydrogenase (6PGDH, EC 1.1.1.44), and phosphoglucomutase (PGM, EC 2.7.5.1). The two reference clones, SO34 cl4 and MN cl2 were included in each electrophoresis procedure.

Estimation of overall genetic and clustering procedure. The clonal diversity in the populations was measured by WHITTAM index $d = n (1 - \Sigma Xi^2) / (n - 1)$, with Xi = frequency of each zymodeme and n = number of individuals.[24] Genetic differences among stocks were estimated by Jaccard' s phenetic distances.[25] A dissimilarity matrix was analyzed by computer and relationships were depicted by the Neighbor-Joining method.[26] The method of Wagner parcimony was also applied to the whole sample using the phylogeny inference package with Mix and Seqboot programs to measure statistical significance of the different branches of the tree; the *Crithidia* species was used as an out-group.[27]

RESULTS

Genetic and genotype diversity. Among the 12 enzyme systems tested, the ME inferred the activity of two different enzyme loci as previously described and therefore 13 enzyme loci were resolved. The phenotype composition of the different zymodemes is given in Table 2. In few stocks, the activity of some loci was missing (allele null) despite several essays (GDH NAD+, ME 1 and PEP 2 enzyme systems) and was quoted 0 in Table 2. In other cases the activity was not readable and the result was quoted as 99 (missing data). All loci proved to be polymorphic and thirty-eight distinct zymodemes were detected. The clonal diversity of the Colombian set of stocks was high, d = 0.98. All stocks referred to *T. rangeli* species (Table 1; 6 Colombian stocks and one from Honduras) exhibited the activity of only 1 locus instead of 2 for the ME system (Table 2, Z2 to Z7 and Z9). Similarly, the *Crithidia* stock (M. Mayorga), used as out-group, exhibited only 1 locus for ME system (Z1).

Phylogenetic diversity. According to the Neighbor-Joining dendrogram computed from Jaccard's distance matrix, a first upper branch (upper part of the dendrogram, Figure 1) included the two *T. cruzi* reference clones, the Bolivian, Chilean and Panamean *T. cruzi,* all the Colombian stocks previ-

TABLE 2
Multilocus genotypes of the 38 zymodemes identified with 13 polymorphic loci

Zymodemes	*G6pd*	*Gpi*	*Gdh Nad*	*Gdh Nadp*	*Got*	*Idh*	*Mdh*	*Me 1*	*Me 2*	*Pep 1*	*Pep 2*	*Pgm*	*6Pgdh*
1	*1*	*1*	*2*	*3*	*2*	*2*	*1*	*0*	*1*	*2*	*4*	*1*	*1*
2	*1*	*5*	*1*	*4*	*1, 2*	*2*	*3*	*0*	*3*	*6*	*0*	*5*	*1*
3	*2*	*6*	*0*	*4*	*1, 2*	*3*	*3*	*0*	*3*	*6*	*0*	*3, 4*	*2*
4	*2*	*6*	*0*	*4*	*1*	*3*	*3*	*0*	*3*	*6*	*0*	*3, 4*	*2*
5	*2*	*5*	*0*	*4*	*1*	*2*	*3*	*0*	*3*	*6*	*0*	*3, 4*	*99*
6	*99*	*5*	*0*	*4*	*1*	*2*	*3*	*0*	*3*	*6*	*0*	*7*	*99*
7	*3*	*5*	*0*	*4*	*1*	*2*	*3*	*0*	*3*	*6*	*0*	*2, 4*	*6*
8	*2*	*5*	*0*	*4*	*2*	*3*	*3*	*2*	*3*	*6*	*0*	*7*	*5*
9	*2*	*6*	*0*	*1*	*4*	*5*	*3*	*0*	*5*	*5*	*0*	*2, 4*	*2, 3*
10	*6*	*5*	*0*	*4*	*2*	*2*	*2*	*1*	*3*	*6*	*2*	*4, 7*	*3*
11	*6*	*4, 7*	*3*	*4*	*2*	*2*	*2*	*2*	*3*	*6*	*2*	*2*	*4*
12	*7*	*4*	*5*	*4*	*2*	*2*	*2*	*2*	*2*	*6*	*3*	*4, 10*	*5*
13	*6*	*1*	*0*	*3*	*99*	*1*	*5*	*99*	*99*	*4*	*1*	*7*	*3*
14	*3*	*6*	*5*	*2*	*3*	*2*	*2*	*2*	*2*	*4*	*0*	*5, 8*	*6*
15	*3*	*6*	*4*	*3*	*3*	*2*	*2*	*2*	*2*	*2*	*0*	*5, 8*	*6*
16	*3*	*6*	*5*	*3*	*3*	*2*	*2*	*2*	*2*	*2*	*0*	*5, 8*	*6*
17	*3*	*99*	*4*	*3*	*3*	*2*	*2*	*2*	*3*	*2*	*0*	*5, 8*	*6*
18	*3*	*6*	*4*	*3*	*3*	*2*	*2*	*2*	*4*	*3*	*0*	*5, 8*	*6*
19	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*3*	*2*	*0*	*5, 8*	*6*
20	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*1*	*3*	*2*	*0*	*5, 8*	*6*
21	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*4*	*2*	*0*	*5, 8*	*6*
22	*99*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*3*	*4*	*0*	*5, 8*	*6*
23	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*3*	*1*	*0*	*5, 8*	*6*
24	*3*	*6*	*4*	*3*	*3*	*2*	*2*	*2*	*3*	*6*	*3*	*5, 8*	*6*
25	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*2*	*5*	*3*	*5, 8*	*6*
26	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*2*	*4*	*5*	*3*	*5, 8*	*6*
27	*3*	*6*	*4*	*2*	*6*	*2*	*2*	*2*	*3*	*4*	*2*	*5, 7*	*6*
28	*3*	*6*	*4*	*3*	*3*	*2*	*2*	*2*	*3*	*4*	*2*	*5, 7*	*6*
29	*3*	*6*	*0*	*3*	*3*	*2*	*2*	*1*	*4*	*4*	*2*	*5, 7*	*6*
30	*5*	*6*	*5*	*3*	*3*	*2*	*2*	*3*	*3*	*2*	*2*	*5, 8*	*6*
31	*4*	*6*	*5*	*3*	*3*	*2*	*2*	*3*	*4*	*5*	*3*	*5, 8*	*6*
32	*3*	*5*	*0*	*3*	*2*	*4*	*2*	*1*	*3*	*6*	*2*	*7*	*6*
33	*3*	*5*	*0*	*3*	*2*	*4*	*2*	*1*	*3*	*6*	*3*	*7*	*6*
34	*3*	*5*	*2*	*3*	*2*	*4*	*2*	*2*	*3*	*6*	*3*	*7*	*6*
35	*3*	*2, 5*	*0*	*3*	*2*	*4*	*2*	*2*	*3*	*5*	*3*	*6, 9*	*3, 6*
36	*3*	*2, 5*	*3*	*3*	*2*	*4*	*2*	*2*	*4*	*5*	*3*	*6, 9*	*3, 6*
37	*99*	*3, 5*	*0*	*3*	*2*	*4*	*2*	*1*	*3*	*4*	*3*	*6, 9*	*3, 6*
38	*3*	*3, 5*	*3*	*3*	*2*	*4*	*2*	*1*	*3*	*5*	*3*	*9*	*6*

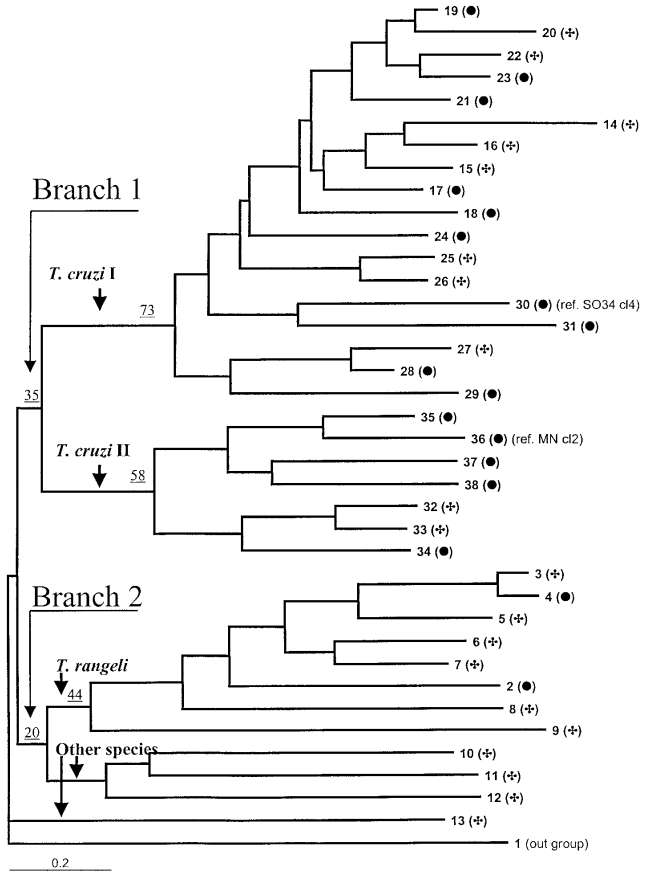

FIGURE 1. Dendrogram constructed using the Neighbor-Joining method with Jaccard's phenetic distance matrix based on a MLEE on 13 polymorphic loci: analysis of 38 different identified zymodemes from 43 trypanosome stocks including two *T. cruzi* reference laboratory clones SO34 cl4 and MN cl2. The numbers at the end of the branches referred to zymodemes (Table 1 and 2). Bootstrap values are underlined. Domestic (●) and sylvatic (✣) cycle origin of the stocks.

ously characterized as belonging to *T. cruzi,* and 14 stocks of *Trypanasoma* sp. Since, this branch corresponded to *T. cruzi* taxon and included 68% of the Colombian stocks. Two subdivisions which included SO34 cl4 and MN cl2 *T. cruzi* reference respectively corresponded to *T. cruzi* I and II. The majority of the *T. cruzi* Colombian stocks (92%), were clustered in *T. cruzi* I. Nevertheless, this group still presented high polymorphism, the Jaccard's distance average between the genotypes was 0.42 ± 0.13. According to the distance matrix (data no shown), Z31 and Z25 were the genotypes mostly related to SO34 cl4 reference (Jaccard's distance = 0.44). Only 2 Colombian stocks isolated from a sylvatic vector (*Panstrongylus geniculatis*) were attributed to *T. cruzi* II and were genetically distant from MN cl2 reference (Jaccard's distance > 0.60). *T. cruzi* II comprised also the Bolivian stock Santa Cruz (Z35) closely related to MN cl2 and the Tulahuen Chilean stock. The Panamean stock (Z34) clustered in *T. cruzi* II was more related to the 2 Colombian stocks included in this group. Branch 2 contained the *T. rangeli* stocks and 4 sylvatic *Trypanasoma* sp. (3 from bats and 1 from *R. prolixus*). These stocks were distinct genotypes and presented high genetic distances from any *T. cruzi* stock. Moreover, among them, several pairs of zymodemes exhibited very important genetic distances and the average distance between pairs of stocks was of 0.65 ± 0.24 (Table 3). One stock isolated from bat (Z13) was distant from any studied stock (the minimal genetic distance from other stocks was 0.69).

The Wagner parsimony analysis using the *Crithidia* sp. stock as out-group, confirmed the general topology of the Neighbor-Joining tree with slight modifications. Zymodeme 13 was clustered apart from all the other stocks and the two following upper subdivisions corresponded to branches 1 and 2. Nevertheless, the corresponding bootstrap values were very low as indicated in Figure 1. Branch 1 was divided into two clusters which correlate with the UPGMA analysis (*T. cruzi* I and II) and presented higher bootstrap values (Figure 1). In order to test the significance of the above results, subset of stocks were also analyzed. Data were analyzed without zymodeme 13 because of missing data at 3 loci that could have introduced artifacts in the construction of the tree. The bootstrap value of branch 1 (*T. cruzi*) was increased to 48 instead of 35 and similarly higher values were registered for *T. cruzi* I and II (0.80 and 0.65 respectively). Among the other stocks any bootstrap value allowed to depict significant cluster. The analysis restricted to the *T. cruzi* Colombian stocks with the two reference clones showed modified bootstrap values of the *T. cruzi* I and II (0.63 and 0.73 respectively). At the lower level of the tree only two pairs of stocks were significantly grouped with bootstrap values > 0.70: Z28 and Z27, Z32 and Z33.

DISCUSSION

Differentiation between American *Trypanasoma* stocks. The current sample of Colombian stocks analyzed by isoenzyme electrophoresis with 13 polymorphic loci showed an im-

TABLE 3
Matrix of Jaccard's distances between zymodemes clustered in the principal branch 2

Zymodemes	2	3	4	5	6	7	8	9	10	11	12
2	0.00										
3	0.62	0.00									
4	0.67	0.07	0.00								
5	0.47	0.31	0.27	0.00							
6	0.36	0.50	0.47	0.23	0.00						
7	0.53	0.55	0.53	0.27	0.23	0.00					
8	0.65	0.53	0.58	0.53	0.43	0.65	0.00				
9	0.88	0.64	0.62	0.70	0.79	0.74	0.83	0.00			
10	0.73	0.74	0.78	0.63	0.56	0.67	0.65	0.88	0.00		
11	0.78	0.84	0.88	0.82	0.79	0.78	0.77	0.96	0.60	0.00	
12	0.83	0.84	0.88	0.82	0.85	0.83	0.77	0.96	0.73	0.67	0.00

portant genetic diversity and high genetic distances between genotypes compatible with the presence of several distinct species. This set of stocks included the two American trypanosomes of man, *T. cruzi* and *T. rangeli* and the cluster analysis performed here failed in drawing a clear distinction between these two species. Moreover the relative taxonomic position of other stocks unrelated to any of these species was not possible to determine. In spite of congruent clustering obtained by numerical and cladistic methods, the statistical significance of the upper branches was low. This result can be explained by the fact that the upper level of resolution of the isoenzyme method was reached. In other words, the theoretical maximum distances are close to be reached between the species and also between members within a single species (high genetic distances between *T. cruzi* I and II up to 0.89 and among *T. rangeli* stocks, see Table 3). Consequently, hierarchization between such set of diverse *Trypanasoma* species is not reliable. In spite of this difficulty, the cluster analysis allowed to detect with best reliability the stocks belonging to *T. cruzi* I and II because these subgroups were statistically supported.

The referred *T. rangeli* stocks were not clearly clustered in a monophyletic group because the bootstrap value was low (0.44). Contrasting with a previous study, great genetic distances were observed among Colombian *T. rangeli* stocks and, geographical diversification of the sample might explain this result.[28,29] Similar clustering difficulties were recorded in an attempt to classify Peruvian trypanosomes isolated from monkey using reference stocks of *T. cruzi* and *T. rangeli*.[18] Furthermore, this study of a reduced number of stocks, showed that the malic enzyme provided distinct patterns between *T. cruzi* (two different loci) and *T. rangeli* species (one locus only). In the current sample, 7 new stocks previously referred to *T. rangeli* according to biological and/or morphological criteria confirmed that the Malic enzyme revealed a potential diagnostic marker. Among the *Trypanasoma* sp. stocks, Z8 that was apparently related to *T. rangeli* (genetic distance from *T. rangeli* Z6 = 0.43) presented 2 loci at the Malic enzyme system. Nevertheless, the actual phylogenetic analysis did not allowed to infer definitively Z8 to *T. rangeli* species.

Prevalence of species of the subgenus *Schizotrypanum* from Colombian bats, showed that bats can harbor several species: *T. cruzi, T. marinkellei* and *Schizotrypanum* sp.[30] Moreover, molecular and biological studies of such trypanosomes suggested that those differing from *T. cruzi* sensus stricto should be treated as distinct subspecies.[31,32] Here, three stocks isolated from bats and one from a sylvatic *R. prolixus* belonged to branch 2 and exhibited high genetic distances to *T. cruzi* and *T. rangeli* species. Therefore they should belong to other species. Also, since these stocks exhibit high genetic distances between them, the results could not supported a monophyletic origin.

MLEE is an excellent marker to depict relationships between *T. cruzi* clones. Nevertheless, this marker is too polymorphic to clearly determine phylogenetic hierarchy among trypanosomatidae species. Relationships between *T. cruzi, T. rangeli, Schizotrypanum* species, and unrelated species like *T. cruzi*-like organisms will have to be assessed by using conservative, slowly evolving genetic characters such as rRNA sequences, for which genetic distances and expected homoplasy are lower. PCR test of mini-exon amplification to differentiate *T. cruzi* and *T. rangeli* species was proposed and Colombian stocks have been successfully typed with this marker; the respective mini-exon PCR products of *T. cruzi* and *T. rangeli* had distinct electrophoretic mobility and the intergenic region harbored specific sequences.[16,19,33] The study of the intergenic region among other species of *Schizotrypanum* could help to determine the taxonomic status of these species.

Genetic variability of *Trypanosomatidae* in Colombia. Interestingly enough was the fact that the studied Colombian stocks were new ones, isolated from various mammals and vector hosts of sylvatic and domestic cycles in different geographical areas. Six stocks, belonging to *T. rangeli* species were isolated in different regions, confirming the high prevalence and large distribution of *T. rangeli* in Colombia. The new set of *T. rangeli* stocks was very heterogeneous contrasting with a previous study of Colombian stocks but high isoenzyme variability of *T. rangeli* taxon has been already recorded.[28,34] Nevertheless, the majority of the stocks were related to *T. cruzi* species. The current results confirmed that the large majority of stocks were clustered in *T. cruzi* I that included Zymodeme I used as reference by others.[12,13] In this group, high residual genetic variability, either in domestic and sylvatic cycles and absence of reliable subdivisions were observed. It was apparent that the stocks characterized by MLEE showed no tendency of host or cycle specificity. Indeed from a given host, such as *Didelphis masupialis,* diverse distant genotypes as Z16 and Z26 were isolated (genetic distance 0.44, data not showed). Conversely, genotypes closely related were isolated from human and sylvatic mammals (Z22 and Z23, 0.14; Z27 and Z28, 0.13). Furthermore, Z25 was isolated in different geographical areas. Consequently, host and geographical specificity of zymodemes were not observed among this limited sample of stocks. *R. prolixus* is the principal vector in Colombia and invade human dwellings but has also a sylvatic habitat in palms. The habitats of palm roof house favors the migration of infected insects from sylvatic to domestic areas and should explain the observed diversity of the stocks maintained by the participation of several vectors and mammals hosts. In a first report, 4 sylvatic stocks and 53 domestic ones did not present zymodeme differences (WIDMER et al. 1985).[12] In other areas, a clear clustering between sylvatic and domestic stocks was observed and should correspond to a different epidemiological situation where the vector domiciliation should be well established and associated with less overlapping between cycles.[13] Several stocks were isolated from *D. masupialis* and this result favors the important role of *D. masupialis* as reservoir in Colombia.

Two Colombian stocks belonged to *T. cruzi* II (Z32 and Z33) and were distant from the reference clone MN cl2, which is very abundant in domestic cycle of the Southern part of South America where these stocks are probably associated with high rate of symptomatic human infections. Several stocks that have a sylvatic tropism have been identified as *T. cruzi* II and clustered in two subdivisions equated to monophyletic groups.[6,9,11] One of these groups included the namely Zymodeme III and Colombian sylvatic stocks related to this zymodeme had been previously identified.[13] In absence of reference stocks, it is difficult to infer Z31 and Z32 to one of these groups but these zymodemes should be specific of sylvatic cycles.

T. cruzi II also comprised the Bolivian stock Santa Cruz (Z35) which was closely related to MN cl2. This result was in agreement with the high prevalence of this clone in Bolivian domestic cycle.[35,36,37] The Tulahuen Chilean stock and the corresponding laboratory clone were more related to MN cl2 than to SO34 cl4 as previously observed.[5] The Panamean stock (Z34) also was clustered in *T. cruzi* II and was more related to the 2 Colombian stocks included in this group.

Financial support: This work was supported by a grant from the World Health Organization Special Program for Research and Training in Tropical Diseases (No, 910273).

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