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ISOZYME VARIABILITY IN *TRYPANOSOMA CRUZI*, THE AGENT OF CHAGAS' DISEASE: GENETICAL, TAXONOMICAL, AND EPIDEMIOLOGICAL SIGNIFICANCE

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Abstract.—A genetic interpretation of the zymograms of 524 *Trypanosoma cruzi* stocks from various hosts and representing a broad geographical range (United States to Southern Brazil) reveals high genetic variability (only one monomorphic locus out of 15) and suggests that this parasite has a diploid structure. The data do not give any indication of Mendelian sexuality, although many opportunities are present for genetic exchange between extremely different genotypes.

The population structure of *T. cruzi* appears to be multiclonal and complex. The natural clones evidenced by isozyme analysis are numerous (43 different ones are recorded among 121 stocks assayed at 15 gene loci) and exhibit a large range of genotypes, in a nonhierarchical structure; it is not possible to cluster them into a few strictly delimited groups which could represent natural taxa.

The available data suggest that the genetic variability of *T. cruzi* reflects the long separate evolution of multiple clones. It is suggested that long clonal evolution may explain the present biological and medical variability of the causative agent of Chagas' disease.

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Trypanosoma (*Schizotrypanum*) *cruzi* is a flagellate protozoon of major medical importance. It is the causative agent of Chagas' disease, which affects several million people in Central and South America. A striking feature of this parasite is its large variation in medical properties such as immunology, pharmacology, and pathogenicity (Dvorak, 1984).

Toyé (1974) first attempted to survey the genetic variability of *T. cruzi* by means of isozymes. Miles et al. (1977, 1978, 1980) and Ready and Miles (1980) used a phenetic interpretation of the zymograms to distinguish three distinct isozyme groups in Northern Brazil, which they called "zymodemes." Other studies have similarly confirmed the heterogeneity of isozyme phenotypes in *T. cruzi* (Romanha et al., 1979; Kreutzer and Souza, 1981; Ebert, 1982; Goldberg and Silva Pereira, 1983; Zillmann and Ebert, 1983).

Since 1980, we have undertaken an extensive electrophoretic survey of isozyme variability in *T. cruzi*. We have interpreted the data in genetic and populational terms, aiming to clarify the life cycle and mating system of *T. cruzi* and to elucidate the evolutionary origin and taxonomic status of the zymodemes. The present paper summarizes our main results.

MATERIALS AND METHODS

Experimental Conditions.—Methods for isolating, growing, harvesting, and storing the stocks have been previously described (Tibayrenc and Le Ray, 1984). Cellulose-acetate electrophoresis was performed under conditions already described (Tibayrenc et al., 1985). Fourteen enzyme systems were assayed in total: aconitase (E.C.4.2.1.3., ACON), adenylate kinase (E.C.2.7.4.3., ADK), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PD), glucose-6-phosphate isomerase (E.C.5.3.1.9, GPI), glutamate dehydrogenase NAD^+ (E.C.1.4.1.2, GDH-NAD^+), glutamate dehydrogenase $NADP^+$ (E.C.1.4.1.4, GDH-$NADP^+$), isocitrate dehydrogenase (E.C.1.1.1.42, IDH), leucine aminopeptidase (cytosol aminopeptidase) (E.C.3.4.11.1, LAP), malate dehydrogenase (E.C.1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, $NADP^+$) or malic enzyme (E.C.1.1.1.40, ME), peptidase 1 (Ficin) (E.C.3.4.22.3 [formerly E.C.3.4.4.12], PEP-1); substrate: leucyl-leucyl-leucine), peptidase 2 (Bromelain) (E.C.3.4.22.4 [formerly E.C.3.4.4.24], PEP-2); substrate: leucyl-L-alanine), phosphoglucomutase (E.C.5.4.2.2 [formerly E.C.2.7.5.1], PGM), and 6-phosphogluco-

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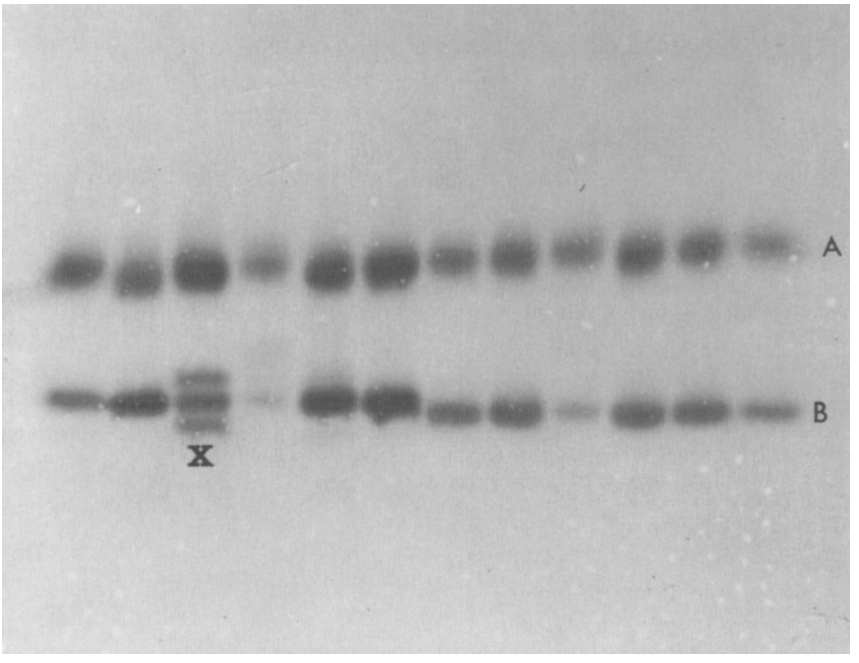

FIG. 1. Electrophoresis of malic enzyme in *Trypanosoma cruzi*. The two sets of bands, A and B, represent the activity of two separate loci (*Me-1* and *Me-2*, respectively). A typical, three-banded heterozygous pattern (labelled X) for a dimeric enzyme can be seen at *Me-2*.

nate dehydrogenase (E.C.1.1.1.44, 6PGD). MDH and ME each exhibited two zones of activity (Fig. 1), which are believed to be determined by two separate gene loci (*Mdh-1* and *Me-1* for the faster migrating enzymes, and *Mdh-2* and *Me-2* for the slower ones), giving a total of 16 genetic loci. However, we have discarded the data for *Mdh-1*, because the patterns were not always well resolved in the gels. Hence, our data incorporate no more than 15 different loci.

Origins of the Stocks.—A total of 524 *T. cruzi* stocks were surveyed throughout this work. We isolated more than 400 of them from 1980 to 1984 in Bolivia from domestic specimens of *Triatoma infestans*, the main vector in that country; most of the others were obtained from various colleagues. Table 1 summarizes the origin of the 524 stocks. Most of these stocks were studied with a restricted range of enzymes (including always GPI, IDH, ME, and PGM), used as markers for testing genetic hypotheses or epidemiological features. Table 2 lists the subset of 121 stocks studied with the total set of 14 enzymes for the purpose of ascertaining the extent of genetic variability and the taxonomic relationship among the variants. These 121 stocks were obtained from various hosts and types of transmission cycle in a broad ecogeographical range. They were isolated in the field between 1938 and 1984 and amount to a well diversified sample. Nevertheless, this is not a randomized sample; it cannot give an exact picture of the actual frequences of the different isozyme variants in each country, transmission cycle or host type. The most reliable sample for statistical analyses is the one obtained from Bolivian domestic transmission cycles.

Nomenclature.—We used the term "isozyme strain" (IS) to refer to any set of *T. cruzi* stocks sharing the same isozyme pattern, without regard to the taxonomical or medical significance of this pattern (Tibayrenc et al., 1983). The term "zymodeme" has been used with two different meanings: first, to designate any isozyme variant (Gibson et al., 1980); and second, to describe "main" variants (Ready and Miles, 1980). The first definition is synonymous with our term "isozyme strain." Herein, we use "zymodeme" in this context, but, when warranted, we use the term "iso-

TABLE 1. Geographic origin of the 524 stocks of *Trypanosoma cruzi* studied at four gene loci and their composite isozyme genotype or zymodeme (see Table 3).

Locality	Zymodeme	Number of stocks
Colombia		
Puerto Ele	2	5
Ecuador		
Guayaquil	10	1
Peru		
Ucayali	10	1
Bolivia		
Yungas	2	10
	39	11
Chiwisivi	2	141
	39	2
Cochabamba	2	15
	39	10
	40	1
Comarapa	2	9
	39	8
	32	4
Santa Cruz	2	7
	16	1
	28	2
	39	24
	40	3
Sucre	2	23
	10	1
	32	8
	39	16
Camiri	2	4
	37	2
	38	1
	39	6
	40	7
Tupiza	2	45
	32	9
	39	31
	40	14
Tarija	2	8
	38	1
	39	20
	40	4
Alto Beni	2	1
	25	1
Potosi	39	1
Vallegrande	2	8
Venezuela		
Miranda	2	1
Portuguesa	2	1
Cojedes	2	4
Aragua	2	1
Barinas	2	1
Carabobo	2	1
French Guiana		
Montjoly	1	1
Montsinery	2	5
Cacao	2	2
	3	1
	8	1
Paramana	2	1
Chile		
Cachicuyu	2	2
	39	3
Cucumen	32	1
Monte Patria	32	1
	39	1
Arrayan	32	1
Chanaral	32	1
Locality *X**	40	1
Brazil		
Goias Goiana	2	1
	31	2
Locality *X**	3	1
Belém	2	2
	27	1
	35	1
	36	1
Espirito Santo	2	1
São Paulo	2	4
	31	3
Minas Gerais	2	1
Locality *X**	10	1
Valle Grande	39	1
Rio Grande	40	2
Bahia	30	1
Honduras		
Tegucigalpa	10	1
Mexico		
Locality *X**	2	1
USA		
Locality *X**	14	2

TABLE 1. Extended.

* Exact location is not known.

zyme genotype." We use the expressions zymodeme I, II, and III to refer to the three main isozyme patterns described by Ready and Miles (1980).

RESULTS

The Ploidy of Trypanosoma cruzi

We present the data for *Gpi* as an illustrative example of our results concerning the ploidy of *Trypanosoma cruzi*. This locus exhibits either one-banded patterns or three-banded ones (Fig. 2). In the three-banded patterns, the bands are equidistant, and they stain in approximately a 1:2:1 ratio, as expected of a diploid heterozygote for a di-

TABLE 2. Geographic origin, zymodeme pattern, date of collection, and host of the 121 *Trypanosoma cruzi* stocks studied at 15 gene loci (see Table 3 for the genotypes and numbering of the 43 zymodemes inferred from these 15 loci).

Stock	Zymodeme	Host	Locality	Date
1) A107	1	*Rhodnius prolixus*	Montjoly, French Guiana	?
2) XE941	2	human	Goias Goiana, Brazil	?
3) A68	2	*Didelphis marsupialis*	Montsinery, French Guiana	4.82
4) A97	3	*Didelphis marsupialis*	Cacao, French Guiana	3.83
5) XE450	3	human	Brazil	?
6) A82	4	*Didelphis marsupialis*	Montsinery, French Guiana	7.82
7) A88	4	*Didelphis marsupialis*	Montsinery, French Guiana	9.82
8) A99	5	*Didelphis marsupialis*	Cacao, French Guiana	3.83
9) PB6	6	*Rhodnius pictipes*	Alto Beni, Bolivia	10.82
10) Chile 5	7	*Triatoma infestans*	Cachicuyu, Chile	?
11) A105	8	*Didelphis marsupialis*	Cacao, French Guiana	4.83
12) C8Cl1*	9	*Triatoma infestans*	Chiwisivi, Bolivia	3.81
13) C37	9	*Triatoma infestans*	Chiwisivi, Bolivia	3.81
14) C83	9	*Triatoma infestans*	Chiwisivi, Bolivia	6.81
15) 19-79	10	human (chronic case)	Sucre, Bolivia	79
16) A98	11	*Didelphis marsupialis*	Cacao, French Guiana	3.83
17) Tehuantepec*	12	Triatominae	Mexico	38
18) Davis	13	*Triatoma dimidiata*	Tegucigalpa, Honduras	5.83
19) FH4	14	*Didelphis* sp.	U.S.A.	?
20) FH5	14	*Didelphis* sp.	U.S.A.	?
21) 20R16	15	*Didelphis* sp.	Yapacani, Bolivia	83
22) 31R26	16	human (acute case)	Santa Cruz, Bolivia	83
23) X10Cl1 Z1*	17	human	Belém, Brazil	?
24) A6	18	*Philander opossum*	Paramana, French Guiana	11.81
25) A55	18	*Philander opossum*	Montsinery, French Guiana	3.82
26) A66	18	*Philander opossum*	Montsinery, French Guiana	3.82
27) Chile 4	19 + 39	*Triatoma infestans*	Cachicuyu, Chile	?
28) Cutia	19	*Dasylprocta aguti*	Espirito Santo, Brazil	?
29) Gamba	19	*Didelphis azarae*	São Paulo, Brazil	?
30) Mico	19	*Callithrix geoffroyi*	Minas Gerais, Brazil	?
31) OPS1	19	*Rhodnius prolixus*	Miranda, Venezuela	11.76
32) OPS12	19	*Canis familiaris*	Portuguesa, Venezuela	2.77
33) OPS13	19	*Rattus rattus*	Cojedes, Venezuela	3.77
34) OPS21	19	human	Cojedes, Venezuela	6.77
35) OPS31	19	human	Cojedes, Venezuela	7.77
36) OPS49	19	human	Aragua, Venezuela	2.78
37) OPS53	19	*Rhodnius robustus*	Barinas, Venezuela	4.78
38) MC50	19	*Rhodnius prolixus*	Puerto Ele, Colombia	?
39) MC52	19	*Rhodnius prolixus*	Puerto Ele, Colombia	?
40) MC53	19	*Rhodnius prolixus*	Puerto Ele, Colombia	?
41) MC60	19	*Rhodnius prolixus*	Puerto Ele, Colombia	?
42) MC61	19	*Rhodnius prolixus*	Puerto Ele, Colombia	?
43) 26R26	19	*Triatoma infestans*	Santa Cruz, Bolivia	?
44) 16R34	19	*Triatoma infestans*	Santa Cruz, Bolivia	80
45) 33R2	19	human (chronic case)	Vallegrande, Bolivia	83
46) 34R2	19	human (chronic case)	Vallegrande, Bolivia	83
47) 35R2	19	human (chronic case)	Vallegrande, Bolivia	83
48) 37R2	19	human (chronic case)	Vallegrande, Bolivia	83
49) 39R2	19	human (chronic case)	Vallegrande, Bolivia	83
50) 133-79	19	human (acute case)	Santa Cruz, Bolivia	79
51) CO14	20	*Triatoma infestans*	Cochabamba, Bolivia	11.82
52) 14R30	20	*Triatoma infestans*	Cochabamba, Bolivia	82
53) 19R26	20	*Triatoma infestans*	Cochabamba, Bolivia	82
54) Y46	20	*Triatoma infestans*	Yungas, Bolivia	5.83
55) Y52	20	*Triatoma infestans*	Yungas, Bolivia	7.83
56) CO26	20 + 39	wild *Triatoma infestans*	Cochabamba, Bolivia	9.83
57) CO30	20 + 39	wild *Triatoma infestans*	Cochabamba, Bolivia	9.83
58) CO33	20	wild *Triatoma infestans*	Cochabamba, Bolivia	9.83
59) CO36	20	wild *Triatoma infestans*	Cochabamba, Bolivia	4.84
60) CO37	20	wild *Triatoma infestans*	Cochabamba, Bolivia	4.84

TABLE 2. Extended.

Stock	Zymodeme	Host	Locality	Date
61) 8R21	20	human (chronic case)	Vallegrande, Bolivia	82
62) 38R2	20	human (chronic case)	Vallegrande, Bolivia	83
63) 41R2	20	human (chronic case)	Vallegrande, Bolivia	83
64) Cuica	20	*Opossum cuica philander*	São Paulo, Brazil	?
65) Esquilo	20	*Sciurus aestuans ingrami*	São Paulo, Brazil	?
66) Rato 1584	20	*Akodon lasiotis*	São Paulo, Brazil	?
67) OPS4	21	*Didelphis marsupialis*	Carabobo, Venezuela	12.76
68) OPS22	21	*Panstrongylus geniculatus*	Cojedes, Venezuela	5.77
69) OPS89	21	*Triatoma maculata*	Guatico, Venezuela	?
70) MIL3	22	*Philander* sp.	Ucayali, Peru	?
71) ITMAP 943	23	*Aotus* sp.	Brazil	?
72) Ectd	24	*Triatoma dimidiata*	Guayaquil, Ecuador	3.77
73) PB3	25	*Rhodnius pictipes*	Alto Beni, Bolivia	?
74) Espirito Santo	26	*Triatoma vitticeps*	Espirito Santo, Brazil	?
75) CanIII cl1 Z3*	27	human	Belém, Brazil	?
76) 27R27	28	*Aotus* sp.	Santa Cruz, Bolivia	80
77) 10R26	29	*Aotus* sp.	Santa Cruz, Bolivia	80
78) Esmeraldo cl3 Z2*	30	human	Bahia, Brazil	?
79) XE625	31	human	Goias, Goiana, Brazil	?
80) CX395	31	human	Goias, Goiana, Brazil	?
81) TU15	32	*Triatoma infestans*	Tupiza, Bolivia	12.81
82) TU107	32	*Triatoma infestans*	Tupiza, Bolivia	12.81
83) MXCH88	32	human	Cucumen, Chile	?
84) XHCH56	32 + 39	human	Monte Patria, Chile	1.83
85) MCH3	33	*Triatoma infestans*	Arrayan, Chile	?
86) XHCH80	33	human	Chanaral, Chile	1.83
87) Y*	34	human (acute case)	São Paulo, Brazil	?
88) Morcego 1354	34	*Tadarida laticaudata*	São Paulo, Brazil	?
89) Morcego MPB	34	*Eumops auripendulus*	São Paulo, Brazil	?
90) M6241	35	human (acute case)	Belém, Brazil	?
91) M5631	36	*Didelphis novemcinctus*	Belém, Brazil	?
92) CA34	37	*Triatoma infestans*	Camiri, Bolivia	11.82
93) CA8	38	*Triatoma infestans*	Camiri, Bolivia	11.82
94) SC43Cl2*	39	*Triatoma infestans*	Santa Cruz, Bolivia	5.81
95) SC7	39	*Triatoma infestans*	Santa Cruz, Bolivia	5.81
96) SU13	39	*Triatoma infestans*	Sucre, Bolivia	11.82
97) SU34	39	*Triatoma infestans*	Sucre, Bolivia	11.82
98) Y5	39	*Triatoma infestans*	Yungas, Bolivia	7.81
99) Y48	39	*Triatoma infestans*	Yungas, Bolivia	6.83
100) Y50	39	*Triatoma infestans*	Yungas, Bolivia	6.83
101) PO26	39	*Triatoma infestans*	Potosi, Bolivia	?
102) C50	39	*Triatoma infestans*	Chiwisivi, Bolivia	3.81
103) 23R25	39	*Triatoma infestans*	Cochabamba, Bolivia	82
104) COM14	39	*Triatoma infestans*	Comarapa, Bolivia	11.82
105) Chile 1	39	*Triatoma infestans*	Cachicuyu, Chile	1.77
106) Chile 2	39	*Triatoma infestans*	Cachicuyu, Chile	1.77
107) CO19	39	wild *Triatoma infestans*	Cochabamba, Bolivia	7.83
108) CO27	39	wild *Triatoma infestans*	Cochabamba, Bolivia	9.83
109) CO28	39	wild *Triatoma infestans*	Cochabamba, Bolivia	9.83
110) Bug 2149	39	Triatominae	Valle Grande do Sul, Brazil	?
111) 11R23	39	human (acute case)	Santa Cruz, Bolivia	82
112) 15R35	39	human (acute case)	Santa Cruz, Bolivia	82
113) 9R21	39	human (chronic case)	Santa Cruz, Bolivia	82
114) 9280Cl1*	39	human (chronic case)	Santa Cruz, Bolivia	80
115) 18R41	40	human (acute case)	Santa Cruz, Bolivia	82
116) 7R72	40	human (acute case)	Santa Cruz, Bolivia	82
117) FI	40	*Triatoma infestans*	Valle Grande do Sul, Brazil	?
118) 3R21	41	human (acute case)	Santa Cruz, Bolivia	81
119) CA15	42	*Triatoma infestans*	Camiri, Bolivia	11.82
120) MR	42	*Triatoma infestans*	Valle Grande do Sul, Brazil	?
121) Tulahuen FKIIA*	43	human	Chile	?

* Laboratory cloned stock.

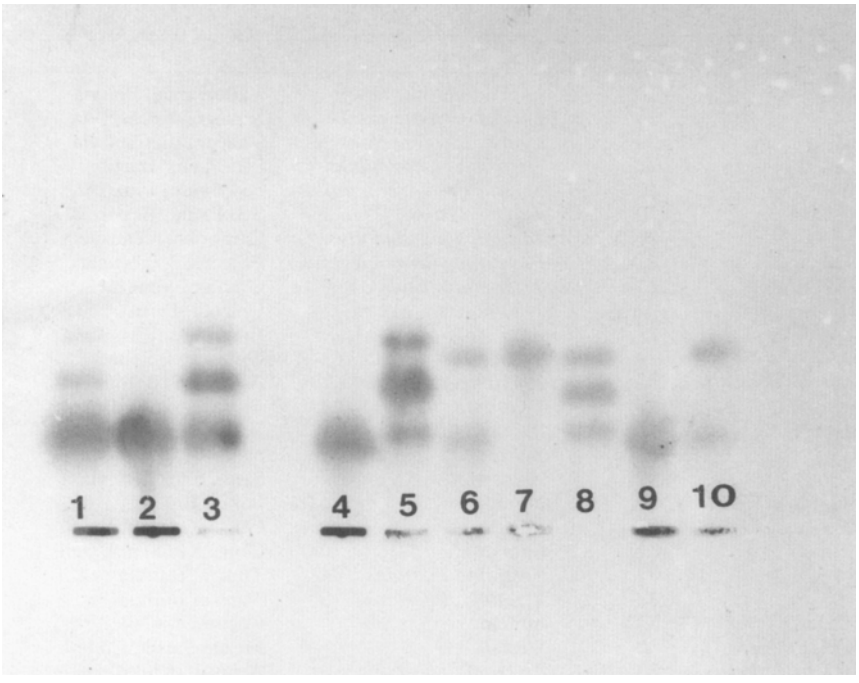

FIG. 2. Electrophoresis of glucose-6-phosphate isomerase in *Trypanosoma cruzi*. 1, 3) mixtures of genotypes *2/4* and *5/5*; 2, 4, 9) genotype *5/5*; 5) genotype *2/4*; 6, 10) mixtures of genotypes *3/3* and *5/5*; 7) genotype *3/3*; 8) genotype *3/4*.

meric enzyme. Similar three-banded patterns are observed for *Mdh-2, Idh, Me-1* (Fig. 1A), *Me-2* (Fig. 1B), and *6Pgd*. On the other hand, the zymograms of *Pgm* and *Gdh-NADP*$^+$ are consistent, respectively, with monomeric and hexameric structures (two- and seven-banded heterozygous patterns). It is worth noting that the quaternary structure of enzymes inferred from the isozyme patterns agrees with the conclusions reached by other authors on the basis of other biochemical data (Walter and Ebert, 1979; Jeremiah et al., 1982; Chapman et al., 1984). Measurement of the amount of DNA in *T. cruzi* zymodemes suggests that all zymodemes have the same ploidy (Lemesre and Tibayrenc, 1983). These results led us to propose a diploid constitution for *T. cruzi* (Tibayrenc et al., 1981*a*, 1981*b*, 1985, 1986*b*; Tibayrenc and Miles, 1983). Moreover, a diversified ensemble of results, both from isozyme and DNA data, suggests that diploidy is a general feature of kinetoplastid flagellates (Gibson et al., 1980; Tait, 1980; Castro et al., 1981; Lanar et al., 1981; Maazoun et al., 1981; Borst et al., 1982; Gibson et al., 1985; Gibson and Miles, 1986).

If we assume that *T. cruzi* is diploid, we can attribute the isozyme patterns to alleles. Table 3 gives the inferred genotypes of the 43 different zymodemes identified using all 15 loci, in the 121 *T. cruzi* stocks from a broad geographical range (Tibayrenc et al., 1986*b*). The use of 15 loci makes it possible to distinguish zymodemes that appear to be identical when only four loci are used. Hence, several of the 20 zymodemes listed in Table 1 are each split into several zymodemes in Table 3. Specifically, zymodeme 2 in Table 1 splits into zymodemes 2, 4–7, 9, 11, 12, 15, and 17–20; zymodeme 10 (Table 1) splits into zymodemes 10, 13, and 22–24; zymodeme 28 (Table 1) splits into zymodemes 28 and 29 (Table 3); zymodeme 31 (Table 1) splits into zymodemes 31 and 34 (Table 3); zymodeme 32 (Table 1) splits into zymodemes 32 and 33 (Table 3); and zymodeme 40 (Table 1) splits into zymodemes 40 and 43 (Table 3).

The Question of Sexual Recombination

In the African species *T. brucei*, Tait (1980) proposed Mendelian sexuality on the basis of isozyme studies of natural popu-

TABLE 3. Genotype of the 43 zymodemes identified by assaying 15 isozyme loci in 121 stocks of *Trypanosoma cruzi*. For each locus, allele *1* codes for the fastest electromorph. *Adk* is not listed, because it is monomorphic (genotype *1/1*); *Acon* is not listed either, because the only variant is zymodeme 27, which has the genotype *2/2*.

	Locus and genotype												
Zymodeme	*G6pd*	*Gpi*	*Gdh-1*	*Gdh-2*	*Idh*	*Lap*	*Mdh*	*Me-1*	*Me-2*	*Pep-1*	*Pep-2*	*Pgm*	*6Pgd*
1	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	3/3	1/1	2/7	4/4
2	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	3/3	1/1	3/3	4/4
3	6/6	5/6	3/3	2/2	1/1	1/1	2/2	2/2	4/4	3/3	1/1	3/3	4/4
4	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	4/4	1/1	3/3	4/4
5	6/6	5/5	3/3	2/6	1/1	1/1	2/2	2/2	4/4	4/4	1/1	3/3	4/4
6	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	2/2	1/1	3/3	2/4
7	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	3/3	4/4
8	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	8/8	4/4
9	6/6	5/5	3/3	2/2	1/1	1/1	2/2	3/3	4/4	1/1	1/1	3/3	4/4
10	6/6	5/5	3/3	3/3	1/1	1/1	2/2	3/3	4/7	1/1	1/1	3/3	2/2
11	6/6	5/5	3/3	2/2	1/1	1/1	2/2	2/4	4/4	1/1	1/1	3/3	4/4
12	4/4	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	3/3	4/4
13	4/4	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/7	1/1	1/1	3/3	4/4
14	4/4	5/5	3/3	2/2	1/1	1/1	2/2	2/2	7/7	1/1	1/1	3/3	4/4
15	4/4	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	3/3	3/4
16	4/4	6/6	3/3	2/2	1/1	1/1	2/2	3/3	2/4	1/1	1/1	1/3	4/4
17	7/7	5/5	3/3	2/2	1/1	2/2	2/2	3/3	4/4	4/4	1/1	3/3	4/4
18	7/7	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	4/4	1/1	3/3	2/4
19	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	3/3	4/4
20	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	1/1	1/1	3/3	2/4
21	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/4	5/5	1/1	3/3	4/4
22	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	4/7	4/4	1/1	3/3	4/4
23	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	7/7	4/4	1/1	3/3	4/4
24	5/5	5/5	3/3	2/2	1/1	1/1	2/2	2/2	7/7	2/2	1/1	3/3	4/4
25	5/5	5/5	3/3	2/2	1/1	1/1	2/3	2/2	1/7	2/2	1/1	3/3	1/4
26	9/9	4/4	2/2	5/5	2/2	6/6	2/2	2/2	3/3	6/6	2/2	8/8	5/5
27	8/8	4/4	2/2	5/5	2/2	5/5	2/2	2/2	3/3	6/6	2/2	9/9	5/5
28	9/9	4/4	2/2	5/5	2/2	4/4	2/2	2/2	3/3	7/7	2/2	4/4	6/6
29	9/9	4/4	2/2	5/5	2/2	4/4	2/2	2/2	3/3	8/8	2/2	4/4	6/6
30	1/1	1/3	2/2	5/5	2/2	3/3	2/2	2/2	6/6	3/3	1/1	11/11	1/1
31	2/2	3/3	2/2	5/5	2/2	3/3	2/2	2/2	6/6	5/5	1/1	11/11	1/1
32	2/2	3/3	2/2	5/5	2/2	3/3	2/2	2/2	6/6	5/5	1/1	10/12	1/1
33	2/2	3/3	2/2	5/5	2/2	3/3	2/2	2/2	6/6	4/4	1/1	10/12	1/1
34	2/2	3/3	2/2	3/3	2/2	3/3	1/2	2/2	6/6	5/5	1/1	11/11	1/1
35	5/5	4/4	1/1	2/2	2/2	4/4	2/2	1/1	5/5	6/6	1/1	5/5	4/4
36	5/5	4/4	1/1	2/2	2/2	4/4	2/2	1/1	5/5	6/6	1/1	9/9	4/4
37	4/4	4/4	1/1	1/1	2/2	3/3	2/2	1/1	5/5	5/5	1/1	6/10	1/4
38	4/4	2/4	1/1	1/1	2/2	3/3	2/2	1/1	5/5	5/5	1/1	10/10	1/4
39	4/4	2/4	1/1	1/1	2/2	3/3	2/2	1/1	5/5	5/5	1/1	6/10	1/4
40	4/4	3/4	1/1	1/1	2/2	3/3	2/2	1/1	5/5	4/4	1/1	4/11	1/4
41	4/4	3/4	1/1	1/1	2/2	3/3	2/2	1/1	5/5	4/4	1/1	4/11	1/4
42	3/3	3/4	1/1	4/4	2/2	3/3	2/2	1/1	5/5	4/4	1/1	4/11	1/4
43	3/3	3/4	2/2	4/4	2/2	3/3	2/2	1/1	5/5	4/4	1/1	4/11	1/4

lations. Recombination can occur in *T. brucei* under defined experimental conditions (Jenni et al., 1986; Paindavoine et al., 1986; Zampetti-Bosseler et al., 1986). On the other hand, our extensive survey strongly suggests that recombination is severely restricted or absent in natural populations of *T. cruzi*.

Some zymodemes exhibit a striking "fixed heterozygosity" at several loci over broad geographical ranges and long periods of time, which is incompatible with the occurrence of meiotic segregation (Tibayrenc et al., 1981*b*, 1984*a*, 1986*a*, 1986*b*). For example, zymodeme 39 is heterozygous at the *Gpi*, *Pgm*, and *6Pgd* loci (Table 3); it remains unchanged over a geographical range that includes Chile, various localities of Bolivia, and Southern Brazil (Table 2); and it was isolated numerous times from 1977 to 1984 (Table 2).

If we consider the observed genotypes as

potential parental genotypes, a high number of the possible recombinants (offspring genotypes different from the parental ones) are lacking. An illustrative example is given by the *Gpi* locus. Most of the possible recombinants were never observed in a Bolivian sample of 457 stocks isolated in domestic cycles (Table 1), although *2/5* and *3/5,* for example, would be expected to occur with a nonnegligible frequency, given the high incidence of the potential parental genotypes. Moreover, these potential parental genotypes occur very frequently in close sympatry, which provides maximum opportunity for mating (Tibayrenc, 1985). The relevant zymodemes have even been found in the same house (Tibayrenc and Desjeux, 1983; Tibayrenc et al., 1984*a,* 1986*a*), in the same human host (Brénière et al., 1985), and in the same insect (vector) host. In Bolivia, about 10% of infected *Triatoma infestans* (the main domestic vector in that country) yield mixed stocks of two different zymodemes (Tibayrenc et al., 1985, 1986*a*). The most easily identifiable case includes the mixture of the *Gpi* genotypes *3/3* (zymodeme 32) and *5/5* (zymodemes 9, 19, and 20). The pattern obtained in cases of mixed stocks is double-banded, whereas the expected heterozygote *3/5* should show three bands, since this enzyme is a dimer (Fig. 2). Yet this heterozygote has never been observed, neither in Bolivia nor elsewhere (Tables 1 and 3). In all the cases interpreted as mixed zymodemes, the *Gpi* data are confirmed by examination of the other loci. The hypothesis of two different zymodemes mixed within the same host without recombination between them might explain some of the complex patterns observed in some *T. cruzi* stocks by other authors (Zillmann and Ebert, 1983).

The absence of a majority of the possible recombinants is confirmed by chi-square tests showing highly significant deviations from Hardy-Weinberg expectations in Bolivian domestic populations of *T. cruzi.* This result is obtained either by considering the whole set of zymodemes or only a subset of relatively closely related ones among which sexual recombination could be considered as more probable than among radically different zymodemes (Tibayrenc et al., 1984*a*). It is worth noting that, at the same geographical scale and with similar isozyme methods, the populations of the vector *Triatoma infestans* do not depart from Hardy-Weinberg expectations (Dujardin et al., 1987) and so appear to be panmictic.

Absence of recombination is also supported by considering the entire set of loci. Genotypes at different loci are very often systematically associated in strong linkage disequilibrium (Tibayrenc et al., 1986*b*). Forty-three different genotypes (zymodemes) are distinguishable in the 121 stocks from various countries (Table 2). If the alleles at each of the 15 loci combined randomly, the total number of possible genotypes would be enormous (7×10^{15}), so that even the most probable genotypes would occur with a very low frequency. It would be quite unlikely to sample repeatedly identical (or even very similar) zymodemes in a set of 121 stocks. Nevertheless, we found only 43 zymodemes, 16 of which differ by only one allele. Indeed, a given zymodeme is often sampled in geographically distant sites and from different types of transmission cycles over long periods of time (see particularly zymodemes 19, 20, and 39 [Table 2]; see also the striking example of the laboratory stock Tehuantepec (zymodeme 12), which was isolated in 1938 and is almost identical to zymodemes 11 and 13 [which were isolated in 1983]).

We have analyzed this linkage disequilibrium (Zhang et al., unpubl.) using the methods of Ohta (1982). The levels of significance are extremely high, close to the maximum possible. This may be emphasized by pointing out that in wild barley, *Hordeum spontaneum,* a predominantly self-fertilizing species (selfing rate above 99%), Q. Zhang, M. A. Saghai-Maroff, and R. W. Allard (pers. comm.) have found that, in spite of large linkage disequilibria, the observed number of different genotypes is much greater for any given number of loci than the number observed in the present study. This result strongly supports the hypothesis that recombination is exceptional or absent in the populations of *T. cruzi* we have examined.

Our results do not rule out the possibility of mating in *T. cruzi,* but they show that mating is at least severely restricted and that, for the ecotopes examined (representing a

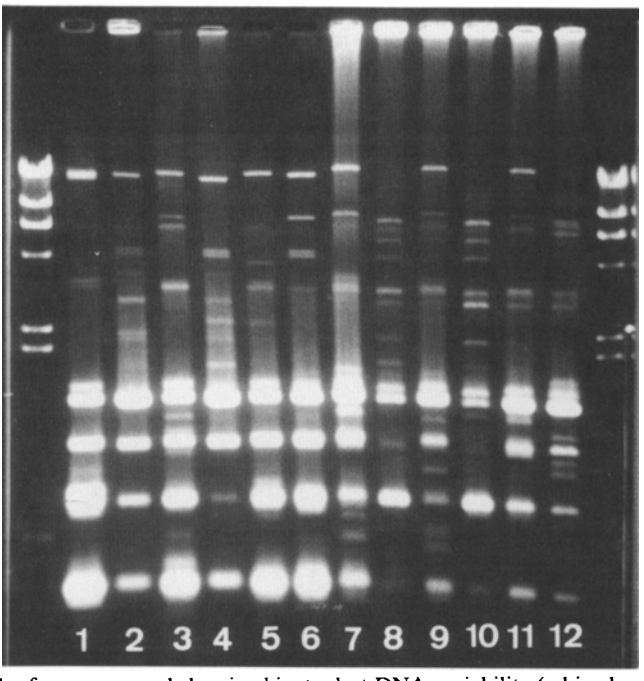

FIG. 3. Photograph of an agarose gel showing kinetoplast DNA variability (schizodeme analysis) in a set of *T. cruzi* isozyme genotypes (zymodemes). Samples at either end are markers (phage λ digested with the restriction enzyme *Hind* III). Samples 1–6 were digested with the restriction enzyme *Hae* III, and samples 7–12 were digested with the enzyme *Eco*R I. Samples 1 and 7 = zymodeme 12; samples 2 and 8 = zymodeme 37; samples 3 and 9 = zymodeme 17; samples 4 and 10 = zymodeme 39; samples 5 and 11 = zymodeme 9; samples 6 and 12 = zymodeme 29 (see Fig. 5). One can see that the closely related zymodemes 37 and 39 share a high number of common restriction fragments (after Tibayrenc and Ayala [1987]).

large ecogeographical range, and various hosts), *T. cruzi* populations exhibit a predominantly clonal structure.

It is worth comparing our results with the "clone concept" developed in bacteriology. Bacterial cultures isolated from different sources, in different locations, and often at different times are often so similar to one another that they must have derived essentially from a common ancestor by virtually complete asexual reproduction (Ørskov and Ørskov, 1983). A clonal structure with little if any chromosomal recombination has been postulated in natural populations of *Escherichia coli* by Ochman and Selander (1984) on the basis of isozyme analyses analogous to ours, even though some recombination (parasexuality) readily occurs in this bacterium under certain laboratory conditions. In the same way, even if genetic recombination could be experimentally induced in *T. cruzi*, its absence or scarcity in nature is apparent.

The clone concept suggests that any two sets of genetic characteristics should result in similar inferred genetic relationships among a group of natural isolates. This has been verified in the case of *E. coli* between biotyping and isozyme analysis (Miller and Hartl, 1986). Similarly, we have recently compared isozyme data for *T. cruzi* with the fragment patterns obtained by restriction endonuclease digestion of kinetoplast DNA ("schizodemes"; see Morel et al., 1980). The isozyme and kinetoplast DNA variabilities are highly correlated (Figs. 3, 4; see Tibayrenc and Ayala, 1987), which favors the hypothesis of clonal structure in *T. cruzi*. We, therefore, hypothesize that the zymodemes and schizodemes are natural clones of the parasite, as they can be identified by isozyme techniques and the restriction-endonuclease patterns of kinetoplast DNA, respectively.

The apparent lack of mating in *T. cruzi*, contrasting with the Mendelian sexuality inferred for *T. brucei* (Tait, 1980), may be explained as a result of the ancient adap-

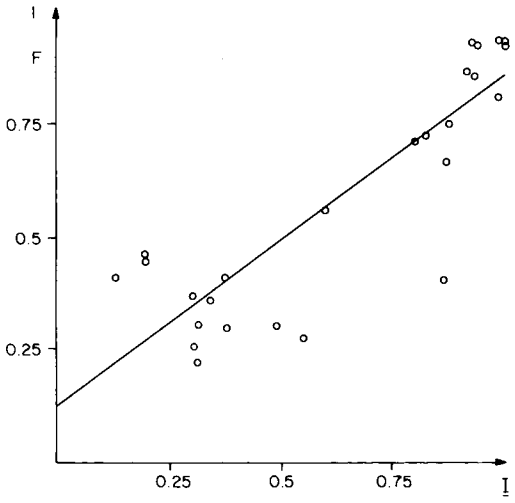

FIG. 4. Correlation between genetic identify I (Nei, 1972), calculated from 15 isozyme loci, and coefficient F (proportion of common kDNA fragments; Boursot and Bonhomme, 1986), based on six restriction enzymes. The correlation involves 26 pairwise comparisons between 21 *T. cruzi* stocks representing 19 different isozyme genotypes. The equation for the regression line is $F = 0.124 + 0.718\ I$ (after Tibayrenc and Ayala [1987]).

tation of *T. cruzi* insect vectors to human habitats, which made possible a dramatic increase of the populations of these insects (Dujardin and Tibayrenc, 1985), and hence of *T. cruzi* populations (Tibayrenc et al., 1983), in association with the spread of human populations in the American continent. Mating in these circumstances could have become "superfluous." We suggest that, if a residual sexuality is to be discovered in *T. cruzi*, this will be in selvatic cycles (rather than in domestic cycles) and among closely related zymodemes (rather than among radically different ones).

Amount of Variability

Given the hypotheses of diploidy and clonal structure, some population-genetic parameters can be estimated. The level of polymorphism is 93.3% (14 loci variable out of 15); the number of alleles at the polymorphic loci ranges from two to 12, with an average of 5.14 per locus. The level of observed heterozygosity among the zymodemes is 0.059, while the expected heterozygosity is 0.47. Expected heterozygosity is calculated as $H = 1 - \Sigma x_i^2$, where x_i is the frequency of the i^{th} allele (Ayala, 1982). This measure is mathematically similar to the genetic diversity calculated by Selander and Levin (1980) in their isozyme survey of *E. coli* natural populations. These authors observed high H values, which they explained as consequence of a basically clonal structure in natural populations of *E. coli*. The striking difference between observed and expected heterozygosity in *T. cruzi* may be explained in the same way. Nei's (1972) standard genetic distance among the 43 zymodemes is often very high, ranging from 0.017 to 2.015, with an average of 0.757 and a standard deviation of 0.478 (Tibayrenc et al., 1986*b*). The stocks surveyed represent a very genetically heterogeneous group of organisms.

Taxonomic Clustering

The phylogenetic relationships among the zymodemes show a nonhierarchical structure, which makes the UPGMA clustering method (Sokal and Sneath, 1963) previously used by Tibayrenc and Miles (1983) unsuitable. Minimum-length Wagner networks (Farris, 1970; Felsenstein, 1978) appear best suited for the purpose. Figure 5 shows an unrooted Wagner network depicting the phylogenetic relationships among the 43 isozyme genotypes (zymodemes) representing 121 *T. cruzi* stocks (Tibayrenc et al., 1986*b*). The patristic (evolutionary) distances obtained with this method (Fig. 5) are highly correlated to Nei's (1972) genetic distances (Table 4).

Figure 5 shows that the 43 isozyme genotypes (zymodemes) cannot be grouped into a few natural clusters. The only group that might be considered "natural," although still showing much heterogeneity, would be constituted by zymodemes 1–25 (those zymodemes more or less related to the zymodeme I of Miles and colleagues). The other zymodemes exhibit a large range of genotypes. This dispersion remains even if we discard possible "*T. cruzi*-like" organisms by considering only those stocks that were isolated either from human hosts or from triatomine bugs. The overall dispersion pattern shown by the network in Figure 5 is confirmed by the genetic and patristic distance values, which exhibit a continuum of intermediates between the extreme values (Table 4). If there were a few

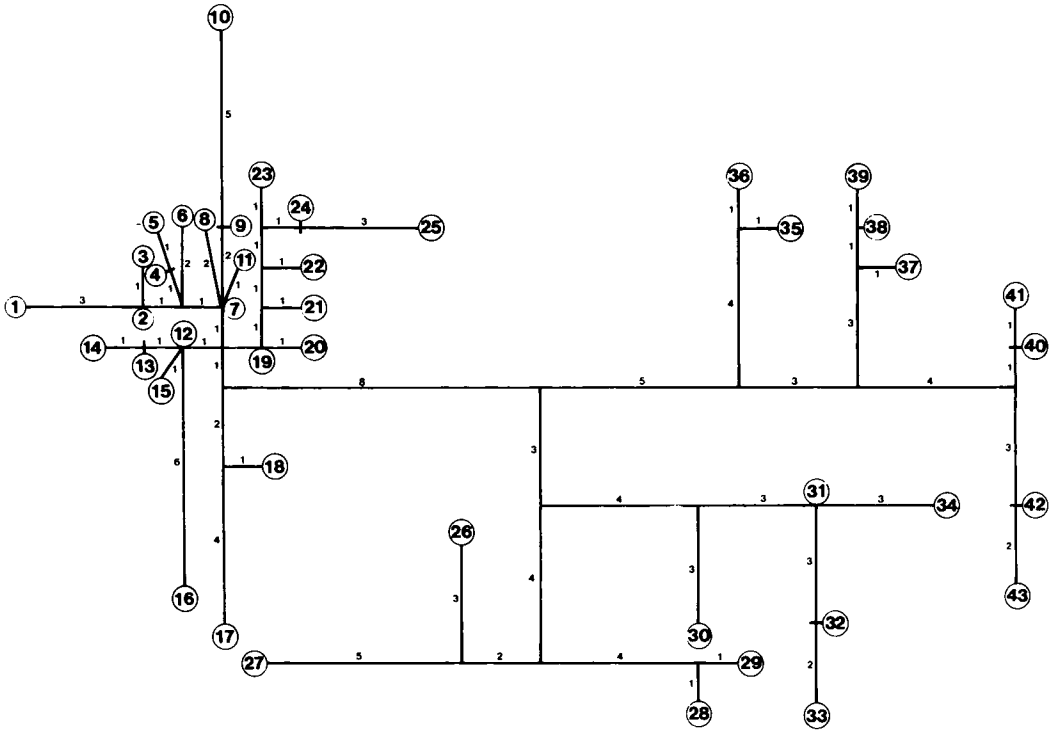

FIG. 5. A minimum-length unrooted Wagner network of 131 steps linking 43 zymodemes of *Trypanosoma cruzi*. The numbers identifying the zymodemes (each surrounded by a circle) are given at the terminal points of branches. The numbers along the branches are the patristic (evolutionary) distances for each segment (after Tibayrenc et al. [1986*b*]).

clusters separated by wide gaps, one would expect such gaps to be reflected in the genetic and patristic distance values. The dispersion pattern of the *T. cruzi* genotypes is also supported by schizodeme analysis, which shows a similar continuum of values for the proportion of common restriction bands among a set of zymodemes (Tibayrenc and Ayala, 1987).

A typological approach for describing the variability of *T. cruzi* stocks seems, therefore, misleading. A classification into three main, strictly delimited, zymodemes had been proposed by Ready and Miles (1979). Subsequently, additional variability was recognized within each main zymodeme (Tibayrenc and Miles, 1983; Gibson and Miles, 1985). The present data, based on

TABLE 4. Matrix of patristic distances (above the diagonal) and genetic distances (below the diagonal) between ten representative zymodemes identified by assaying 15 isozyme loci in *Trypanosoma cruzi* (after Tibayrenc et al. [1986*b*]).

	Zymodeme									
Zymodeme	12	10	17	39	43	41	30	35	28	27
12	—	9	8	23	27	24	20	20	22	24
10	0.34	—	15	30	34	31	27	27	29	31
17	0.31	0.44	—	27	31	28	24	24	26	28
39	0.95	1.25	1.15	—	14	11	23	13	25	27
43	1.15	1.25	0.95	0.43	—	7	27	17	29	31
41	0.88	1.27	0.88	0.16	0.27	—	24	14	26	28
30	1.08	1.29	1.30	0.77	0.53	0.75	—	20	16	18
35	0.92	1.30	0.92	0.46	0.57	0.42	1.08	—	22	24
28	1.32	1.59	1.61	1.15	0.86	1.06	0.75	0.92	—	12
27	1.61	1.99	2.01	1.40	1.15	1.42	0.90	1.09	0.51	—

TABLE 5. The correspondence between the zymodeme identifications used in previous papers (columns A and B) and the zymodemes defined by 15 isozyme loci (column C): A) Tibayrenc and Le Ray (1984) and Tibayrenc et al. (1984*a*); B) Tibayrenc and Miles (1983); C) Tibayrenc et al. (1986*b*) and the present paper (see Table 3).

A	B	C
1	BOL Z1	9
1	BRAZ Z1	17
1e	—	12
1f	—	23
2	BOL Z2	39
2a	—	43
2b	—	38
2c	—	32
2d	—	34
2e	BRAZ Z2	30
3	BRAZ Z3	27

more extensive samples, suggest that the formerly proposed classification gives an insufficient account of the actual variability of the parasite, although the three formerly described zymodemes remain valuable as reference strains that are radically dissimilar from one another. Zymodemes I, II, and III correspond respectively to our zymodemes 17, 30, and 27 (see Fig. 5 and Table 5).

We have defined 43 natural clones of *T. cruzi*. Additional isozymes or other biochemical data would certainly demonstrate heterogeneity within each clone; indeed, this has been confirmed by schizodeme analysis (Tibayrenc and Ayala, 1987). Other authors have already predicted that the combination of several methods of biochemical characterization will yield a higher number of clones within a given asexual species than will any single method (Steiner and Levin, 1977; Ochman et al., 1980). In order to determine the actual number of clones present in a given taxon, it might well be necessary to survey its entire genome (Jaenike et al., 1980). This means that it is impossible to give a definitive enumeration of the *T. cruzi* zymodemes (Table 5 gives the correspondence between the zymodeme numbering used in the present work and those used in previous studies). It is significant, however, that comparison of the isozyme classification with a quantitative schizodeme analysis suggests that the 15 enzyme loci used here provide a well defined phylogenetic classification, suitable for medical or epidemiological studies (Tibayrenc and Ayala, 1987). The use of fewer isozyme loci would be less satisfactory (Avise and Aquadro [1982] chose a lower limit of 14 loci for accepting the phylogenetic value of genetic distances in vertebrates).

DISCUSSION

Evolutionary Origin

We have recently proposed alternative hypotheses about the evolutionary origin of *T. cruzi* zymodemes, all compatible with the information then available (Tibayrenc et al., 1983, 1984*b*). The hypotheses are: i) ancient divergence and independent evolution of multiple clones; ii) ancient divergence of three (or some other number) biological species, with consequent loss of sexuality, and followed by additional divergence among the clones derived from each inferred species; and iii) recent origin of multiple clones derived from a sexual ancestral population, as in the situation observed for parthenogenetic cockroaches (Parker et al., 1977; see also Vrijenhoek et al., 1977, 1978).

The data presented here favor the first hypothesis. It is not possible to group the *T. cruzi* zymodemes into a few sharply separated clusters that could represent the ancestral biological species (second hypothesis). The third hypothesis would require an excessively large degree of genetic variability in the hypothetical sexual ancestor in order to account for the high numbers of alleles recorded for some loci (12 in the case of *Pgm*) and for the large value of many genetic distances (Table 4). According to this third hypothesis, the genetic distances measured between the *T. cruzi* zymodemes would not reflect evolutionary times of divergence but, rather, the polymorphisms present in the sexual populations from which the particular clones have derived. Yet, the hypothesis that genetic distances serve as phylogenetic markers that are roughly correlated with time (molecular clock) is supported by the high correlation observed between isozyme and kinetoplast DNA data (Tibayrenc and Ayala, 1987). This correlation shows that the two lines of genetic characters corroborate each other, and it

strongly suggests that they both provide suitable phylogenetic information.

It seems likely, therefore, that clonal evolution in *T. cruzi* is ancient and that numerous clones have been evolving independently for a long time; the present distribution of *T. cruzi* genotypes would result from recombination being severely restricted or absent, stochastic extinction of lines, dispersion factors (vector and vertebrate host migrations, geographic characteristics), and selective differences among clones.

It is possible that both local and general selective pressures affect zymodeme distribution. We have evidence of a striking correlation among three environmental parameters (altitude, longitude, and climate) and zymodeme frequency in Bolivia (Tibayrenc et al., 1984*a*). In addition, some zymodemes have been isolated several times, but only within restricted areas (see zymodeme 34 in the São Paulo region), which is consistent with either founder effects or local adaptation. Lastly the overall zymodeme distribution suggests the existence of "general purpose genotypes," since the vast majority of the zymodemes were sampled only one or a few times, while a limited number (e.g., zymodemes 19, 20, and 39) are widespread over large ecogeographical areas, a pattern frequently observed in other asexual organisms (Parker and Selander, 1976; Jaenike et al., 1980). It is possible that such successful natural clones have been the origin of sets of zymodemes more or less related to one another (this would be the case for the group 1–25). Such a hypothesis was proposed by Whittam et al. (1983) to explain a loose tendency for *E. coli* isozyme genotypes to subdivide.

Relationships between Isozyme Classification and Other Biological and Medical Data

The results summarized above make it possible to replace descriptive concepts (i.e., zymodeme, schizodeme) with a predictive one (natural clone) and to propose a falsifiable model of *T. cruzi* intraspecific variability. The working hypothesis we propose is that the clonal structure of the parasite is the major factor controlling its general variability. Because the natural clones behave largely as independent genetic entities ("agamospecies"), it is reasonable to expect the biochemical variability of the parasite to be statistically correlated with other features, such as medical characteristics (e.g., the clone concept in bacteriology [Ørskov and Ørskov, 1983]). This would have obvious medical applications, because the biochemical (isozymic) characterization of clones would provide a good indication of their medical properties.

It is also conceivable, however, that the medically important properties have little or no correlation with the biochemical characteristics for at least two possible reasons. i) The medical properties could have a genetical determination that has nothing to do with the one responsible for the biochemical characteristics. For example, no correlation was found in *E. coli* between isozyme variability and resistance to five common antibiotics (Miller and Hartl, 1986), which is due to the fact that antibiotic resistance is mostly plasmidic, while isozyme variability is driven by chromosomal genes. ii) The medical and other biological properties could be the result of different selective pressures in different environments and so would have no correlation with the biochemical characteristics. If this were the case, two stocks belonging to the same zymodeme or to closely related zymodemes isolated, say, one from a domestic cycle and the other one from a wild cycle, could have dissimilar biological and medical characteristics; whereas two stocks isolated both from a domestic cycle could be biologically and medically similar, even if they belong to quite different zymodemes. It should be noted that the two hypotheses are not mutually exclusive, given that some medical characteristics but not others could be correlated with the biochemical classification.

Some results favor our working hypothesis, in that they suggest some correspondence between the biochemical classification and other biological properties, such as growth kinetics (Dvorak et al., 1980), development in the insect vector (Garcia and Dvorak, 1982), vector specificity in some cases (Miles et al., 1981*b*), general biological behavior (Andrade et al., 1983), pharmacological properties in vitro (Barnabé et al., 1983), specificity of monoclonal antibodies

(Flint et al., 1984), and pathogenic properties (Miles et al., 1981*a*). However, the results are at best scanty, as none of the studies cited above has considered an ample and diversified set of *T. cruzi* zymodemes.

A resolution between the two hypotheses proposed will only come about from the study of representative samples of similar and dissimilar zymodemes, isolated from various types of cycles, in order to ascertain whether the correlation proposed by the working hypothesis obtains for all, or at least part, of the relevant medical properties of the parasite. Such studies would answer a long-open question and contribute decisively toward clarifying the epidemiology and pathogenicity of Chagas' disease.

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