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Evolution

Natural populations of *Trypanosoma cruzi*, the agent of Chagas disease, have a complex multiclonal structure

(linkage disequilibrium/speciation/evolution/enzyme variation/Wagner network)

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ABSTRACT **We have studied 15 gene loci coding for enzymes in 121 *Trypanosoma cruzi* stocks from a wide geographic range—from the United States and Mexico to Chile and southern Brazil. *T. cruzi* is diploid but reproduction is basically clonal, with very little if any sexuality remaining at present. We have identified 43 different clones by their genetic composition; the same genetic clone is often found in very distant places and in diverse hosts. There is much genetic heterogeneity among the different clones, and they cannot be readily classified into a few discrete groups that might represent natural taxa. These findings imply that the biological and medical characteristics need to be ascertained separately for each natural clone. The evidence indicates that clonal evolution is very ancient in *T. cruzi*. We propose two alternative hypotheses concerning the relationship between the biochemical diversity and the heterogeneity in other biological and medical characteristics of *T. cruzi*. One hypothesis is that the degree of diversity between strains simply reflects the time elapsed since their last common ancestor. The second hypothesis is that biological and medical heterogeneity is recent and reflects adaptation to different transmission cycles. A decision between the two hypotheses can be reached with appropriate studies, with important medical consequences.**

Isozyme studies of *Trypanosoma cruzi*, the causative agent of Chagas disease, were initiated in 1974 (1). Analysis of the zymograms revealed substantial isozymic variability among stocks (2–7), which were classified into three groups or "zymodemes" (2, 3, 8).

We studied *T. cruzi* from Bolivian populations and proposed a genetic interpretation of the zymograms that lead to the following hypotheses. (*i*) *T. cruzi* is a diploid organism (9, 10), a conclusion supported by a DNA study (11). (*ii*) Mendelian sexuality is absent or very rare (10, 12, 13). (*iii*) The genetic distances between the strains characterized by the isozyme patterns are often very large (14, 15). These results made it possible to propose alternative hypotheses concerning the evolutionary origin and taxonomic status of the strains (15, 16).

We have now conducted an extensive isozyme study of 121 *T. cruzi* stocks, isolated from the whole geographic range of the Chagas disease agent. The results confirm and extend the conclusions proposed on the basis of the limited data from Bolivia.

MATERIALS AND METHODS

Information on the geographic origins of the 121 stocks studied is given in Table 1. The methods used for growing, harvesting, and storing the stocks have been described (17).

Electrophoresis was carried out on cellulose acetate plates (Helena Laboratories). The 14 enzyme systems assayed were as follows: aconitase (aconitate hydratase, EC 4.2.1.3), adenylate kinase (EC 2.7.4.3), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose-6-phosphate isomerase (EC 5.3.1.9), glutamate dehydrogenase (EC 1.4.1.2), glutamate dehydrogenase ($NADP^+$) (EC 1.4.1.4), isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42), leucine aminopeptidase (cytosol aminopeptidase, EC 3.4.11.1), malate dehydrogenase (EC 1.1.1.37), malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) or malic enzyme (EC 1.1.1.40), peptidase 1 (ficin, EC 3.4.22.3, formerly EC 3.4.4.12, substrate: leucylleucylleucine), peptidase 2 (bromelain, EC 3.4.22.4, formerly EC 3.4.4.24, substrate: leucyl-L-alanine), phosphoglucomutase (EC 5.4.2.2., formerly EC 2.7.5.1), and phosphogluconate dehydrogenase (EC 1.1.1.44). The assays for aconitase and adenylate kinase were described (18), with slight modifications; all other enzymes were assayed (19) with the modifications given (17).

RESULTS

The genetic interpretation of the enzyme data is as given in ref. 9. Fifteen gene loci are revealed by the 14 enzyme systems assayed (*Me-1* and *Me-2* are the only two loci obtained from the same enzyme assay). Fourteen of the 15 loci, or 93.3%, are polymorphic in our sample; the exception is *Adk*. The number of alleles among the polymorphic loci ranges from 2 to 12, with a mean of 5.14 per locus. The average frequency of heterozygous loci per strain is 0.059.

Stocks from the same or from different localities often have the same genetic constitution at all 15 loci; each particular pattern of the 15 loci is called an isozyme strain without prejudging the medical or taxonomic significance of this classification (15). Among the 121 stocks, there are a total of 43 isozyme strains, or genotypic constitutions, 27 of which are represented by only one stock. In four cases, two from Bolivia and two from Chile, two different isozyme strains were present in the same stock. The isozyme strains found in each country and the number of stocks of each strain are given in Table 1.

Genetic distances among the strains are calculated using Nei's *D* statistic (20). Table 2 gives these genetic distances for the pairwise comparisons between ten representative isozyme strains. Although some isozyme strains are genetically quite similar to each other (e.g., isozyme strains 39 and 41, Table 2), a majority are genetically quite different, indicating a substantial amount of biochemical (genetic) divergence among the *T. cruzi* strains. The average genetic distance between pairs of isozyme strains is, for all 43

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Table 1. *T. cruzi* isozyme strains found in each country

Country	Isozyme strain (no. of stocks)				Total isozyme strains	Total stocks
	Triatoma infestans	Other Triatominae	Humans	Other mammals		
Bolivia	9 (3), 19 (2), 20 (10), 32 (2), 37, 38, 39 (16), 42	6, 25	10, 16, 19 (6), 20 (3), 39 (4), 40 (2), 41	15, 28, 29	17	57*
Brazil	40, 42	26, 39	2, 3, 17, 27, 30, 31 (2), 34, 35	19 (3), 20 (3), 23, 34 (2), 36	16	23
Chile	7, 19, 33, 39 (3)	—	32 (2), 33, 39, 43	—	6	9*
Colombia	—	19 (5)	—	—	1	5
Ecuador	—	24	—	—	1	1
French Guiana	—	1	—	2, 3, 4 (2), 5, 8, 11, 18 (3)	8	11
Honduras	—	13	—	—	1	1
Mexico	—	12	—	—	1	1
Peru	—	—	—	22	1	1
USA	—	—	—	14 (2)	1	2
Venezuela	—	19 (2), 21 (2)	19 (3)	19 (2), 21	2	10
Total isozyme strains	11	10	18	18	43†	
Total stocks	40*	17	35	29		121

No number is given in parentheses when only one stock of a given strain has been found.
*Two mixed stocks, each containing two strains were found in each of two countries, Bolivia and Chile.
†This number is smaller than the addition of the isozyme strains found in the various countries, or in different hosts, because several isozyme strains are each found in more than one of them.

isozyme strains, $D = 0.757$, with a range from 0.017 to 2.015 and a standard deviation of 0.478.

We have tried several clustering methods to summarize the similarities and differences among the isozyme strains. The minimum-length Wagner networks (21, 22) appear to be the most informative for our purposes. This method has been used to classify isozyme strains in a parthenogenetic snail (23) and to resolve the biochemical taxonomy of *Leishmania* (24). We have used the MIX computer program (Wagner parsimony algorithm) from J. Felsenstein's PHYLIP package. Each allele is treated as an independent character and is coded as either present or absent. We used eight different input sequences of the 43 isozyme strains and obtained eight unrooted Wagner networks; the most parsimonious one is shown in Fig. 1. The patristic, or evolutionary, distances between pairs of 10 representative isozyme strains are given in the upper triangle of Table 2. These patristic distances are significantly correlated with the genetic distances shown in the same table ($r = 0.76$, $df = 43$, $P < 0.01$). The patristic distances along each branch of the minimum-length network are given in Fig. 1 for all 43 isozyme strains.

DISCUSSION

Diploidy. In comparison with our results (9, 10), several new heterozygous patterns are present in our sample, for loci at which heterozygotes either had not been found (*Mdh*) or were already known (*Gpi, Me, Pgm, 6Pgd*). This supports the hypothesis advanced (9–11) of diploidy for the whole taxon *T. cruzi*.

Lack of Sexual Reproduction. The present data corroborate the hypothesis of a basically clonal population structure with rare or absent recombination and genetic segregation (10, 12, 13). Indeed, a strong linkage disequilibrium is apparent. If the allozymes at each of the 15 loci would combine randomly, the total number of possible genotypes is enormous (7×10^{15}), so that even the most probable genotypes would occur with a very low frequency. Hence, it would be quite unlikely to observe repeatedly identical, or even very similar, allozyme patterns in a sample of 121 stocks. Yet, we have found in our sample only 43 genotype patterns, 16 of which differ from each other by only one allele. It is notable that a given isozyme strain can appear in geographically distant sites. For example, isozyme strain 39 is present in Bolivian localities quite far apart from each other (20 stocks), in Chile (4 stocks), and in southern Brazil (1 stock). Natural selection favoring only certain genotypic combinations could account for a limited number of isozyme strains, but it would be difficult to explain, for example, the presence of isozyme strain 20 in domestic transmission cycles in Bolivia and in sylvatic transmission cycles in Brazil (Sao Paulo). Moreover, some

Table 2. Matrix of patristic distances (above diagonal) and genetic distances (below diagonal) among ten representative isozyme strains

Isozyme strain	Isozyme strain									
	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	

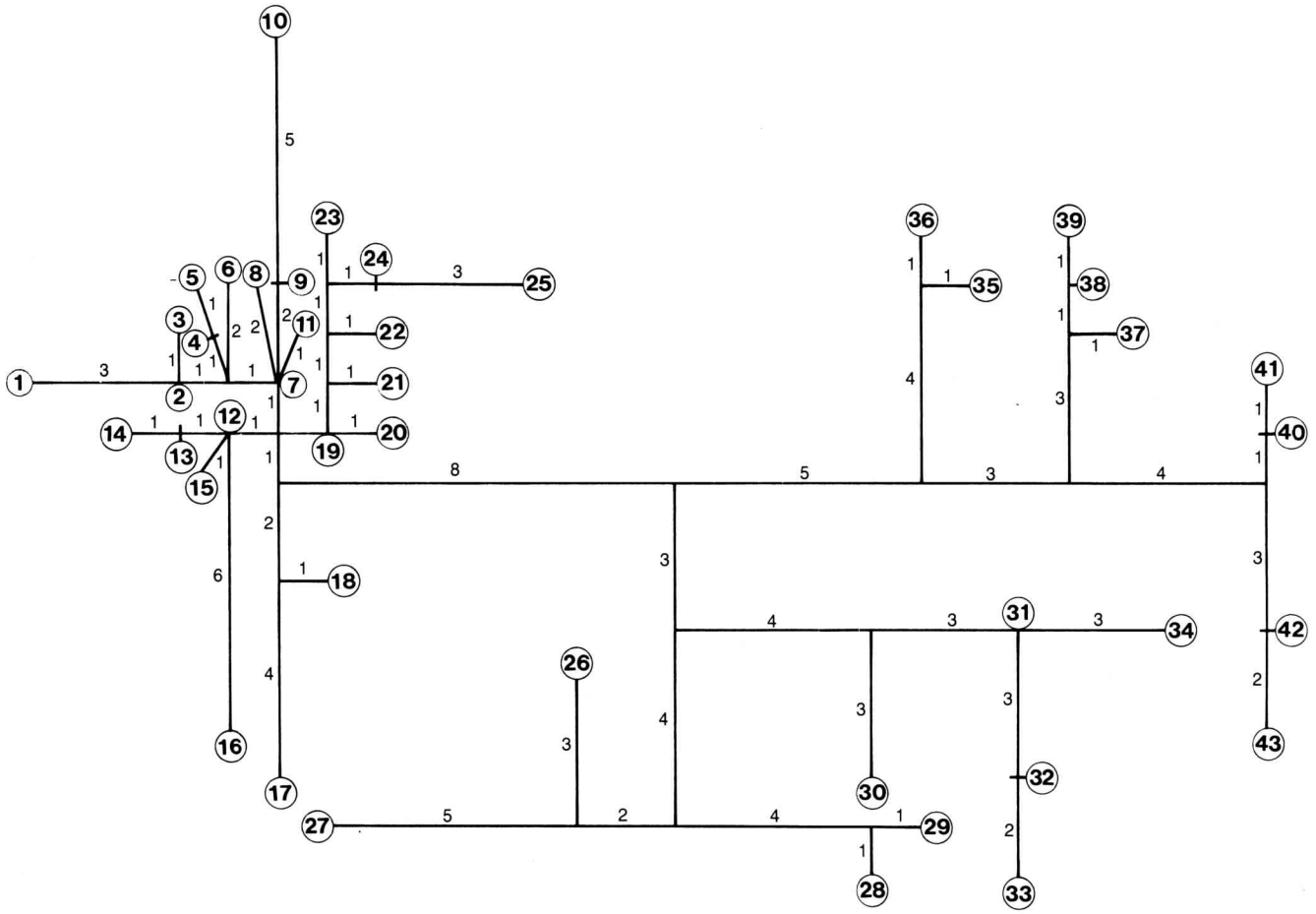

FIG. 1. A minimum-length unrooted Wagner network of 131 steps linking the 43 isozyme strains of *Trypanosoma cruzi*. The numbers identifying the strains (each surrounded by a circle) are given at the terminal points of branches. The numbers along the branches are the patristic, or evolutionary, distances for each segment. These distances are only approximate measures of allelic differences among the strains in that they ignore additional changes necessary to avoid internal nodes at which some loci lack alleles.

isozyme strains are ubiquitous, and many possible genotypes remain absent, even if we ignore the Bolivian sample, which is the most extensive and could represent a special case (see Table 1). This situation also obtains if we consider only the stocks isolated from humans and Triatomine bugs (this would help to eliminate the possible inclusion in our sample of "*T. cruzi*-like" organisms, which might be isolated from nonhuman mammals).

The working hypothesis of a lack of mating is difficult to test in those regions, such as French Guiana, where only similar isozyme strains have been recorded, because there are not sufficient markers to exclude genetic recombination. Nevertheless, the widespread distribution of certain isozyme strains (with no allozyme differences among the loci assayed) together with the striking linkage disequilibrium suggests strongly that reproduction is predominantly or exclusively asexual and that the population structure is clonal as concluded on the basis of data obtained from Bolivian ecosystems (10, 12, 13). Selander and Levin (25; see also refs. 26 and 27) relied on similar evidence to conclude that *Escherichia coli* populations have a clonal structure, although some genetic recombination (parasexuality) is known to occur in these bacteria. Like these authors (25–27), we do not rule out occasional mating in *T. cruzi*: the diploid constitution suggests indeed the existence of a sexual cycle at least in the past.

A recent study (28) has argued that there is little opportunity for mating among certain *T. cruzi* isozyme strains because of founder effects and the weak dispersal tendency of the vectors and, hence, that the evidence for absence of mating must be considered with caution. But even if there are founder effects and the vectors have low vagility, this could only reduce the opportunity for mating and cannot account for our results: ample opportunity for mating and for its detection exists in many cases. We have shown that different isozyme strains are often found without recombination in the same house (13, 29), as well as in the same Triatomine bug (about 10% of all infected *Triatoma infestans* in Bolivia, refs. 10 and 29), and occasionally, even in the same human patient (30). The presence in the same individual host of mixed stocks without recombination is evidenced by the dimeric enzyme glucose-6-phosphate isomerase: recombinants between two isozyme strains homozygous for different alleles would exhibit the three-banded pattern characteristic of dimeric heterozygotes, rather than the two-banded pattern, which was repeatedly observed whereas the recombinant pattern was never observed. Moreover, the vast majority of the possible recombinant genotypes are totally lacking, even in the southern part of the geographic range studied (Bolivia, Brazil, and Chile; see Table 1) where the sympatric occurrence of very different isozyme strains is common. It should be noticed that few (four) mixed stocks occur in our present sample relative to our previous field studies. This may be due to the fact that the stocks now surveyed had been kept in culture for several months before studying them: there is a tendency for one isozyme strain to eliminate the other under such conditions (31).

Although the occurrence of rare mating cannot be excluded, the prevalence of clonal reproduction in *T. cruzi* is most consistent with the data, and it has important biological and medical implications. If there is no mating, the *T. cruzi* isozyme strains are natural clones and, hence, independent genetic entities, that need to be studied separately in order to establish their biological and medical characteristics. A set of isolates that belong to the same natural clone may share common biological or medical properties that will not be intermingled with those characteristics of other clones. This situation stands in contrast with the one proposed for the African species, *Trypanosoma brucei*, which apparently exhibits Mendelian sexuality (ref. 32; however, additional experimental data do not corroborate the inference of Mendelian sexuality in *T. brucei*, see ref. 33).

Taxonomic Clustering. Miles and his collaborators (2, 3, and 8) have proposed, on the basis of more limited sampling of *T. cruzi* stocks, that these can be classified into three main isozyme strains (zymodemes I, II, and III). Our data (14–16) were consistent with this classification, although they showed a high intragroup diversity. The three zymodemes correspond, respectively, to isozyme strains 17, 30, and 27 of this work. It can be seen in Fig. 1 that these three isozyme strains are quite different from one another. But it is also apparent that the 43 isozyme strains identified in our study do not fall into three natural clusters. One could, perhaps, separate them into three groups (isozyme strains 1–25, 26–34, and 35–43), but these groups would be very heterogeneous, particularly the second and third ones. Except for isozyme strains 1–25, there is no evidence of substantial clustering in our data. Rather than three or any other fixed number of taxa, our data suggest that *T. cruzi* strains are broadly spread over the field of possible genotypes (which remains even if we discard possible "*T. cruzi*-like" organisms. Whether additional sampling will increase the clustering around certain foci or will continue to show a widespread and disparate distribution remains to be seen; our experience leads us to anticipate the second of these two alternatives). This view has again important biological and medical implications, because it rejects the notion that study of each one of the three postulated zymodemes would be sufficient to characterize the taxon *T. cruzi*.

Evolutionary Origin. We have recently proposed alternative hypotheses about the evolutionary origin of *T. cruzi*, all compatible with the then available information (15, 16). The alternatives are as follows: (*i*) ancient divergence and independent evolution of multiple clones; (*ii*) ancient divergence of three (or some other number) biological species with some heterogeneity among the clones of each species; and (*iii*) recent origin of multiple clones derived from a sexual ancestral population.

Our present results favor the first hypothesis. As argued in the previous section, these data do not justify grouping the isozyme strains into a few relatively homogeneous taxa, separated by wide gaps (second hypothesis). The third hypothesis seems also unlikely. If the present isozyme strains have all diverged by clonal reproduction from a recent sexual ancestral population (species), one would have to postulate an incredibly high degree of genetic variation in that population. It seems likely, therefore, that clonal evolution in *T. cruzi* is ancient and that numerous clones have been evolving independently for a long time.

We have shown extreme biochemical heterogeneity in *T. cruzi*; there also is extensive heterogeneity with respect to medical and other biological properties (34). What is the relationship between these two kinds of variation? Two alternative hypotheses seem possible. One is that long clonal evolution has led to the incorporation in particular clones of given biochemical and other biological characteristics, so that the degree of differentiation between any two clones simply reflects the length of time elapsed in their separate evolution. If this is the case, one would find sets of biochemically related clones to be also related with respect to other biological properties; that is, there would be a statistical correlation between the biochemical and the biological characteristics of the clones. This would have significant medical implications, because the biochemical (enzymatic) characterization of clones would provide a good indication of their medical properties.

The second hypothesis is that natural selection has promoted the adaptation of particular clones (or particular stocks of a given clone) to man and to domestic cycles, while other clones (or other stocks of the same clone) would be adapted to other hosts and to wild cycles. The biochemical (enzymatic) diversity would be of more ancient origin and would have evolved largely independently, so that the biochemical characteristics of a clone would provide no clue to its human pathogenicity and other biological properties. That is, two biochemically related (or even identical) isozyme strains isolated, say, one from a domestic cycle and the other from a wild cycle, could have divergent biological and medical characteristics; whereas two isozyme strains isolated from, say, a domestic cycle could be biologically and medically similar, even though biochemically quite different.

The two hypotheses are not completely exclusive and might be both partially true. Some results are consistent with the first hypothesis, in that they show correlation between biochemical classification and biological properties (35–40). But the data are at best scanty and additional evidence must be sought. The resolution between the two hypotheses proposed would come from the biological and medical characterization of a representative set of biochemically similar and dissimilar isozyme strains isolated some from domestic and some from wild cycles to see whether the correlation proposed by the first hypothesis is indeed the case.

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