Impact of clonal evolution on the biological diversity of *Trypanosoma cruzi*

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SUMMARY

Trypanosoma cruzi, the agent of Chagas' disease, exhibits considerable biological variability. Moreover, it has been postulated that populations of this protozoan are subdivided into natural clones, which can be separated from each other by considerable levels of evolutionary divergence. The authors have proposed that this long-term clonal evolution may have a profound impact on *Trypanosoma cruzi* biological diversity. In order to test this hypothesis, 16 *T. cruzi* stocks representing 3 major clonal genotypes of the parasite were analysed for 8 di erent *in vitro* biological parameters. The overall results show a strong statistical linkage between genetic and biological di erences. This is in agreement with the working hypothesis, although a notable biological variability is observable among the stocks of each of the 3 major clonal genotypes. The authors propose that *T. cruzi* genetic variability must be taken into account in any applied study dealing with this parasite.

Key words: *Trypanosoma cruzi*, clonality, biodiversity, evolution, Chagas' disease.

INTRODUCTION

Trypanosoma cruzi, a parasitic protozoan, is the agent of Chagas' disease, a major health problem in Latin America. Chagas' disease presents various clinical forms and levels of gravity, which could be due to the variability of its causative agent. *T. cruzi* presents indeed a considerable biological variability, as verified by various experimental studies (Dvorak, 1984). By population genetic analyses relying mostly on Multilocus Enzyme Electrophoresis (MLEE), we have proposed (Tibayrenc *et al*. 1986) that *T. cruzi* presents a typical clonal population structure. Populations of this parasite are subdivided into natural clones, which can be separated from each other by considerable evolutionary levels of divergence. We have also proposed (Tibayrenc *et al*. 1986) that long-term clonal evolution, through the accumulation of random mutation in separate clonal lines, could lead to divergence in the biological features of *T. cruzi* natural clones, including relevant medical properties such as virulence or resistance to anti-chagasic drugs.

In order to test this working hypothesis, a sample of 16 stocks, corresponding to 3 major clonal genotypes of the parasite, has been analysed for 8 di erent *in vitro* biological parameters. The possible links between genetic variability and clonal diversity on the one hand, and biological variability on the other hand, have been explored statistically.

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MATERIALS AND METHODS

The stocks were selected, according to previous MLEE characterization, in order to represent 3 groups of clonal genotypes (19, 20 and 39) that appear to be widespread and very frequent in South America, so called major clones (Tibayrenc & Brénière, 1988). Clonal genotypes 19 and 20 are closely related to each other, while clonal genotype 39 is distantly related to either 19 or 20 (Tibayrenc *et al*. 1986).

Table 1 indicates the origin of the stocks, and their identification according to previous MLEE analysis based on the use of 15 enzyme loci. All stocks have been cloned by micromanipulation, and actual cloning has been verified under the microscope.

The stocks were cultured and harvested as described by Tibayrenc & Le Ray (1984) and analysed by 20 di erent enzyme systems, corresponding to 22 genetic loci, according to methods described by Ben Abderrazak *et al*. (1993). The 20 enzyme systems surveyed are as follows: aconitase (EC 4.2.1.3, ACON), alanine aminotransferase (EC 2.6.1.2, ALAT), diaphorase (EC 1.6.–.–, DIA), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPD), glutamate dehydrogenase NAD^+ (EC 1.4.1.2, GDH–NAD^+), glutamate dehydrogenase $NADP^+$ (EC 1.4.1.4, GDH–$NADP^+$), aspartate amino transferase (EC 2.6.2.1, GOT), glucose-6-

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Table 1. Origin of the 16 *Trypanosoma cruzi* stocks under study

(Clonet numbering is according to Tibayrenc *et al*. (1986).)

Stocks	Clonet	Host	Place
SP104 cl1	19	*Triatoma spinolai*	Chile/Region IV
Cutia cl1	19	*Dasylprocta aguti*	Brazil/Espiritu santo
Gamba cl1	19	*Didelphis azarae*	Brazil/São Paulo
13379 cl7	19	Man; acute phase	Bolivia/Santa Cruz
OPS21 cl11	19	Man; ?	Venezuela/Cojedes
SO34 cl4	20	*Triatoma infestans*	Bolivia/Potosi
Cuica cl1	20	*Opossum cuica philander*	Brazil/São Paulo
P209 cl1	20	Man; chronic phase	Bolivia/Sucre
Esquilo cl1	20	*Sciurius aestuans ingrami*	Brazil/São Paulo
P11 cl3	20	Man; chronic phase	Bolivia/Cochabamba
SC43 cl1	39	*Triatoma infestans*	Bolivia/Santa Cruz
Bug2148 cl1	39	*Triatoma infestans*	Brazil/Rio Grande do sul
SO3 cl5	39	*Triatoma infestans*	Bolivia/Potosi
MN cl2	39	Man; chronic phase	Chile/Region IVa
Bug2149 cl10	39	*Triatoma infestans*	Brazil/Rio Grande do sul
NR cl3	39	Man; chronic phase	Chile/Region IIIa

phosphate dehydrogenase (EC 1.1.1.49, G6PD), glucose-6-phosphate isomerase (EC 5.3.1.9, GPI), isocitrate dehydrogenase (EC 1.1.1.42, IDH), leucine aminopeptidase (cytosol aminopeptidase) (EC 3.4.11.1, LAP), malate dehydrogenase EC 1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating, $NADP^+$) or malic enzyme (EC 1.1.1.40, ME), mannose-phosphate isomerase (EC 5.3.1.8, MPI), nucleoside hydrolase (EC 2.4.2.–.–, NHi); substrate: inosine, peptidase 1 (Ficin) (EC 3.4.22.3, PEP-1); substrate: leucyl-leucyl-leucine), peptidase 2 (Bromelain) (EC 3.4.22.4, PEP-2; substrate: leucyl-L-alanine), 6-phosphoglucomutase dehydrogenase (EC 1.1.1.44, 6PGD), phosphoglucomutase (EC 5.4.2.2, PGM), and superoxide dismutase (EC 1.15.1.1, SOD).

The multi-enzyme identification for each stock has been verified every 6 months throughout the study, in order to detect any possible cross-contamination.

All the stocks were maintained in LIT culture medium supplemented with 10% heat-inactivated foetal calf serum in exponential growth condition.

Growth kinetics of epimastigote forms

A total of 25×10^6 parasites was inoculated in a final volume of 50 ml. The cultures were incubated at 27 °C. The kinetics were followed for 24 days. The culture concentration was estimated 3 times every 2 days by counting in a Thoma chamber. Two parameters were taken into account (i) the doubling time (DT), estimated in hours, was calculated from the log phase and (ii) the parasite concentration in 10^{-6} parasites per ml (10^{-6} P/ml) at the end of the log phase (Celp).

Differentiation of epimastigotes from trypomastigotes

The *in vitro* metacyclogenesis was performed as previously described (Contreras *et al*. 1985). Briefly, the parasites were harvested from an epimastigote LIT culture at the end of the log phase. They were incubated at room temperature in TAU medium for 2 h and then transferred and incubated in TAU 3AAG medium at 27 °C, at the final concentration of 5×10^6 P/ml to follow the di erentiation.

The kinetics were followed for 10 days. Every day, a triplicate was stained in eosin–methylene blue. Metacyclic trypomastigotes and epimastigotes were counted. Two parameters were analysed (i) the highest rate (percentage) of di erentiation, expressed by the number of metacyclic trypomastigotes divided by the number of total cells multiplied by 100 (DIF) and (ii) the time in days at which DIF was recorded (TDIF).

Susceptibility of epimastigotes to complement-mediated lysis

Mid-log phase epimastigotes were harvested by centrifugation and inoculated in LIT medium containing 25% fresh human AB^+ serum. The suspension was incubated at 37 °C for 1 h. The remaining cells were then counted in a Thoma chamber. Each experiment was replicated 4 times. The complement susceptibility of epimastigotes (CS) was estimated by the percentage of lysed cells.

Experimental infection in mice

The experimental infection was performed in female, 4 or 5-week-old Balb/c mice (IFFA CREDO,

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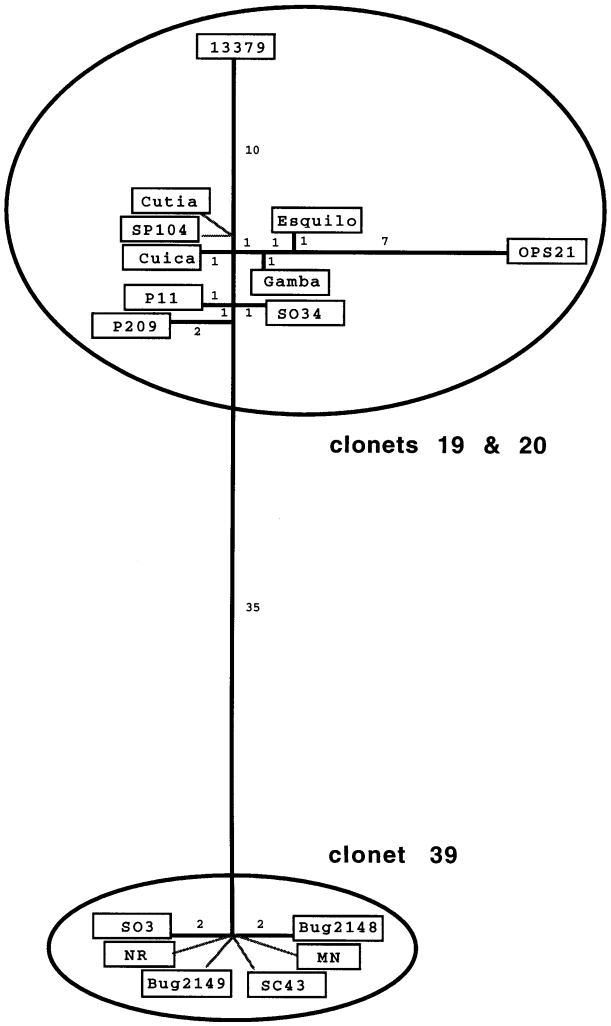

Fig. 1. A Wagner phylogenetic tree depicting the evolutionary relationships among the 16 *Trypanosoma cruzi* stocks under study. The numbers along the branches represent the evolutionary (patristic) distances for each segment. Numbering of the clonal genotypes or clonets is according to Tibayrenc *et al*. (1986).

France). A group of at least 6 mice was used for each infection by a given stock.

Metacyclic trypomastigotes obtained from a late stationary phase LIT medium culture were separated from epimastigotes by complement-mediated lysis at 37 °C overnight. Trypomastigotes were washed 3 times in sterile PBS and 3×10^6 parasites were injected intraperitoneally in mice in a final volume of 0·25 ml of PBS.

Mice were immunosuppressed 60 h after parasite inoculation (Calabrese *et al*. 1991) by injection of 8 mg of cyclophosphamide (endoxan-Asta®). Parasitaemia was estimated every 2 days for 50 days, according to a variation of Pizzi's technique (Brener, 1965). Five μl of fresh blood were compressed between a slide and a 20×20 mm² cover-slip. A total of 50 microscopic fields was examined by light microscopy under $\times 720$ magnification. Parasitaemia was evaluated as 10^{-4} P/ml by scoring the number of parasites observed and multiplying the figure recorded by 10^4.

Three parameters were taken into account (i) the maximum level of parasitaemia (MP), (ii) the infectivity (INF), evaluated by the number of mice showing a parasitaemia at any time, divided by the total number of mice surveyed and multiplied by 100 and (iii) the mortality (MOR), evaluated by the total number of mice killed on the 70th day divided by the total number of mice surveyed multiplied by 100.

Controls were performed as follows: 2 groups of 10 mice were injected intraperitoneally with PBS, pH 7·2, without parasites, and were immunosuppressed like the infected mice by injection of 8 mg of cyclophosphamide, 60 h after injection.

Statistical analysis

Phylogenetic divergence between the stocks was estimated from MLEE data according to Jaccard's distance (Jaccard, 1908), with the following formula:

$$D = 1 - [a/(a+b+c)],$$

where a is the number of bands that are common to the 2 compared genotypes, b is the number of bands present in the 1st genotype and absent in the 2nd and c is the number of bands absent in the 1st genotype and present in the 2nd. The overall evolutionary relationships among the 16 stocks is represented in Fig. 1 by a Wagner phylogenetic tree (Felsenstein, 1978, 1982).

Comparison of genetic and biological data

In a first step, for each experimental parameter, means obtained for the 3 groups of stocks, corresponding to 3 major clonal genotypes, were compared by a Student's t-test.

In a second step, biological variability for each parameter was quantified as follows. If Xa is the value of the stock a for the parameter X, if Xb is the value of the stock b for the same parameter,

$$Xab = |Xa - Xb|,$$

the absolute value of the di erence is taken into account as a 'biological distance' for the pair of stocks a and b (120 pairs in total). Jaccard's distances are also given for pairs of stocks. For each parameter (8 in total), the correlation between Jaccard's genetic distances and the biological distances was estimated by a non-parametric Mantel test (Mantel, 1967). In contrast to the classical correlation test, this is a randomization procedure and does not need any assumptions about the number of degrees of freedom.

In a third step, an 'overall biological distance' was evaluated as follows. For each of the 8 parameters (X, Y, Z, $\cdots$), the weakest value of Xab (as defined

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above) was quoted 0, the highest value of Xab was quoted 1, and the other values were evaluated as percentages of the highest value ($= X'ab$). The overall biological distance between 2 stocks a and b was then calculated as the arithmetic mean of $X'ab$, $Y'ab$, $Z'ab$, ⋯ The correlation between these overall biological distances and Jaccard's genetic distances was computed for all pairs by the Mantel test (Mantel, 1967).

Lastly, the biological diversity of all stocks was evaluated by a multivariate discriminant analysis. The goal of this analysis was to find the combination of biological parameters which provides the best discrimination of the di erent genotypes. MP (maximum of parasitaemia) had very little discriminant power in the best combination, meaning that it has no influence on the discriminant test. Furthermore, as its frequencies distribution was far away from normality, MP was discarded from this analysis. For each category (genotype), a centre of mass and an inertia were calculated defining ellipses as the extension of each of the 3 clonal genotypes on the 2 first discriminant axes.

RESULTS AND DISCUSSION

Genetic diversity

As seen in Fig. 1, the phylogenetic picture obtained from 22 isoenzyme loci was in general agreement with the one previously obtained from 15 loci (Tibayrenc *et al*. 1986). All stocks previously identified as either clonal genotype 19 or clonal genotype 20 appeared as closely related to each other, and the same result was observed in all stocks previously attributed to clonal genotype 39, while stocks 19/20 were distantly related to stocks 39. As predicted, due to the higher discriminatory power of the methods used in the present work, additional genetic variability was apparent within each of the previously characterized isoenzyme genotypes, which should be considered as families of closely related clones rather than true clones (Tibayrenc & Ayala, 1988). We have previously proposed the term of 'clonet' to refer to all stocks that appear as identical for a given set of genetic markers in a clonal species (Tibayrenc & Ayala, 1991). Genotypes 19, 20 and 39 can be considered as clonets by using 15 enzyme loci with the techniques previously used by us (Tibayrenc *et al*. 1986). On the other hand, the distinction between genotypes 19 and 20, which was based on 1 allele di erence at the 6PGD locus only (Tibayrenc *et al*. 1986), became unclear with the present methods of genetic analysis. Additional studies will be required to decide whether the distinction between genotypes 19 and 20 remains phylogenetically informative. In the present work, in some statistical analyses (see further), stocks attributed to either genotype were plotted together in a unique 19/20 group. This is all the more justified since (i) all these stocks are anyway genetically close to each other and (ii) no statistical di erences were recorded between stocks related to genotype 19 on the one hand and stocks related to genotype 20 on the other hand, for any of the biological parameters under study (see further).

Biological diversity

As previously recorded by other authors (Dvorak, 1984), a considerable biological diversity was shown in the present sample of stocks. Table 2 gives the arithmetic means and the standard deviations of the means obtained for the 16 stocks and the 8 biological parameters under study.

Comparison of these means by a Student's t-test (Table 3) shows significant di erences for several biological parameters between the group '19/20' and genotype 39. No significant di erences are seen between genotype 19 and genotype 20.

When correlation between genetic and biological distances is tested by the Mantel test for all pairs of stocks, significant results are obtained for the following biological parameters: DT, Celp and CS, with levels of significance of $< 10^{-4}$, 6×10^{-4} and $< 10^{-4}$, respectively.

When correlation is tested between genetic distance and the overall biological distance of all pairs of stocks (see Materials and Methods section), the level of significance is 7×10^{-4}.

Lastly, according to the discriminant analysis, the probability of misclassifying (Pm) a stock in an improper genetic group by its biological parameters is $Pm = 0{\cdot}23$ while analysing genotype 19 and genotype 20; Pm is less than 0·04 while comparing the group 19/20 to genotype 39. This is reflected in the graphical illustration of this discriminant analysis (Fig. 2): the ellipses corresponding to genotypes 19 and 20 overlap, while the ellipse corresponding to genotype 39 falls apart.

These results reveal 2 major facts. (i) When considering stocks attributed to a given clonal genotype (either 19 or 20 or 39), they obviously do not represent a homogeneous set, and their biological variability, as revealed by the experimental parameters under survey, is important. (ii) Nevertheless, in the whole set of stocks, links between evolutionary divergence (genetic distance) and biological di erences are statistically highly significant for several of the experimental parameters considered apart. When plotted together all biological parameters in an 'overall biological distance' (see Materials and Methods section), the correlation with genetic distances is very significant ($P = 7 \times 10^{-4}$).

Result (i) suggests that overall evolutionary divergence is not the only parameter that influences the biological parameters under survey. Result (ii) is totally corroborated by further results from our team

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Table 2. Means and standard deviations for each clonet for the 8 biological parameters

(DT, doubling time; Celp, concentration at the end of the log phase; DIF, maximum of di erentiation; TDIF, time of the DIF; CS, complement sensitivity; MP, maximum of parasitaemia; INF, infectivity; MOR, mortality.)

Clonet	DT (h)	Celp (10^{-6} P/ml)	DIF (%)	TDIF (Day)	CS (%)	MP (10^{-4} P/ml)	INF (%)	MOR (%)
19								
Mean	36·6	25·3	34·5	8·4	66·6	177·7	30	28·6
S.D.	6·9	12·5	29·4	1·8	28·5	396·9	42	27·8
20								
Mean	33·6	32	39·3	8·5	92·8	500·5	74·4	43·5
S.D.	10·5	11·6	24·2	1	4·2	705·3	39·2	41·8
39								
Mean	61	17·6	21·1	7·6	32·1	2	14·3	32·6
S.D.	10·8	7	19·6	2	13·3	4·7	30	30·7

Table 3. Comparison of the means of the parameters under study for the 3 clonal genotypes by a Student's *t*-test

	19–20	19–39	20–39	19/20–39
DT	N.S.*	$0{\cdot}001 < P\dagger < 0{\cdot}01$	$0{\cdot}001 < P < 0{\cdot}01$	$P < 0{\cdot}001$
Celp	N.S.	N.S.	$0{\cdot}02 < P < 0{\cdot}05$	N.S.
DIF	N.S.	N.S.	N.S.	N.S.
TDIF	N.S.	N.S.	N.S.	N.S.
CS	N.S.	$0{\cdot}02 < P < 0{\cdot}05$	$P < 0{\cdot}001$	$P < 0{\cdot}001$
MOR	N.S.	N.S.	N.S.	N.S.
MP	N.S.	N.S.	$0{\cdot}01 < P < 0{\cdot}02$	N.S.
INF	N.S.	N.S.	N.S.	N.S.

* Non-significant.

† Probability of having no di erences between the 2 groups. For $P < 0{\cdot}05$, the di erence between groups has been considered significant.

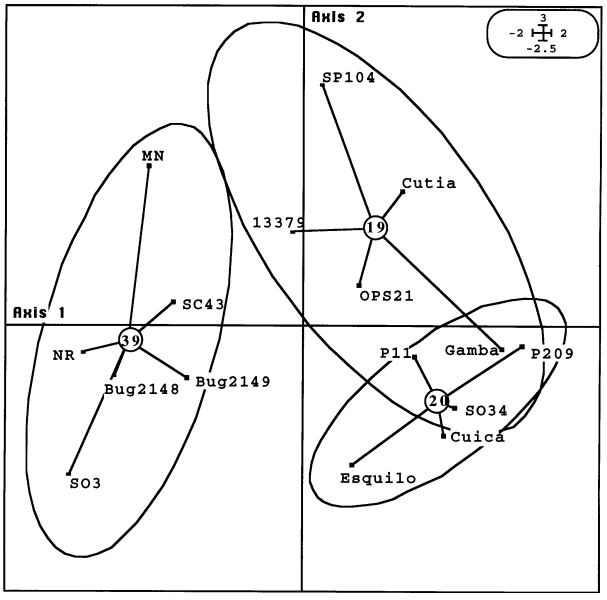

Fig. 2. Diagrammatic illustration of the discriminant analysis of 7 biological parameters on the 2 first discriminant axes.

dealing with a broader range of stocks and additional biological parameters, including cellular infection and *in vitro* drug sensitivity (Revollo, 1995), and is in agreement with the working hypothesis of the present study, that long-term clonal evolution has an important impact of the biological diversity of *T. cruzi*.

It should be noticed that this result corroborates the former work by Andrade, Brodskyn & Andrade (1983), although this last study was based on a phenetic analysis of *T. cruzi* isoenzyme variability rather than interpretation in terms of phylogenetic diversity.

An immediate recommendation which comes from the result of the present work is that *T. cruzi* genetic diversity should be taken into account in any applied study dealing with this parasite, at least in a negative way: for example, when assaying a new vaccine candidate or a new anti-chagasic drug, it appears indispensable to perform a control trial on a set of cloned stocks representative of the whole phylogenetic variability of *T. cruzi*.

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