Detection of linkage disequilibrium in *Trypanosoma brucei* isolated from tsetse flies and characterized by RAPD analysis and isoenzymes

J. R. STEVENS* *and* M. TIBAYRENC

UMR CNRS/ORSTOM 9926 : Génétique Moléculaire des Parasites et des Insectes Vecteurs, ORSTOM, 911 Avenue Agropolis, BP 5045, 34032, Montpellier, France

(*Received 20 April 1994 ; revised 25 July 1994 ; accepted 31 July 1994*)

SUMMARY

This study analyses the different populations of *Trypanosoma brucei* spp. which may coexist within the midgut of wild tsetse flies (Stevens *et al.* 1994). Cloned trypanosome populations characterized by multilocus enzyme electrophoresis (MLEE) were further analysed by the random amplified polymorphic DNA (RAPD) technique, allowing detection of genetic variation at a finer level than that possible by MLEE. Genetic distance matrices derived from the results of each of the two biochemical methods were calculated and compared using a computer program based on the method of Mantel (1967). The observed correlation was used to investigate the degree of linkage disequilibrium (LD) in the data, association between unrelated polymorphic markers providing a measure of the departure from panmixia. The potential of each biochemical method to detect linkage was evaluated by an extended Mantel test. The MLEE/RAPD correlation test evidenced significant LD within the population, suggesting a predominantly clonal method of reproduction for these West African trypanosomes. Analysis of RAPD data by the extended Mantel test also showed significant LD, while the results with MLEE data were less conclusive, providing an indication of the relative potential of the two techniques to detect fine genetic variation.

Key words: *Trypanosoma brucei*, random amplified polymorphic DNA, isoenzymes, linkage disequilibrium, population structure.

INTRODUCTION

Genetic variation in cloned populations of *Trypanosoma brucei* spp. from individual tsetse flies (*Glossina palpalis palpalis*) from Côte d'Ivoire has been described by Stevens *et al.* (1994). In previous studies of genetic variation in *T. brucei* isolated from tsetse (Letch, 1984; Godfrey *et al.* 1990) – the probable site of genetic exchange for trypanosomes (Jenni *et al.* 1986) – primary isolates were grown in mice prior to cloning. Thus certain populations less well adapted for growth in laboratory rodents may have been undetected or lost (Schütt & Mehlitz, 1981).

In a recent study of *T. cruzi*, Tibayrenc *et al.* (1993) examined the degree of correspondence between 2 independent biochemical methods: multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990). Results were used as the basis for an investigation of the population structure and the mode of propagation in the parasite.

The current study extends the approach of Tibayrenc *et al.* (1993) to analyse the genetic structure of cloned populations of *T. brucei* naturally present in tsetse flies (Stevens *et al.* 1994), trypanosome populations studied by MLEE being further characterized by RAPD analysis. Genetic distance matrices based on each of the 2 biochemical methods were calculated and compared using the method of Mantel (1967). The correlation observed was used to investigate linkage disequilibrium (LD) between the populations. The potential of each biochemical method to detect linkage was also evaluated using a new extended Mantel test.

* Reprint requests to: Dr J. R. Stevens, School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK.

MATERIALS AND METHODS

Trypanosomes

The origins of the 3 stocks (GPAP/CI/89/KP 13, GPAP/CI/89/KP 14, GPAP/CI/89/KP 33) and the clones made from them have been described by Stevens *et al.* (1994). Briefly, trypanosomes were isolated from wild tsetse flies (*Glossina palpalis palpalis*) trapped in Côte d'Ivoire; isolates were collected by the research teams of Professor D. Mehlitz and Dr I. Maudlin. A series of clones was then made directly from laboratory midgut isolates; this prevented the possible loss of less virulent

Table 1. RAPD amplified DNA fragment data scored from Polaroid photographs of ethidium bromide-stained gels

(All amplified fragments, up to 20 for certain primers, are identified by a unique code letter (A–T) for each of the 8 primers; only reproducible fragments between 0·3 and 2·7 kb were scored. The coded data were input directly to the distance matrix and linkage disequilibrium computer programs.)

	Operon 10-mer primer							
Stock	OPA-01	OPA-02	OPA-04	OPA-05	OPA-07	OPA-08	OPA-09	OPA-10
KP14 par.	CDEFGHJKMNOP	CGJLPRST	ADGKLOPQR	ADFGO	ABDEHK	ADEFGJ	CFGIJK	BDEGK
KP14 cl.1	CDEFGHJLMNOP	ACGJLNT	BJOPQRT	ADFGOS	ABDEGHIK	ADEFGJ	BDGIJK	BDEFGK
KP14 cl.2	CDEFGHJKMNOP	CGJLPRST	ADGKLOPQR	ADFGO	ABDEHK	ADEFGJ	ACFGIJK	BDEGK
KP14 cl.3	CDEFGHJKMNOP	CGJLPRST	ADGJKLOPQR	ADFGO	ABDEHK	ADEFGJ	ACFGIJK	BDEGK
KP14 cl.4	CDEFGHJKMNOP	ACDGJLNPRST	BDJOPQRT	ADFGIO	ABDEHK	ADEFGJ	ACFGIJK	BDEGK
KP13 par.,2,4	ADEFGJKMNOP	CGJLMQRST	ADFILOPQ	ADFGKLQ	ACEHK	DEFGJ	ACEFGIJK	BDEGK
KP13 cl.1	ADEFGJKMNOP	CGJLMQRST	ADFILOPQ	ACDFGKLQ	ACEHK	DEFGJ	ACEFGIJK	BDEGK
KP13 cl.3	ADEFGJKMNOP	GJLMQRST	ADFILOPQ	ADFGKLQ	ACEHK	DEFGJK	ACEFGIJK	BDEGK
KP33 par.	DEFKMNOP	ADGJLMNOT	BIJOQRT	ADFGKLOPQ	AHK	ABFGIK	CEIJK	DEGK
KP33 cl.1	DFGIJMNOP	BEFK	CEHNOS	BEFGHPR	FJ	CDFGIJKL	EFH	ABCDEGHIK
KP33 cl.2	DEFJKMNOP	GJLMQRST	ADFGILOPQR	ADFGKLOP	ADEHK	ADEFGJ	AFGIJK	BDEGJK
KP33 cl.3	ADEFGJMNOP	CGJLMQRST	AFIKLOQ	ADFGKMPQ	AEHK	DEFGJ	ACEFGIJK	BDEGK
KP33 cl.4	DEFJKMNOP	ADGHJNOT	BJOQRT	ADFGIKLOP	AEHK	AEFGJK	AEFGIJK	BDEGJK

trypanosome strains from mixed infections in culture. Clones were then grown *in vitro* as procyclic forms, prior to characterization. After harvesting, water-soluble enzymes and DNA were separated. All populations, parental and cloned, were confirmed as *T. brucei* using DNA polymerase chain reaction (PCR) primers (Masiga *et al.* 1992; Stevens *et al.* 1994).

Fifteen of the sample populations characterized by MLEE (Stevens *et al.* 1994) were analysed by RAPD PCR (Table 1). For each of the 3 primary isolates these comprised the uncloned parental population and 4 clones. Cloned populations 1–4 were selected independently of enzymic variation for each primary isolate.

DNA preparation

Total DNA was prepared from lysed trypanosomes by treating with proteinase-K, phenol extraction, chloroform/isoamyl alcohol and ethanol precipitation, according to standard methods (Van der Ploeg *et al.* 1982).

RAPD analysis

RAPD analysis was performed according to the protocol of Williams *et al.* (1990), using TEBU *Taq* polymerase (TEBU Corporation, France). Eight 10-mer primers (Operon kit A, Primers: 1, 2, 4, 5, 7, 8, 9, 10; Operon Technologies, USA), which had previously been shown to be suitable for the characterization of parasitic protozoa (Tibayrenc *et al.* 1993), were used. All RAPD PCR were performed with a Techne PHC-2 thermal cycler. The resulting PCR-amplified DNA fragments were separated in 1·2% agarose gels at 150 V for 4 h. Fragments were viewed under UV light after staining with ethidium bromide. Gels were photographed for scoring and numerical analysis. The presence of some faint bands complicated the scoring procedure and necessitated repeat amplification of certain samples; only bands reproducible between amplifications were scored (Graham *et al.* 1994).

Enzyme range

Electrophoresis was carried out on cellulose acetate plates (CAE) (Ben Abderrazak *et al.* 1993). Fifteen enzyme systems were examined as described by Stevens *et al.* (1994): EC 1.6.-.-, diaphorase (DIA); EC 1.1.1.10, threonine dehydrogenase (TDH); EC 1.1.1.37, malate dehydrogenase (MDH); EC 1.1.1.40, 'malic' enzyme (ME); EC 1.1.1.42, isocitrate dehydrogenase (ICD); EC 1.1.1.44, phosphogluconate dehydrogenase (6PGD); EC 1.1.1.49, glucose-6-phosphate dehydrogenase (G6PDH); EC 1.2.1.12, glyceraldehyde-3-phosphate dehydrogenase (GAPDH);

EC 2.6.1.1, aspartate aminotransferase (ASAT); EC 2.7.5.1, phosphoglucomutase (PGM); EC 3.2.2.1, two nucleoside hydrolases using different substrates; the enzyme utilizing inosine was coded NHI, and that utilizing deoxyinosine, NHD; EC 3.4.11.-. or .13.-, leucine aminopeptidase (LAP); EC 3.4.11.-. or .13.-, peptidase, substrate: L-leucyl-L-alanine (PEP_B); EC 5.3.1.9, glucose phosphate isomerase (GPI).

Interpretation of biochemical data

Analysis was based on a total of 17 putative loci from MLEE and 8 RAPD primers. Reproducible RAPD fragments and isoenzyme bands detected by electrophoresis were scored in the same manner, presence or absence, for the calculation of a distance measure; distance analyses were based on Jaccard's method (1908). For RAPD patterns, each gel band was coded with a letter from A to T (Table 1); isoenzymes were similarly coded. Similarity values were calculated using all bands for each primer/enzyme, between each pair of populations. Distance matrices were produced using a program developed by Stevens & Cibulskis (1990); dendrograms were derived from the matrices by the unweighted pair-group method using arithmetic averages (UPGMA; Sokal & Michener, 1958).

Numerical analyses: correlation between markers

Association between unrelated polymorphic markers constitutes a striking case of linkage disequilibrium, and hence suggests that the population under study may not be undergoing random mating. Correlation between isoenzymes and kDNA RFLP (schizodeme analysis) has been taken as additional evidence for clonal propagation in *T. cruzi* by Tibayrenc & Ayala (1987). In the present study, the correspondence between the two observed distance matrices (RAPD and MLEE) was evaluated using the approach of Tibayrenc *et al.* (1993) (Fig. 1), whereby the distance between each pair of populations contributes to the calculation of an overall correlation coefficient. Such a situation is analogous to the comparison of environmental distance matrices as described by Mantel (1967). Accordingly, a significance value was derived using a computer program to perform a Mantel test; the program was written in Fortran 77 and was designed to run on a PC.

In brief, by random reassortment of the observed genotypes, the Mantel test allows a significance value to be attached to a correlation coefficient calculated between two sets of genetic distances in cases where the number of degrees of freedom cannot be readily calculated. Since in genetic distance matrices many distances are calculated with respect to single populations, the cells of such matrices are not independent and the usual test for a significant correlation is therefore inappropriate. Manly (1985) discusses two approaches for deriving a significance value for an r value produced by a Mantel test when the number of individuals being compared is too large for all permutations of the distance matrices to be calculated. Either the mean and variance of the randomized distribution of r values can be calculated and can be treated as a standard normal deviate, or a large number of randomized matrices can be computer generated and the resulting distribution of r values used in place of the true randomized distribution. This latter, Monte Carlo approach has the major advantage of being unaffected by the shape of the distribution of r values which may become highly skewed, particularly as sample (matrix) size decreases; consequently, this approach was used in the current study.

Numerical analysis: linkage within markers

Linkage disequilibrium in *T. brucei* was studied further using a modified version of the above Mantel program to investigate linkage *within* a data set. In this method a single data set is considered, and the loci (primers or enzymes) are shared into two groups at random. The analysis then proceeds as before; firstly, genetic distance matrices are calculated, one relating to each group of loci. A correlation coefficient is then calculated between the two groups of distances for this, the observed arrangement of genotypes. Next the genotypes are reassorted a given number of times, a new correlation coefficient being calculated for each reassortment. Following the required number of genotype reassortments, a probability value can be attached to the observed correlation coefficient. The process then begins again; the loci are once more randomly redistributed into two groups, the observed correlation coefficient is calculated, and so forth. When the number of loci is small all combinations may be evaluated; however, with large numbers of loci, as for this study, a random sample of reshuffles is performed.

RESULTS

Zymodemes and RAPD electrophoretic types

The complete set of isoenzyme results has been presented by Stevens *et al.* (1994). The sample populations included in the current analysis of correlation between independent markers were classified into 8 zymodemes based on 17 loci and 13 RAPD electrophoretic types (ETs) using 8 primers.

Three of the 15 sample populations were identical by both MLEE and RAPD, thus correlations between the 2 sets of markers were calculated between distance matrices of 13 ETs. Populations identical by both MLEE and RAPD were assumed to be the product of repeat sampling of one particular

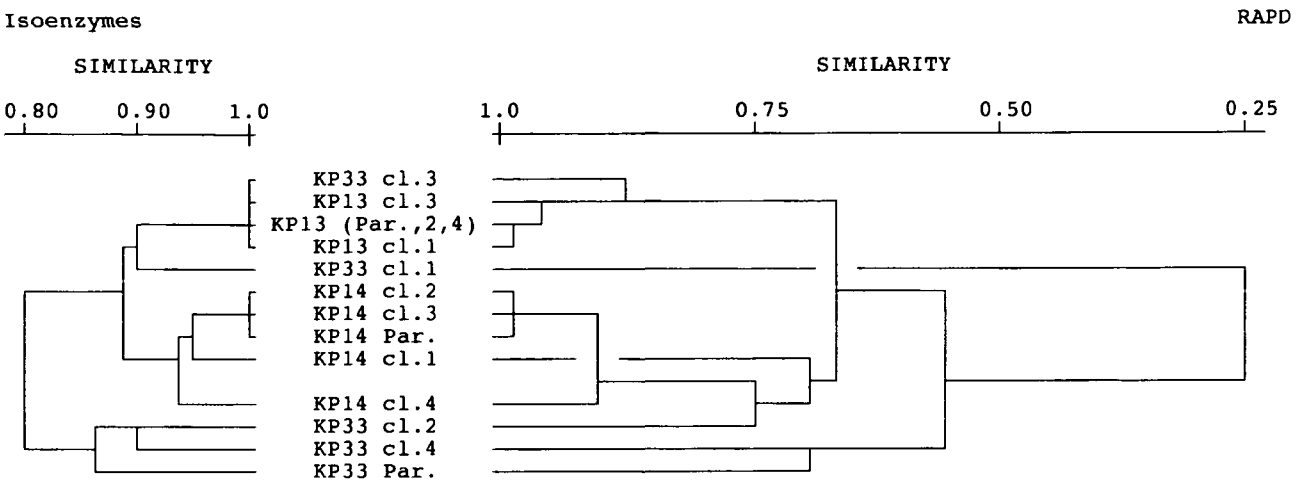

Fig. 1. The observed dendrograms calculated using data from MLEE and RAPD analysis of 13 ETs (15 populations) of *Trypanosoma brucei*. Association between unrelated polymorphic markers provides evidence of linkage disequilibrium; the correspondence between the 2 observed distance matrices was evaluated using a computer program to perform a Mantel test, producing a correlation of $r = 0{\cdot}4993$, significance $P < 0{\cdot}0005$.

population within the primary isolate. Moreover, the consistency with which small data sets yield results in agreement with a hypothesis of random mating (Cibulskis, 1988) enhances the significance of any departures from panmixia detected in the current data set.

The observed dendrograms calculated using data from MLEE and RAPD analysis of the 15 populations (13 ETs) are presented in Fig. 1.

Numerical analysis : correlation between markers

Comparison of the observed similarity matrices, from RAPD analysis and MLEE, produced a correlation of $r = 0{\cdot}4993$. This value was significant, with a probability of $P < 0{\cdot}0005$ (5000 Monte Carlo reassortments). Such a result indicates a strong correspondence in the relationships defined between trypanosome populations by the 2 biochemical methods.

Numerical analysis : linkage within isoenzyme data

The presence of linkage within the isoenzyme data set was tested using (*a*) the 13 populations used in the MLEE/RAPD analysis, and (*b*) the 29 cloned populations analysed by Stevens *et al.* (1994). In both analyses, 10 random combinations of 2 groups of loci (9 and 8 loci; 2000 repeats of each combination) showed significant linkage ($P < 0{\cdot}01$) in 70% of combinations.

Numerical analysis : linkage within RAPD data

Analysis of RAPD data was performed for the 13 populations used in the MLEE/RAPD analysis. Ten random combinations of 2 groups of primers (4 primers/group; 2000 repeats of each combination) showed significant linkage ($P < 0{\cdot}001$) in 100% of combinations. Thus random assortment of the genotypes defined by RAPD analysis produced LD more extreme than that evidenced in the observed data only twice out of 2000 reassortments.

DISCUSSION

In many previous characterization studies of *Trypanozoon* parasites (Gibson, Marshall & Godfrey, 1980; Tait, 1980; Godfrey *et al.* 1990; Stevens & Godfrey, 1992) the large majority of isolates were characterized without being cloned, probably leading to a gross underestimate of the true level of genetic variation. The present study together with the work of Stevens *et al.* (1994) provides evidence of the range of genetically different *T. brucei* populations which may coexist within the midgut of individual tsetse flies in nature. In particular, use of RAPD analysis permits the detection of genetic variation at a finer level than that possible by MLEE characterization. For example, while 4 cloned populations and the parental stock of GPAP/CI/89/KP 13 were enzymically identical (Stevens *et al.* 1994), RAPD analysis identified 3 distinct electrophoretic types. Moreover, while the validity of a relatively new technique such as RAPD analysis still remains to be fully established, our results indicate that RAPD fragments are useful as genetic markers. The importance of the existence of such variation to parasite population genetics (Tait, 1980; Tibayrenc, Kjellberg & Ayala, 1990; Cibulskis, 1992; Stevens & Welburn, 1993) remains to be explored, though it now seems probable that many previous characterization studies may have over-simplified the degree of genetic variation present.

The degree of linkage disequilibrium in a given population can be measured by the association between unrelated polymorphic markers; the pres-

ence of a significant degree of LD suggests that the population under study may not be undergoing random mating. In the current study, the highly significant correlation observed between the two independent sets of genetic markers (RAPD and MLEE) suggests the presence of considerable LD. Moreover, as there is no *a priori* reason for expecting that the two sets of genetic markers are in any way linked on the same chromosomes, it is highly unlikely that they are similarly distributed throughout the genome; thus, the degree of correspondence observed between the markers indicates that they are not randomly reassorting as would be the case for a sexually reproducing population, but rather, large parts of the genome appear to be being passed on completely unchanged. Such linkage disequilibrium has provided circumstantial evidence for clonal reproduction in a range of microorganisms (Tibayrenc *et al.* 1990, 1991, 1993; Maynard Smith *et al.* 1993; Pujol *et al.* 1993; Truc & Tibayrenc, 1993).

The significant LD evidenced by analysis of the RAPD data is in good agreement with the high degree of linkage detected by the MLEE/RAPD correlation test. In contrast, while MLEE data showed significant linkage in 70% of the random loci combinations tested, 30% showed insignificant LD. Such a result, however, is not unexpected when the overall level of polymorphism in a population is low, as is the case for the isoenzyme data. In such a situation, the chances to evidence any departure from panmixia diminish proportionally. Similar results have been reported from MLEE studies of other protozoan parasites. For example, a large-scale study of *Leishmania* by Guerrini (1993) identified in some cases little polymorphism within given species, and was thus able to draw only limited conclusions on linkage. Similarly, a study of small samples of *Leishmania* from Peru (A.-L. Bañuls and M. Tibayrenc, unpublished data) identified significant LD by RAPD analysis, while MLEE results were inconclusive. At the extremes of the sexual/clonal spectrum (Tibayrenc *et al.* 1991) in the parasitic protozoa, results are less equivocal. *T. cruzi* shows extreme linkage by both RAPD and MLEE analyses (Tibayrenc *et al.* 1993); in addition to the highly clonal structure reported for *T. cruzi* (Tibayrenc & Ayala, 1988), other possible reasons for the improved ability to investigate linkage are increased sample size and, probably most importantly (see Guerrini, 1993), higher genetic diversity. In contrast, population analyses of *Plasmodium* by MLEE show levels of LD lower than that detected in trypanosomes (Ben Abderrazak, 1993), while by RAPD analysis no significant departures from panmixia are evidenced (D. Walliker, personal communication). Whatever, it should be borne in mind that while, due to limited levels of genetic diversity and small sample sizes, it may sometimes be impossible to reject the null hypothesis (i.e. no linkage), such a rejection does not constitute an *acceptance* of it.

Thus, considering the results of the current study together with the findings of previous researchers, it is apparent that mixed populations of trypanosomes in tsetse are not uncommon. However, while much genetic variation exists the high degree of LD detected indicates that the populations of trypanosomes circulating in tsetse flies are not undergoing random mating. This result is enhanced by the fact that linkage was evidenced by counting stocks sharing the same genotype (as defined by both isoenzymes and RAPD) only once, which decreases the possibility that the results can be explained simply by *epidemic* clonality (see Maynard Smith *et al.* 1993). Rather, the population overall appears to consist of a range of genetic variants, with distinct groups of closely related genotypes present in individual flies, even within the same village. Moreover, even if some genetic exchange is occurring, the population as a whole remains genetically subdivided due to the nature of the disease cycle and, in particular, to the role of the tsetse fly.

In conclusion, the predominant reproductive process in *T. brucei* in this region of West Africa, particularly during epidemic periods, appears to be clonal. Thus, while studies of *T. brucei* in East Africa, notably the Lambwe Valley (Gibson, 1990; Cibulskis, 1992), have reached somewhat different conclusions, the relative impact of sex and clonality in natural populations of *T. brucei* is still a point of debate and may vary between geographical regions and ecological cycles.

We thank Dr B. Oury, Dr B. Basentra and Mr C. Barnabé for technical assistance and all colleagues at ORSTOM, Montpellier for support and encouragement. Primary isolates were kindly provided by the research teams of Professor D. Mehlitz, Free University of Berlin and Dr I. Maudlin, Tsetse Research Laboratory, Bristol. This study received financial support from the Ministère Français des Affaires Etrangères, WHO TDR EPiAF Grant No.s 910020 and 920172, EC 'Science' Grant No. SCI*CT 92-0761 and British/French 'Alliance' Grant No. 91202.

REFERENCES

BEN ABDERRAZAK, S. (1993). Variabilité génétique des populations de *Plasmodium falciparum*. Thèse de Doctorat d'Université, Université des Sciences et Techniques du Languedoc, Montpellier II.

BEN ABDERRAZAK, S., GUERRINI, F., MATHIEU-DAUDÉ, F., TRUC, P., NEUBAUER, K., LEWICKA, K., BARNABÉ, C. & TIBAYRENC, M. (1993). Isoenzyme electrophoresis for parasite characterization. In *Methods in Molecular Biology, Vol 21 : Protocols in Molecular Parasitology* (ed. Hyde, J. E.), pp. 361–382. Totowa, NJ: Humana Press Inc.

CIBULSKIS, R. E. (1988). Origins and organization of genetic diversity in natural populations of *Trypanosoma brucei*. *Parasitology* **96**, 303–22.

CIBULSKIS, R. E. (1992). Genetic variation in *Trypanosoma brucei* and the epidemiology of sleeping sickness in the Lambwe Valley, Kenya. *Parasitology* **104**, 99–109.

GIBSON, W. (1990). Trypanosome diversity in Lambwe Valley, Kenya – sex or selection? *Parasitology Today* **6**, 342–3.

GIBSON, W. C., MARSHALL, T. F. DE C. & GODFREY, D. G. (1980). Numerical analysis of enzyme polymorphism: a new approach to the epidemiology of trypanosomes of the subgenus *Trypanozoon*. *Advances in Parasitology* **18**, 175–246.

GODFREY, D. G., BAKER, R. D., RICKMAN, L. R. & MEHLITZ, D. (1990). The distribution, relationships and identification of enzymic variants within the subgenus *Trypanozoon*. *Advances in Parasitology* **29**, 1–74.

GRAHAM, J., MCNICOL, R. J., GREIG, K. & VAN DE VEN, W. T. G. (1994). Identification of red raspberry cultivars and an assessment of their relatedness using fingerprints produced by random primers. *Journal of Horticultural Science* **69**, 123–30.

GUERRINI, F. (1993). Génétique des populations et phylogénie des *Leishmania* du Nouveau-Monde. Thèse ès Sciences, Université des Sciences et Techniques du Languedoc, Montpellier II.

JACCARD, P. (1908). Nouvelles recherches sur la distribution florale. *Bulletin de la Société Vaudoise de Science Naturelle* **44**, 223–70.

JENNI, L., MARTI, S., SCHWEIZER, J., BETSCHART, B., LE PAGE, R. W. F., WELLS, J. M., TAIT, A., PAINDAVOINE, P., PAYS, E. & STEINERT, M. (1986). Hybrid formation between African trypanosomes during cyclical transmission. *Nature, London* **322**, 173–5.

LETCH, C. A. (1984). A mixed population of *Trypanozoon* in *Glossina palpalis palpalis* from Ivory Coast. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **78**, 627–30.

MANLY, B. F. J. (1985). *The Statistics of Natural Selection on Animal Populations*. London: Chapman and Hall.

MANTEL, N. (1967). The detection of disease clustering and a generalised regression approach. *Cancer Research* **27**, 209–20.

MASIGA, D. K., SMYTH, A. J., HAYES, P., BROMIDGE, T. J. & GIBSON, W. C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* **22**, 909–18.

MAYNARD SMITH, J., SMITH, N. H., O'ROURKE, M. & SPRATT, B. G. (1993). How clonal are bacteria? *Proceedings of the National Academy of Sciences, USA* **90**, 4384–8.

PUJOL, C., REYNES, J., RENAUD, F., RAYMOND, M., TIBAYRENC, M., AYALA, F. J., JANBON, F., MALLIE, M. & BASTIDE, J.-M. (1993). The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected HIV+patients. *Proceedings of the National Academy of Sciences, USA* **90**, 9456–9.

SCHÜTT, I. D. & MEHLITZ, D. (1981). On the persistence of human serum resistance and isoenzyme patterns of *Trypanozoon* clones in experimentally infected pigs. *Acta Tropica* **38**, 367–73.

SOKAL, R. R. & MICHENER, C. D. (1958). A statistical method for evaluating systematic relationships. *University of Kansas Scientific Bulletin* **38**, 1409–38.

STEVENS, J. R. & CIBULSKIS, R. E. (1990). Analysing isoenzyme band patterns using similarity coefficients: a personal computer program. *Computer Methods and Programs in Biomedicine* **33**, 205–12.

STEVENS, J. R. & GODFREY, D. G. (1992). Numerical taxonomy of *Trypanozoon* based on polymorphisms in a reduced range of enzymes. *Parasitology* **104**, 75–86.

STEVENS, J. R., MATHIEU-DAUDÉ, F., MCNAMARA, J. J., MIZEN, V. H. & NZILA, A. (1994). Mixed populations of *Trypanosoma brucei* in wild *Glossina palpalis palpalis*. *Tropical Medicine and Parasitology* **45** (in the Press).

STEVENS, J. R. & WELBURN, S. C. (1993). Genetic processes within an epidemic of sleeping sickness in Uganda. *Parasitology Research* **79**, 421–7.

TAIT, A. (1980). Evidence for diploidy and mating in trypanosomes. *Nature, London* **287**, 536–8.

TIBAYRENC, M. & AYALA, F. J. (1987). Forte corrélation entre classification isoenzymatique et variabilité de l'ADN kinétoplastique chez *Trypanosoma cruzi*. *Comptes-Rendus de l'Académie des Sciences, Paris* **304**, 89–92.

TIBAYRENC, M. & AYALA, F. J. (1988). Isozyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical and epidemiological significance. *Evolution* **42**, 277–92.

TIBAYRENC, M., KJELLBERG, F., ARNAUD, J., OURY, B., BRENIÈRE, S. F., DARDE, M.-L. & AYALA, F. J. (1991). Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. *Proceedings of the National Academy of Sciences, USA* **88**, 5129–33.

TIBAYRENC, M., KJELLBERG, F. & AYALA, F. J. (1990). A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences, USA* **87**, 2414–18.

TIBAYRENC, M., NEUBAUER, K., BARNABÉ, C., GUERRINI, F., SKARECKY, D. & AYALA, F. J. (1993). Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proceedings of the National Academy of Sciences, USA* **90**, 1335–9.

TRUC, P. & TIBAYRENC, M. (1993). Population genetics of *Trypanosoma brucei* in Central Africa: taxonomic and epidemiological significance. *Parasitology* **106**, 137–49.

VAN DER PLOEG, L. H. T., BERNARDS, A., RIJSEWIJK, F. & BORST, P. (1982). Characterization of the DNA duplication–transposition that controls the expression of two genes for variant surface glycoproteins in *Trypanosoma brucei*. *Nucleic Acids Research* **10**, 593–609.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFOLSKI, J. A. & TINGEY, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531–5.