The detection of geographical substructuring of *Trypanosoma brucei* populations by the analysis of minisatellite polymorphisms

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SUMMARY

Analysis of natural populations of *Trypanosoma brucei* has shown that there is linkage disequilibrium between alleles at pairs of loci in isolates taken from the field. This disequilibrium can occur as a result of a low frequency of genetic exchange, the masking of frequent genetic exchange by the rapid expansion of a few genotypes or by the treatment of 2 (or more) genetically isolated populations as a single population. We have analysed stocks from 2 geographically separate locations using 3 minisatellite markers to determine the frequencies of the alleles in each area and the frequency and nature of the multilocus genotypes. The results show that many alleles and multilocus genotypes are unique to each geographical location, supporting the conclusion that these populations are genetically isolated with limited or no gene flow between them. This geographical substructuring needs to be taken into account in considering the origins of the linkage disequilibrium in a number of populations.

Key words: *Trypanosoma brucei*, minisatellites, population genetics.

INTRODUCTION

The analysis of the role of genetic exchange in generating diversity within natural populations is important in understanding the population genetic structure of African trypanosomes as well as having implications at a more practical level, in terms of the spread of drug resistance, the epidemiology of the disease and the identification of the source of outbreaks of human disease. The role of genetic exchange in natural populations is controversial, with arguments being presented for a clonal population structure (Tibayrenc, Kjellberg & Ayala, 1990), an epidemic structure where the rapid expansion of a few multilocus genotypes masks underlying frequent genetic exchange (Maynard Smith *et al*. 1993) and a panmictic population structure (Tait, 1980) where mating is frequent. One of the key arguments for both clonal and epidemic population structures is the observation of linkage disequilibrium between alleles at pairs of unlinked loci when the frequency of different genotypes is analysed in samples from a single population. The cause of such linkage disequilibrium can be due to an absence of frequent genetic exchange, the rapid expansion of a few genotypes as in an epidemic or the treatment of 2 (or more) genetically isolated populations as a single population (Maynard Smith *et al*. 1993).

Relatively limited research has been undertaken to examine whether trypanosome populations are genetically substructured and yet this could be one of the reasons for linkage disequilibrium. As *T. brucei* infects several species of hosts and is distributed over a wide geographical range, it is possible that natural populations are substructured in terms of host and geography. A detailed analysis of the population structure, using iso-enzyme data from a large number of stocks from the Lambwe Valley in Kenya, has been undertaken (Gibson & Welde, 1985) and it was shown (Cibulskis, 1992) that the frequency of different zymodemes (equivalent to multilocus enzyme electrophoretic types-MLEE) is distinct between hosts (man and cattle) as has also been observed using repetitive DNA analysis in stocks from Busoga, Uganda (Hide *et al*. 1994). These results suggest that there are distinct populations in different host species. The analysis of further stocks from the Lambwe Valley by isoenzyme electrophoresis has shown that particular clusters of zymodemes are associated with particular hosts (Mihok, Otieno & Darj, 1990). In addition to this evidence for host-determined population substructuring, analysis of the data from Lambwe Valley (Cibulskis, 1992) also showed differences in zymodeme frequency between different villages within the Lambwe Valley suggesting that geographical

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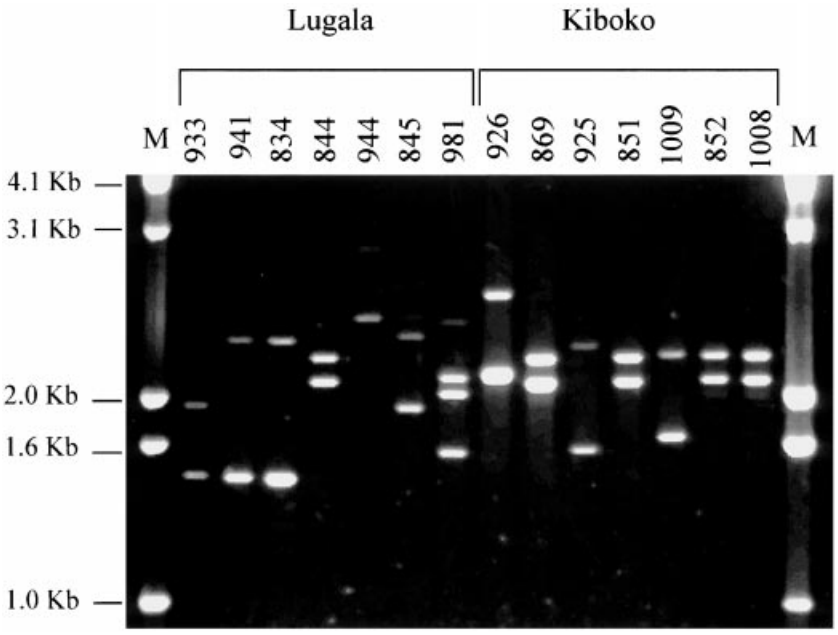

Fig. 1. Amplification by PCR of a range of *CRAM* alleles from *T. brucei* stocks from Kiboko and Lugala. The PCR conditions have been described previously (MacLeod *et al*. 1999). PCR products were separated on a 1% Seakem agarose gel, stained with ethidium bromide and visualized by ultraviolet transillumination. The stock number is given for each lane. Lane M, lambda *Hin*dIII markers (Advanced Biotechnologies).

substructuring of *T. brucei* populations also occurs. However, it was difficult to disentangle the effects of substructuring due to host and geography in that study.

Based on considerations of the distances that tsetse could travel (Leak, 1998), it seems likely that geographical substructuring of populations could occur and indeed, differences in MLEE patterns have been observed between stocks from different regions (Godfrey *et al*. 1990). However, the population genetic analysis of such substructuring – the Wahlund effect (Tibayrenc, 1999) has not been tested formally. With the availability of highly polymorphic markers such as minisatellites (MacLeod, Turner & Tait, 1999), it is possible to address such questions more readily and so determine whether treating geographically separate populations as a single population could lead to linkage disequilibrium. To address the question of substructuring due to the effects of geography, we have analysed 2 populations of trypanosome stocks from the same type of host (tsetse) isolated at the same time from 2 areas in East Africa separated by some 400 km. We have used 3 recently described hypervariable minisatellite markers (MacLeod *et al*. 2000) for this analysis as they provide a level of variation that allows differences to be detected with a greater sensitivity than that obtained by iso-enzyme analysis.

MATERIALS AND METHODS

Parasite stocks

A total of 32 isolates from the salivary glands of tsetse flies caught in either Kiboko, Kenya or Lugala, Uganda by Goebloed *et al*. 1973 in 1969–1970 were analysed. All isolates were from *Glossina pallidipes* except 1 (L941) from *G. fuscipes*. These stocks were subsequently passaged 3–10 times in mice before transfer to our laboratory. The details of these stocks and the derivation of clones from them has been described previously (MacLeod *et al*. 1999; MacLeod *et al*. 2001). A total of 21 stocks from Kiboko and 11 stocks from Lugala were analysed.

Preparation of crude lysates and DNA

Lysates derived from infected mouse blood or purified DNA were prepared from each stock as described in detail previously (MacLeod *et al*. 1999).

PCR-based genotyping with minisatellite markers

Three minisatellite markers (*MS42, 292, CRAM*) were scored for each stock primarily using differences in repeat length determined by migration on agarose gels; the nomenclature is based on the number of repeats per allele as previously described (MacLeod *et al*. 1999, 2000). For the *CRAM* and *292* markers, alleles were identified as follows: the allele size was determined on the basis of mobilities relative to a reference standard run on the same agarose gel. Repeated measurements of the size of the same allele were used to calculate a mean size and standard deviation with 2 standard deviations corresponding to 2·3% of the estimated allele size. This measurement was then taken as the window size for each band and alleles were considered identical if

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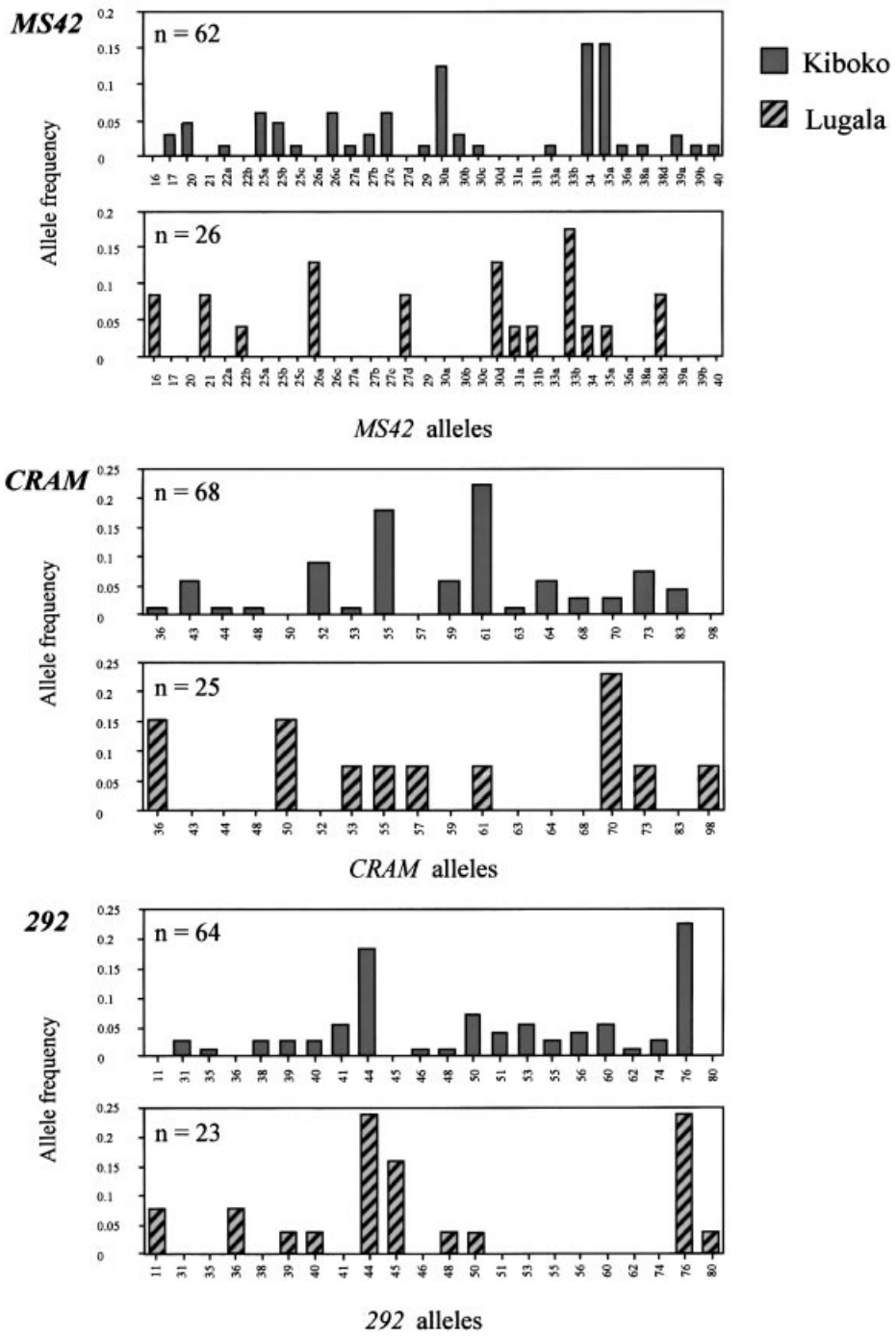

Fig. 2. The frequency of *MS42, CRAM* and *292* alleles in the Kiboko and Lugala populations. *n*, Number of alleles, from 24 Kiboko and 11 Lugala stocks.

they were within this window. For alleles of the *MS42* minisatellite, variation in the sequence of the repeat array as well as the size variation in alleles was used to define different alleles. Minisatellite variant repeat mapping (Jeffreys *et al.* 1991) was used to determine this variation in repeat structure using oligonucleotides specific to each of the 2 common sequence variants in the repeats (MacLeod *et al.* 2001). The nomenclature used to designate the *MS42* alleles is based on the number of repeat units within an allele and a letter designating the variant repeat structure e.g. alleles 30a and 30c indicate 2 alleles with 30 repeat units which differ in their repeat structure while alleles with a different number indicate a different number of repeat units. The repeat unit sequence is 3′ ragacggaaccgaccaagtccc-tgacggtgaccaaagtggtca 5′ where r can either be a or g.

Allele and multilocus genotype frequencies

A number of the uncloned stocks from both Kiboko and Lugala contained more than 2 alleles per locus and this has been shown to be due to the stocks containing more than 1 trypanosome genotype

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Table 1. The number of different (and unique) alleles detected in each population is given for minisatellites, *MS42, CRAM* and *292*

	No. of different alleles (No. of alleles unique to each population)		
Population	*MS42*	*CRAM*	*292*
Lugala	12(10)	9(3)	10(4)
Kiboko	22(20)	15(9)	19(12)

(MacLeod *et al*. 1999). Seventeen of the Kiboko stocks have been cloned but only 1 of the Lugala stocks. For the determination of the multilocus genotype frequencies only stocks showing 2 alleles or cloned stocks were used for analysis. For allele frequency determinations, uncloned stocks were used except where a cloned stock differed in genotype from its uncloned progenitor stock. In such rare cases, the uncloned and cloned stocks were treated as 2 samples rather than 1. The reasons why such results might come about have been described previously (MacLeod *et al*. 1999). The number of stocks, defined in this way, on which each data set is based is indicated in the figure legends.

The genetic distance between the Lugala and Kiboko populations was measured using Nei's genetic distance (Nei, 1972) and the Index of Fixation, F_{ST} (Hartl & Clark, 1997).

RESULTS

To establish whether geographical population substructuring occurs in *T. brucei*, stocks were analysed from the same host type (tsetse vector) derived at the same time (1969/70) from Lugala (Uganda) and Kiboko (Kenya) for variation at 3 minisatellite loci (*MS42, 292* and *CRAM*). A sample of PCR products of the *CRAM* locus for each stock is shown in Fig. 1 and the data on the frequency of the alleles at each of the 3 loci, is illustrated in Fig. 2, together with the total number of alleles sampled at each locus. Only 2 alleles at the *MS42* locus (alleles 34 and 35a) out of the 32 distinct alleles are common between the two populations and both of these differ substantially in frequency. At the *CRAM* locus, 18 distinct alleles have been identified of which the two populations share 6, with 5 of these differing substantially in frequency. Similarly, out of 22 alleles at the *292* locus only 6 are common between the populations (summarized in Table 1). It is clear that each locus is highly polymorphic thus providing a high level of discrimination between the populations. The distinction between the populations is also apparent when the 3 most frequent alleles at each locus are considered (Fig. 2). Thus, at the *MS42* locus, alleles 30a, 34 and 35a are most frequent in Kiboko but alleles 33b, 30d and 26a are the most frequent in Lugala. Similarly for the *CRAM* locus, the 3 most frequent Kiboko alleles (61, 55 and 52) are distinct from the 3 most frequent alleles (70, 36 and 50) in Lugala. The differences at the *292* locus are not as clear cut as the 2 most frequent alleles (76 and 44) are common to both populations although they are completely distinct for the third most frequent allele. As the sample size of stocks is relatively small, it is critical to assess whether the apparent differences between the two populations could have occurred by chance. The relatively large number of alleles (> 60) sampled in the Kiboko population argues against this, as the most frequent alleles at the *MS42* locus in Lugala (26a, 30d and 33b) would be predicted to be seen at least once if the Lugala and Kiboko stocks were samples of the same homogeneous population. A pairwise measure of genetic distance (Nei, 1972) was calculated using these data and a value of 0·125 obtained, suggesting that these are genetically distinct populations.

To examine these differences in more detail, the multilocus genotype of each stock was determined using the data for each minisatellite marker and only those stocks that were either cloned or were uncloned stocks with only 2 alleles i.e. eliminating uncloned stocks which were mixtures of different genotypes. This approach identified 18 distinct multilocus genotypes by the designation of each allele at the 3 loci. The frequency of each multilocus genotype is illustrated diagrammatically in Fig. 3. A table of multilocus genotypes is available on request. Inspection of Fig. 3 shows that while both populations share 1 quite frequent genotype (M21), they share none of the remaining 17 genotypes. The Kiboko population also differs in that there are a considerable number of low frequency ($< 0{\cdot}1$) distinct genotypes (13 in total) while the Lugala population only contains 4 distinct multilocus genotypes each occurring at a significant frequency. This observation may solely reflect the small sample size in the latter population as inspection of the uncloned mixed genotype stocks shows several alleles at each locus that are not represented in the cloned stocks (MacLeod *et al*. 1999) suggesting that many more multilocus genotypes exist in this population. The conclusion from this analysis is that, as with the loci considered separately, the two populations are genetically distinct with only 1 multilocus genotype out of 18 in common. The amount of inter-population genetic variation can be assessed by estimating the fixation index, F_{ST}, which defines the amount of genetic difference between subpopulations, with a value of zero indicating no divergence and positive deviation from zero indicating the extent of genetic difference. An estimate of F_{ST} was calculated for the Lugala and Kiboko populations and a value of 0·054 obtained. This value indicates

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Table 2. Minisatellite and multilocus genotypes of *Trypanosoma brucei* stocks

(The first column indicates the stock number of cloned (cl) and uncloned (un) stocks. K, Kiboko; L, Lugala, while the second column indicates the year in which each sample was isolated. The third column presents the genotype of each stock for the marker *MS42* based on the number of repeats and on MVR internal maps of each allele (MacLeod *et al*. 2001). * Indicates that estimated allele size (the number of repeats) only was used to genotype these stocks for *MS42*. The fourth and fifth columns present the *CRAM* and *292* minisatellite genotypes presented as an estimate of the number of repeats in each allele, respectively. The sixth column shows the multilocus genotype, i.e. the combined results from the 3 minisatellites. Each genotype was assigned an arbitrary number, prefixed M. N.D., Not determined; N.A., not applicable.)

Stocks	Year	Genotypes *MS42*	*CRAM*	*292*	Multilocus
K 1337cl	1969	40/25a	83/83	48/44	M35
K 1008cl	1970	35a/34	61/55	76/44	M21
K1027cl/un	1969	30a/17	61/52	60/50	M36
K 927cl1B	1970	30c/20	68/61	41/50	M37
K 927cl4B	1970	26c/25a	70/68	44/38	M38
K 984cl	1969	30a/27c	73/55	41/50	M39
K 854cl	1969	30a/27c	73/55	41/50	M39
K 975cl	1970	35a/34	61/55	76/44	M21
K 925cl	1969	29/27b	61/53	76/74	M40
K 925un	1969	38a/20	64/43	46/31	M45
K 982cl	1970	35a/34	61/55	76/44	M21
K 853cl	1969	35a/34	61/55	76/44	M21
K1009cl/un	1970	36a/25b	61/44	76/62	M41
K 851cl	1969	35a/34	61/55	76/44	M21
K 851un	1969	30a/17	61/52	60/50	M36
K 852cl	1969	35a/34	61/55	76/44	M21
K 978cl	1970	30a/27c	73/55	51/39	M42
K 978un	1970	26c/25b	63/55	53/40	M46
K 997cl	1969	39a/25b	68/36	76/56	M43
K 258cl	1969	30a/25c	64/43	56/56	M47
K 936un	1969	35a/34	61/55	76/44	M21
K 926un	1969	30a/27c	83/59	51/39	M44
K 869un	1969	35a/34	61/55	76/44	M21
K 994cl/un	1970	35a/34	61/55	76/44	M21
K 981un	1969	39a/33a/30b/26c	73/59/52/43	76/60/53/44	N.A.
K 854un	1969	39b/26c/25a/20	73/70/64/61	51/44/41/38	N.A.
K 927un	1970	35a/34/30c/26c/25a/20	70/68/61/59/52	76/50/44/41/38	N.A.
K 852un	1969	35a/34/30b/27b	64/52	76/74	N.A.
K 984un	1969	39*/33*/30a/27c	73/59/52/43	76/60/53/41/35	N.A.
K 975un	1970	35a/34	61/55	76/44/41/31	N.A.
K 997un	1969	39a/30a/25a	73/61	76/56/55/53	N.A.
K 258un	1969	30*/26*/25c	48/43	76/56/55/40	N.A.
L 834un	1969	38d/27d	70/36	45/36	M23
L 844un	1969	35a/34	61/55	76/44	M21
L 929un	1969	33*/21	N.D.	45/44	N.D.
L 933un	1970	33b/30d	50/36	76/45	M24
L 934un	1970	26a/22b	61/36	80/44	M25
L 941un	1969	38d/27d	70/36	45/36	M23
L 832un	1969	33b/26a	70/55	76/39	M26
L 845un	1970	31b/26a/21/16	98/73/70/50	76/50/44/11	N.A.
L 791un	1969	33b/30d	70/57/53/50	76/44	N.A.
L 836un	1969	33b/30d	70/57/53/50	76/44	N.A.
L 944un	1970	31a/16	98/73	48/40/11	N.A.

moderate genetic differentiation (Wright, 1978) and strongly supports the conclusion that the two populations are likely to be genetically isolated with limited gene flow between them. The only multilocus genotype which was common to both populations, (M21; Fig. 3) has previously been shown to be the genotype associated with human infectivity in human and cattle stocks from the Busoga region in S. E. Uganda (MacLeod *et al*. 2000), which encompasses Lugala.

DISCUSSION

On the basis of the difference in allele type and multilocus genotype, we have shown that geographically separated populations of *T. brucei* isolated at the same time and from the same host type are genetically distinct, providing evidence for a lack of gene flow between these populations. Geographical substructuring has not previously been formally demonstrated and has significant implications for the

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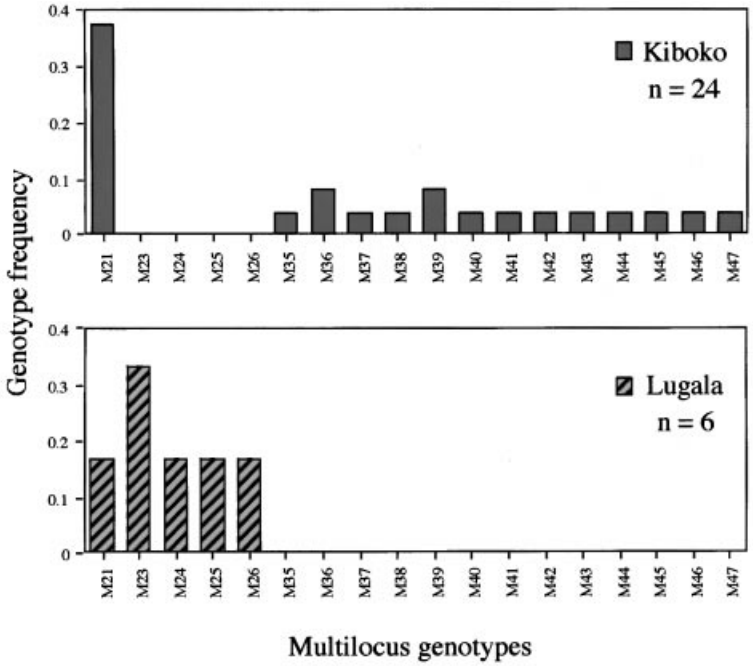

Fig. 3. The frequency of multilocus genotypes in the Kiboko and Lugala populations. *n*, Number of genotypes, from 24 Kiboko and 6 Lugala stocks.

analysis and interpretation of the role of genetic exchange in natural populations because such substructuring would lead to linkage disequilibrium if the populations were combined. Our findings raise the question as to the level of substructuring in trypanosome populations in relation to both host and geography. An analysis of the Kiboko stocks for linkage disequilibrium has been conducted using the Index of Association (Maynard Smith *et al*. 1993); the sample size for the Lugala stocks is too small. This analysis showed that the Kiboko population has an epidemic population structure (data not shown). We propose that this population structure may be due to substructuring in the mammalian hosts as we have shown previously for other populations (MacLeod *et al*. 2000), but can eliminate the possibility that it is an artefact of geographical substructuring.

A key question, based on the conclusions reported here, is whether geographical substructuring could confound the conclusions reached in previous population genetic studies. In the landmark analysis of protozoan population structure by Tibayrenc *et al*. (1990), samples of *T. brucei* from East and West Africa only showed significant linkage disequilibrium when they were combined; our results would support the view that the disequilibrium was created by combining 2 genetically isolated populations. In the analysis of the data of Mihok *et al*. (1990), linkage disequilibrium was demonstrated (Maynard Smith *et al*. 1993) when trypanosome stocks from several hosts were treated as a single population but was insignificant when only 'electrophoretic types' (ETs) were considered. The predominant identical electrophoretic types were isolated from humans and treatment of these as single stocks largely contributed to the reduction of the index of association used as a measure of linkage disequilibrium. If, as has been shown in a sample of human and non-human infective stocks from Busoga in Uganda (MacLeod *et al*. 2000; Hide *et al*. 1994), the population is substructured by host then the removal of the electrophoretic types primarily from 1 host removes the substructuring and thus restores agreement with linkage equilibrium. Using arbitrarily primed PCR, a collection of stocks from East, West and Central Africa has been analysed (Mathieu-Daude *et al*. 1995) and by considering groups of stocks from each of the 3 regions separately, linkage disequilibrium was demonstrated in each group. However, if the geographical origins of the stocks in the East and West African groups are examined, they come from widely separated regions such that geographical substructuring could account for the observed disequilibrium within each group. An extensive analysis of linkage disequilibrium in populations from Cote d'Ivoire, Zambia (Luangwe Valley) and Uganda (Busoga) has been undertaken using published iso-enzyme data and measurement of the index of association (Stevens & Tibayrenc, 1995). These data show that significant linkage disequilibrium occurred in the populations from Zambia and Cote d'Ivoire while the population from Uganda, using only electrophoretic types, did not show disequilibrium. The data from Cote d'Ivoire were subdivided, based on separation into type I and type II *T. b. gambiense* (Gibson, 1986), with the latter group showing an epidemic population structure. These isolates were all collected from within a

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60 km radius where geographical substructuring seems less likely. The isolates from Zambia were collected over a 4 year period primarily from humans (72%) but from different regions within the 400 km long Luangwa Valley. As the collection is from a single area, geographical substructuring seems an unlikely explanation for the observed linkage disequilibrium unless the substructuring is on a very small geographical scale. It is interesting to note that the two populations analysed in the present study are also only 400 km apart. Parasite population substructuring is further highlighted by the detailed analysis of iso-enzyme data from isolates made in the Lambwe Valley in Kenya (Cibulskis, 1992). While a significant association was observed between the MLEE type and host, analysis of association between the MLEE and different localities within the Lambwe Valley did not yield significant associations for each location. However, differences in MLEE type frequency were observed between some regions within the valley, suggesting that transmission may be somewhat localized. While these data are not conclusive in terms of highly local geographical substructuring, it must be remembered that iso-enzyme electrophoresis may lack the resolution to detect such differences.

In this paper, we have demonstrated clear geographical substructuring of *T. brucei* populations separated by a distance of 400 km. This raises the issue of the geographical limits of substructuring in *T. brucei* populations and data from the literature (Cibulskis, 1992) suggest that such substructuring may occur over relatively small distances. While this conclusion requires further analysis to obtain unequivocal evidence, if such localized substructuring occurs it would provide an explanation of the levels of linkage disequilibrium observed in a number of published analyses.

Although there is clearly limited gene flow between the two populations examined in this study, 1 multilocus genotype (M21) was common, found in both Kiboko (9 stocks) and Lugala (1 stock). This genotype has been uniquely associated with human infectivity in Busoga in S. E. Uganda (MacLeod *et al*. 2000). The prediction therefore is that, at the time of sampling, there were human infective trypanosomes in Kiboko. The presence of human infective trypanosomes in tsetse flies in Kiboko is perhaps surprising as no cases of human sleeping sickness have been reported in this area of Kenya. These findings suggest that there is a potential for human sleeping sickness in this area but an alternative interpretation of these data is that the stocks from Kiboko, although genotypically identical to the human infective trypanosomes from Busoga, are not human infective. However, in a recent study of the same stocks from Kiboko but using different markers (Hide *et al*. 2000), it was shown that a subgroup could be defined that showed a high similarity to human stocks from Busoga, again implying that human infective trypanosomes occurred in Kiboko. The subgroup identified by Hide *et al*. (2000), comprises the same set of stocks identified in this study as being of multilocus type M21 and therefore also identical to those from human infections in Busoga. In order to distinguish between the two possible interpretations, the Kiboko stocks should be assayed for their resistance to human serum.

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