

Genetic diversity among *Trypanosoma brucei rhodesiense* isolates from Tanzania

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SUMMARY

We compared 19 stocks of *Trypanosoma brucei rhodesiense* collected in 1991 and 1994 from Tanzania with representative stocks from other foci of Rhodesian sleeping sickness in Zambia, Kenya and Uganda. Stocks were characterized by isoenzyme electrophoresis, restriction fragment length polymorphisms in variant surface glycoprotein genes and random amplification of polymorphic DNA; the banding patterns obtained were coded for numerical analysis. In addition, the Tanzanian stocks were compared by pulsed field gel electrophoresis. Overall the Tanzanian stocks formed a homogeneous group and the predominant genotype isolated in 1991 was still present in the 1994 sample, although at a reduced level. The Tanzanian stocks were distinct from representative stocks from other East African foci. This observation does not support the proposal that there are northern and southern strains of *T. b. rhodesiense*, but is consistent with the view that *T. b. rhodesiense* stocks form a mosaic of different genotypes varying from focus to focus in East Africa.

Key words: sleeping sickness, Tanzania, *Trypanosoma brucei rhodesiense*, human trypanosomiasis.

INTRODUCTION

Both Gambian and Rhodesian forms of African sleeping sickness or human trypanosomiasis have been reported from Tanzania. Gambian sleeping sickness was first recorded around Lake Tanganyika as part of the vast epidemic which ravaged the Congo basin and Uganda at the turn of the century; an active focus persisted until 1958 in Kigoma on the eastern lakeshore (Fairbairn, 1948; Ormerod, 1961). Rhodesian sleeping sickness appeared in epidemic form in the 1920s and 1930s affecting large parts of the country (ibid), and several foci remain (Fig. 1). Currently between 4 and 5 million people are thought to be at risk of infection in Tanzania, but only 1% of these are under regular surveillance. In the past 30 years, the number of new cases reported annually has rarely risen above 500 (Annual Sleeping Sickness Reports for Tanzania, 1965–1995), although this is likely to be an underestimate.

In Tanzania there are considerable differences in the clinical types of human trypanosomiasis encountered. The severity of the disease (anaemia, heart disease, neurological syndromes) varies according to geographical location and this may be

caused by heterogeneity among *Trypanosoma brucei rhodesiense* strains. Throughout the southern part of the country, the disease is endemic and in general has a comparatively mild character, although there are occasional localized epidemics of a more virulent nature. Further north and north-west there are large epidemic areas in which the disease seems to be more virulent. The difference is striking and as a general rule it can be said that the further one goes south the less virulent the disease becomes. This picture agrees with the hypothesis put forward by Ormerod (1967) that there are distinct northern and southern strains of *T. b. rhodesiense* distinguished largely on clinical grounds.

Biochemical and molecular characterization data support this idea, since *T. b. rhodesiense* stocks from Botswana and Zambia have been shown to be quite distinct from those causing disease in Uganda and the neighbouring area of Kenya, both by isoenzyme electrophoresis (Gibson, Marshall & Godfrey, 1980; Godfrey *et al.* 1990; Stevens *et al.* 1992) and by restriction fragment length polymorphisms in ribosomal DNAs (Hide *et al.* 1990, 1991, 1994). However, this result may simply reflect the fact that each epidemic focus of Rhodesian sleeping sickness has its own associated trypanosome strains (Gibson & Welde, 1985; Stevens & Tibayrenc, 1996). Clearly the examination of stocks from Tanzania in the middle of this geographical range would help resolve the issue. Very few trypanosome stocks from Tanzania have been studied, and only from the

† This paper is dedicated to Dr Ernest Komba, who sadly died before he could finish it.

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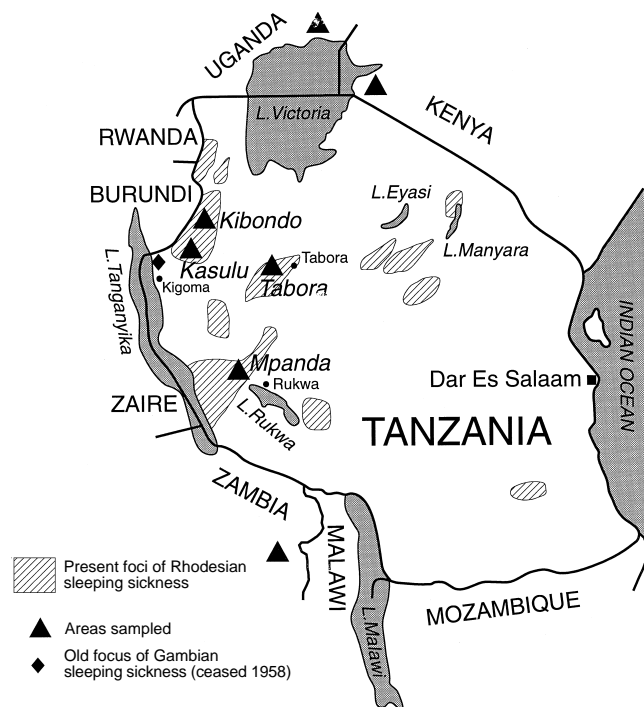


Fig. 1. Map of Tanzania and neighbouring countries showing the main foci of Rhodesian sleeping sickness.

northern foci (Gibson *et al.* 1980; Godfrey *et al.* 1990; Stevens *et al.* 1992; Gashumba *et al.* 1994). In this study *T. b. rhodesiense* stocks isolated in 1991 and 1994 from Tanzanian patients were compared with representative stocks from foci in Uganda, Kenya and Zambia using several biochemical characterization techniques.

MATERIALS AND METHODS

Trypanosomes

For the origin of trypanosome stocks included in this study see Table 1. A total of 25 *T. b. rhodesiense* stocks were compared, 19 from Tanzania and 2 each from Zambia, Kenya and Uganda. For the Tanzanian stocks, the primary isolations were made from patients during 2 surveys conducted in May 1991 and December 1994. Trypanosomes were isolated in 2 ways: by intraperitoneal inoculation of blood into immunosuppressed mice (cyclophosphamide i.p. 200 µg/kg), or by inoculation of 2.5 ml of infected blood directly into a KIVI bottle (Kit for *In Vitro* Isolation of trypanosomes; Aerts *et al.* 1992). Trypanosomes were subsequently cryopreserved from parasitaemic mice and were dispatched to Bristol together with inoculated KIVIs. The bloodstream-form trypanosomes were transformed into procyclics in KIVI or Cunningham's medium (CM; Cunningham, 1977) and were grown on in CM to yield 4×10^9 trypanosomes. Trypanosome stocks which failed to thrive in culture were subpassaged in tsetse flies and midgut procyclics were then grown in CM.

Enzyme electrophoresis

Aqueous extracts for electrophoresis were prepared from washed procyclics using standard methods (Kaukas *et al.* 1990) and were stored in liquid nitrogen (-196°C). Electrophoresis was carried out on cellulose acetate plates (CAE) or on thin-layer starch-gel (TSGE) as described (Lanham *et al.* 1981; Kaukas *et al.* 1990; Stevens *et al.* 1992). Seven enzymes were examined by CAE: malate dehydrogenase (MDH; EC 1.1.1.37); isocitrate dehydrogenase (ICD, EC 1.1.1.42); threonine dehydrogenase (TDH; 1.1.1.103); phosphoglucomutase (PGM; 2.7.5.1); superoxide dismutase (SOD, EC 1.15.1.1); nucleoside hydrolase, substrate inosine (NHI, EC 3.2.2.1); nucleoside hydrolase, substrate deoxyinosine (NHD; EC 3.2.2.1); and a further 2 enzymes by TSGE: alanine aminotransferase (ALAT; EC 2.6.1.2) and aspartate aminotransferase (ASAT; EC 2.6.1.1).

Preparation and analysis of DNA

Total DNA was prepared by standard methods (Van der Ploeg *et al.* 1982). DNAs were restricted with *Pst*I or *Hinc*II and size fractionated in 0.7% agarose gels. Samples for pulsed field gradient gel electrophoresis (PFGE; Schwartz & Cantor, 1984) were prepared by lysing and deproteinizing trypanosomes *in situ* in agarose blocks (Van der Ploeg *et al.* 1984). PFGE was carried out in 1% agarose gels using an LKB Pulsaphor system with a hexagonal electrode and a 5 phase program (900s, 15 h; 300s 15 h; 210s 10 h; 180s 10 h and 40s, 10 h; 130V) (Gibson & Garside, 1991). Gels were stained with ethidium bromide (0.5 µg/ml) and photographed by UV transillumination. Southern transfer (Southern, 1975) was carried out as standard with a preliminary depurination step (0.25 M HCl for 15–30 min). Hybridization was carried out as described by (Gibson, Dukes & Gashumba, 1988) using the following DNA probes from *T. brucei*: (1) VSG 118, 1.2 kb *Eco*RI/*Pst*I fragment from clone TcV-c114 (Bernards *et al.* 1981); (2) VSG 117, 1.5 kb *Pst*I fragment from cDNA clone TcV-117.5 (*ibid*); (3) VSG 121, 0.8 kb fragment from cDNA clone TcV-121.7 (Frasch, Borst & Van den Burg, 1982); (4) α -tubulin, 1.6 kb insert from cDNA clone pTb α T-c1 (Thomashow *et al.* 1983). Probes were labelled with [32 P]dCTP by the random priming method (Feinberg & Vogelstein, 1983). Filters were washed 4×30 min in $3 \times \text{SSC}$, 0.1% SDS at 65°C , followed by 2×30 min stringency washes at $0.1 \times \text{SSC}$, 0.1% SDS at 65°C , unless stated otherwise in figure legends.

RAPD analysis

RAPD (Random Amplification of Polymorphic DNA; Welsh & McClelland, 1990; Williams *et al.*

Table 1. Origin of *Trypanosoma brucei rhodesiense* stocks

(Stocks 001–010 were previously characterized by Gashumba *et al.* (1994). Isolates 002 and 006 came from brothers; 008 and 009 from father and son.)

Stock code	Sex/age	Village	District	Country	Date
TMRS 001	M 15	Kakora	Tabora	Tanzania	22/03/91
TMRS 002	M 45	Ruhita	Kasulu	Tanzania	26/03/91
TMRS 003	M 40	Nyenge	Kasulu	Tanzania	26/03/91
TMRS 005	M 38	Ruhita	Kasulu	Tanzania	26/03/91
TMRS 006	M 20	Ruhita	Kasulu	Tanzania	26/03/91
TMRS 007	M 41	Migunga	Kasulu	Tanzania	26/03/91
TMRS 008	M 14	Mvinza	Kasulu	Tanzania	27/03/91
TMRS 009	M 40	Mvinza	Kasulu	Tanzania	27/03/91
TMRS 010	M 26	Makele	Kasulu	Tanzania	28/03/91
TMRS 106	M 15	Busunzu	Kibondo	Tanzania	11/05/94
TMRS 108	M 30	Kichananga	Kibondo	Tanzania	11/05/94
TMRS 109	F 50	Mvugwe	Kibondo	Tanzania	13/05/94
TMRS 117	M 23	Busunzu	Kibondo	Tanzania	17/07/94
TMRS 119	F 50	Nyankwi	Kibondo	Tanzania	24/08/94
TMRS 120	M 36	Busunzu	Kibondo	Tanzania	23/08/94
TMRS 122	M 38	Busunzu	Kibondo	Tanzania	23/08/94
TMRS 123	M 8	Kanazi	Kasulu	Tanzania	15/09/94
TMRS 124	M 60	Nyarugusu	Kasulu	Tanzania	19/09/94
TMRS 127	M 35	Katumba	Mpanda	Tanzania	18/10/94
TRPZ 274	—	Kasyasya	Luangwa	Zambia	18/02/83
058 clone B	—	Kasempa	Luangwa	Zambia	1974
LVH 56	M 26	Codjope	Lambwe	Kenya	16/08/78
LVH 108	M 34	Nyadenda	Lambwe	Kenya	17/03/81
KETRI 2291	—	Nampongo	Busoga	Uganda	13/09/76
UTRO 2509	—	Namungalwe	Busoga	Uganda	10/12/79

1990) was carried out as described by Kanmogne *et al.* (1996) using approximately 20 ng of total DNA as template. The following 8 primers were used (numbering according to Tibayrenc *et al.* (1993)) 5' to 3': P1, CTTCCCGGAC; P2, GTCGAGCCGT; P4, GTCGGGCTAA; P5, GTTCTGGGGA; P7, GTGGGCAAAG; P8, GGATGCAGTG; P9, CCGCAATGGG; P10, GACGCTAGTG. A total of 15 μ l of each 50 μ l amplification reaction was analysed by electrophoresis in 1.5% agarose. Each PCR reaction was carried out at least twice to ensure reproducibility of results. A no DNA control reaction was carried out for each primer.

Numerical analysis

To evaluate relationships between stocks, dendrograms were constructed using the program of Stevens & Cibulskis (1990) to calculate a phenetic distance measure, Jaccard's coefficient (Dunn & Everitt, 1982), and the unweighted pair-group method using arithmetic averages (UPGMA; Sokal & Michener, 1958), available in SPSS/PC+.

RESULTS

Isoenzyme analysis

The results obtained from the isoenzyme analysis are summarized in Table 2. The majority of the

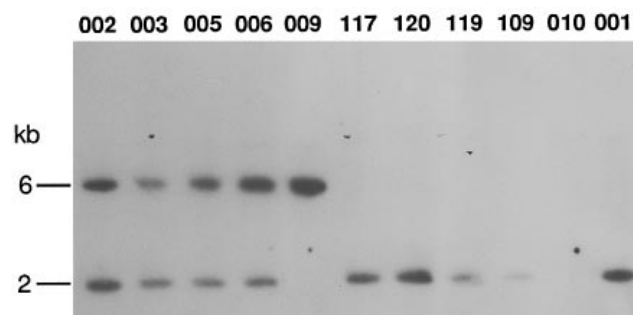
Tanzanian stocks examined were identical or closely similar electrophoretically. Of the 9 enzymes tested, only MDH (stock 010) and NHD (stock 106) showed variation. All stocks possessed ALAT 2 and ASAT 1, typical patterns for *T. b. brucei* and *T. b. rhodesiense*, and all had East African ICD 1 and PGM 1 patterns (Gibson *et al.* 1980; Godfrey *et al.* 1990). The results of isoenzyme analysis were used to group the stocks according to the classification of these authors: 15 stocks belonged to zymodeme 306 and 2 stocks belonged to zymodeme 307; stocks 010 and 106 showed unusual isoenzyme patterns for MDH and NHD and were placed in new zymodemes. To evaluate relationships, UPGMA dendrograms were constructed from Jaccard similarity coefficient matrices. All stocks belonged to a single highly homogeneous group, in agreement with previous results (Gashumba *et al.* 1994).

RFLP analysis

RFLPs were analysed in 3 genes for variant surface glycoproteins (VSGs). Fig. 2 shows the result of hybridizing *HincII* digests with a probe for VSG gene 118. The gene was present in all stocks, except stock 010, and gave either 1 or 2 strongly hybridizing fragments. Rehybridization of the blot with a probe for VSG 117 showed several bands for 010, confirming that sufficient DNA was present in this lane.

Table 2. Electrophoresis results

Zymodeme	Stocks	ALAT	ASAT	ICD	PGM	MDH	TDH	NHI	NHD	SOD
306	001, 002, 003, 005, 006, 007, 008, 009, 108, 109, 117, 119, 122, 123, 127	2	1	1	1	1	1	1	2	1:8
307	120, 124	2	1	1	1	1	1	1	2	1:9
New	010	2	1	1	1	5	1	1	1	1:7
New	106	2	1	1	1	1	1	1	5	1:10



VSG 118

Fig. 2. RFLP analysis of VSG gene 118. Autoradiograph of *HincII* digests hybridized with a DNA probe for VSG 118. No hybridization was visible in the 010 lane even after long exposure. Lanes as indicated; DNA size marker was phage lambda \times *HindIII*.

All the non-Tanzanian stocks had both 2 and 6 kb bands, except TRPZ 274 and LVH 108, which had the 2 kb band only (not shown).

Fig. 3 shows *HincII* digests hybridized with a probe for VSG 117 and washed to low stringency to reveal the family of related genes. Individual stocks had 10–15 hybridizing fragments, some of which were common to different stocks. Three Tanzanian

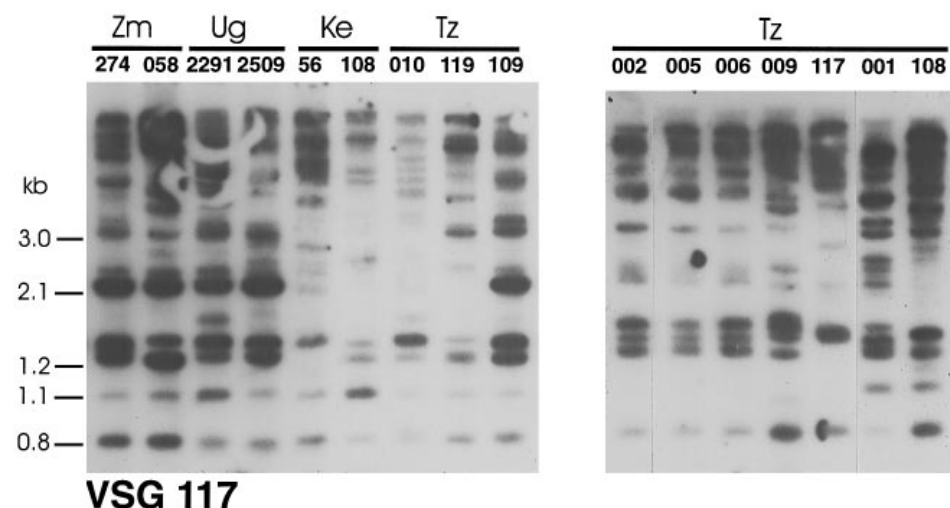
stocks (008, 106, 109) and both Ugandan and Zambian stocks had a strongly hybridizing band of 2.1 kb, representing a conserved copy of the 117 VSG gene; the Kenyan stocks LVH 56 and LVH 108 both lacked this fragment, as did most Tanzanian stocks. DNA fingerprints were generally stock specific, except for 1 fingerprint shared by 6 stocks (002, 003, 005, 006, 007, 123).

This group of stocks, together with 108, 122 and 127, showed only faint hybridization with a DNA probe for VSG 121 (Fig. 4), indicating absence of this VSG gene. Other stocks had one or more strongly hybridizing *PstI* fragments. There appeared to be one conserved VSG 121 gene, yielding a fragment of 850 bp; all the non-Tanzanian stocks had this band, together with Tanzanian stocks 008, 010, 106, 119 and 124. Five other Tanzanian stocks had one or more hybridizing fragments of various sizes (001, 009, 109, 117, 120).

Results for all 3 VSG genes were combined to calculate similarity coefficients and construct a dendrogram of relationships (Fig. 5A).

RAPD analysis

Fig. 6 shows typical results produced with 2 primers with template DNA of 12 stocks. Several DNA fragments were amplified in each sample and



VSG 117

Fig. 3. RFLP analysis of VSG gene 117. Autoradiographs of *HincII* digests hybridized with a DNA probe for VSG 117; blot was washed to low stringency ($3 \times$ SSC at 65°C) to reveal gene family. Lanes as indicated; DNA size markers were phage lambda \times *HindIII* and plasmid Gemini 3 \times *HinfI*.

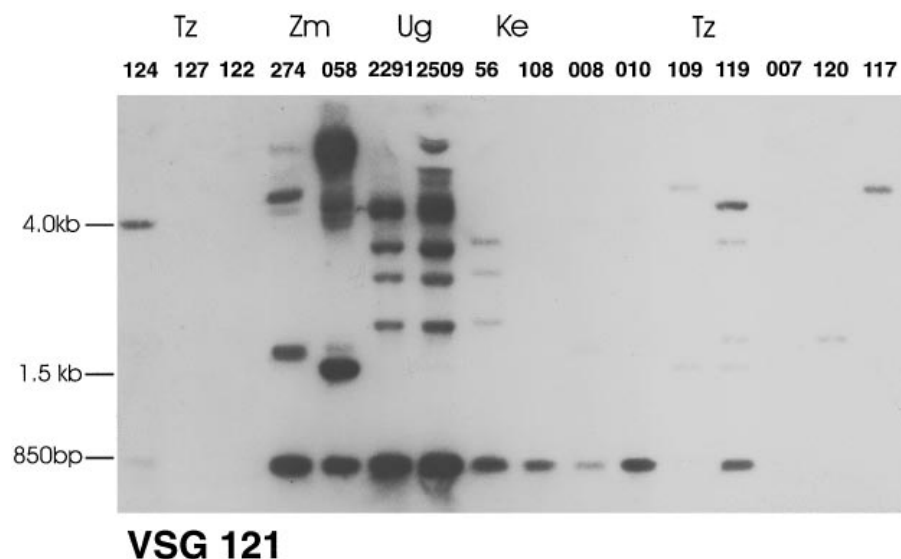


Fig. 4. RFLP analysis of VSG gene 121. Autoradiograph of *Pst*I digests hybridized with a DNA probe for VSG 121. Faint bands were visible in lanes 127, 122 and 007 on the original autoradiograph. Lanes as indicated; DNA size markers were as Fig. 3.

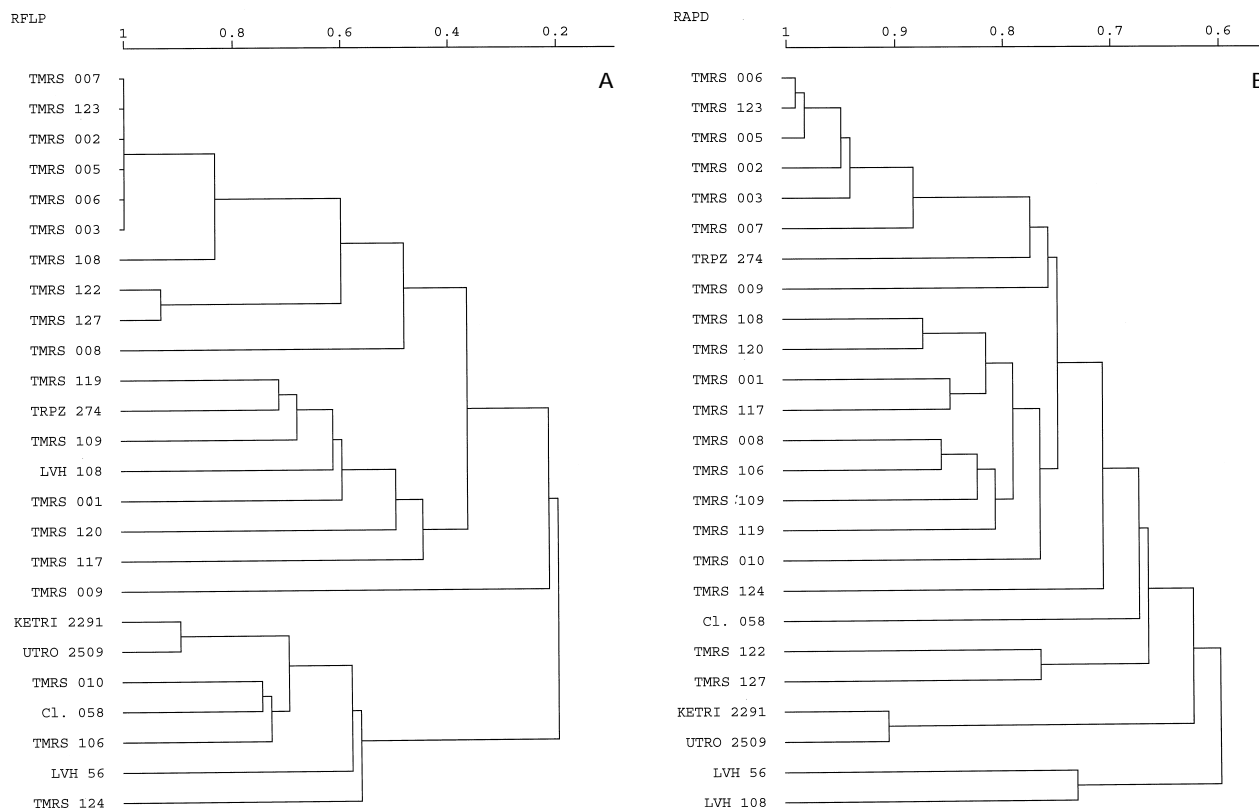


Fig. 5. Dendrograms constructed from (A) RFLP results and (B) RAPD results.

polymorphisms were apparent for most of the primers. Each amplification product was numbered and each sample scored for the presence or absence of each band. Similarity was calculated using the computer program of Stevens & Cibulskis (1990). The rationale for using a phenetic distance for RAPD data has been discussed by Stevens & Tibayrenc (1995). The dendrogram of similarities is shown in Fig. 5B.

PFGE analysis and chromosomal location of the tubulin genes

The molecular karyotypes of the Tanzanian trypanosome stocks were compared by PFGE. Although karyotypes were broadly similar, there was much variation in the size and number of chromosomes between individual isolates (Fig. 7A). Hybridization of a blot of this PFGE gel with a DNA probe for α -

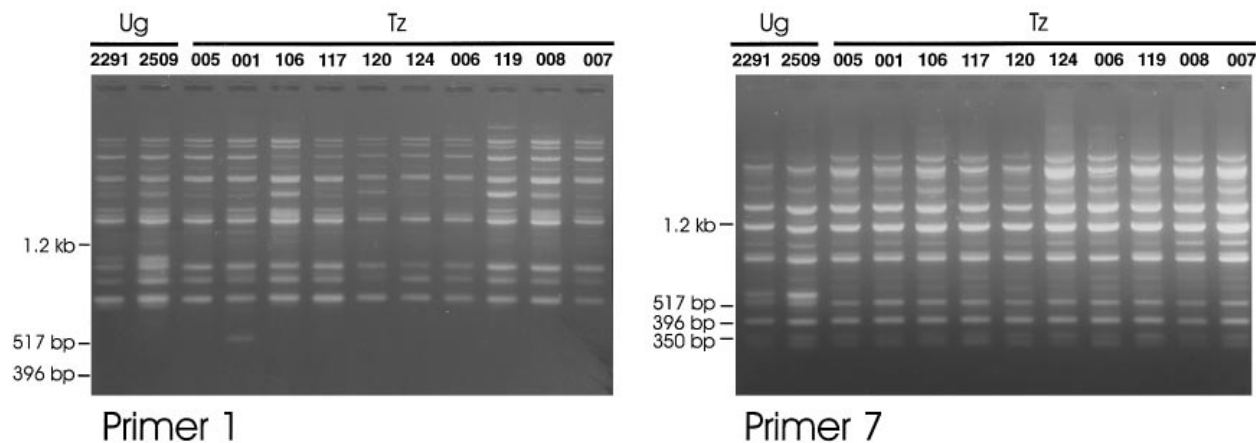


Fig. 6. RAPD analysis of 12 stocks using 2 different primers as indicated. Lanes as shown; DNA size marker was plasmid Gemini 3 \times *Hinf*I.

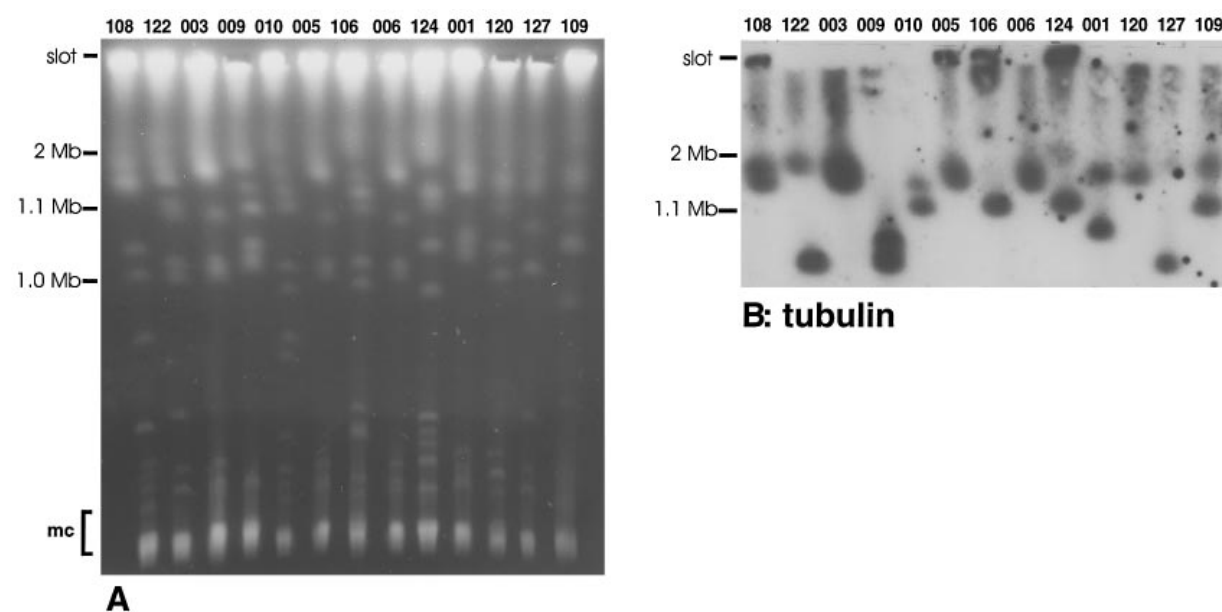


Fig. 7. Molecular karyotypes of Tanzanian *Trypanosoma brucei rhodesiense* stocks. (A) Ethidium-stained PFGE gel; note similarity of karyotypes of stocks 003, 005 and 006. (B) Blot of same gel hybridized with probe for α -tubulin. DNA size marker *Saccharomyces cerevisiae* chromosomes; mc, minichromosomes.

tubulin reveals the extent of size variability for 1 pair of chromosomes (Fig. 7B). Against this general picture of genomic plasticity, several stocks shared a similar karyotype, with minor variation in size and number of small chromosomes (100–250 kb); for example, compare samples 003, 005 and 006 in Fig. 7. This group of stocks was that identified above by VSG 117 fingerprints (i.e. 002, 003, 005, 006, 007, 123), except that the size of the 1 Mb band was slightly reduced in stock 123 (not shown).

DISCUSSION

We have used 4 different biochemical characterization methods to analyse genetic variation in *T. b. rhodesiense* stocks from Tanzania. A previous study using only isoenzyme electrophoresis showed stocks from north-western Tanzania to be highly

homogeneous (Gashumba *et al.* 1994), and isoenzyme analysis of a further 10 stocks collected in 1994 confirmed this picture. However, additional data from RFLP, RAPD and PFGE analyses revealed much greater levels of heterogeneity than predicted from the isoenzyme results. Of the 19 Tanzanian stocks examined, only 6 formed a cohesive group on the basis of RFLPs in VSG genes and molecular karyotypes (002, 003, 005, 006, 007, 123). This group predominated in the 1991 collection, but was also present in 1994 (stock 123), and thus represents a stable and widespread genotype (Tibayrenc, Kjellberg & Ayala, 1990).

Numerical analysis of isoenzyme, RFLP and RAPD bands grouped the majority of Tanzanian stocks, but each technique also identified outliers. Stocks 010 and 106 had unique isoenzyme patterns and were also outliers by RFLP analysis, together

with 009 and 124. By RAPD analysis stocks 122, 124 and 127 were placed apart from the majority of Tanzanian isolates. When the phylogenetic trees derived from the RAPD and RFLP data are compared, it is apparent that the 2 techniques have grouped the stocks in different ways. There are many mechanisms which can reassert the phylogenetic heritability of genetic information and it can be argued that the difference in tree topology reflects different modes of inheritance of the RAPD and VSG markers. While RAPD markers would be expected to provide a broad survey of the trypanosome genome, VSG genes are subject to high levels of gene rearrangement associated with antigenic variation. However, VSG genes constitute a large part of the non-repetitive fraction of trypanosome DNA and would also be surveyed by RAPD analysis. Furthermore, the VSG gene repertoire is sufficiently mitotically stable to provide useful strain fingerprints for *T. b. gambiense* (Paindavoine *et al.* 1986) and *T. b. rhodesiense* as shown here.

Overall then, there is a lack of agreement between the patterns of relatedness described by each of the 3 characterization methods used. Correlation between independent characterization methods is generally interpreted as an indication of clonal propagation or the non-random assortment of the underlying genetic material (Tibayrenc & Ayala, 1988) and has been used previously by a number of authors as evidence of a lack of genetic exchange (Tibayrenc *et al.* 1993; Sanchez *et al.* 1993; Stevens & Tibayrenc, 1995). Conversely, the lack of correlation between different characterization methods demonstrated here may indicate that genetic exchange plays a role in this case.

Recent biochemical characterization results (Godfrey *et al.* 1990; Hide *et al.* 1990, 1991, 1994; Stevens *et al.* 1992) have reopened debate on the existence of northern and southern strains of *T. b. rhodesiense*, as originally proposed by Ormerod (1967). Our results do not support this proposal: the majority of stocks from Tanzania grouped together, while those from Uganda, Kenya and Zambia generally formed outlying groups. One Zambian stock (TRPZ 274) fell into the broad Tanzanian group in both RFLP and RAPD analyses, but was not particularly closely related to the single stock from the southern Tanzanian focus (127 from Mpanda). This stock was most similar to another from the north-western Kibondo focus. Interestingly, the Mpanda patient was a recent immigrant from Burundi, suggesting that refugee movements may also contribute to transfer of trypanosome strains. Until more *T. b. rhodesiense* stocks are examined from the southern Tanzanian foci, we cannot rule out the possibility that there are other *T. b. rhodesiense* genotypes associated with the mild sleeping sickness syndrome in these areas.

In conclusion, rather than the northern and

southern strains proposed by Ormerod (1967), the overall picture would seem to be a mosaic of different genotypes of *T. b. rhodesiense* distributed through the endemic regions of East Africa. The next question is how these different genotypes arise. Considering that genetic exchange can take place between *T. b. rhodesiense* and *T. b. brucei* (Gibson, 1989), and that the 2 subspecies coexist naturally in vectors and reservoir hosts, we can speculate that *T. b. rhodesiense* stocks evolve locally through frequent genetic exchange with their sympatric *T. b. brucei* stocks.

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