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Population genetic structure and cladistic analysis of *Trypanosoma brucei* isolates

Eddy Chukwura Agbo[a,*], Peter-Henning Clausen[b], Philippe Büscher[c], Phelix A.O. Majiwa[d], Eric Claassen[e], Marinus F.W. te Pas[a]

[a] *Division of Animal Sciences, Institute for Animal Science and Health, ID-Lelystad, Edelhertweg 15, 8200 AB Lelystad, The Netherlands*
[b] *Institute for Parasitology and International Animal Health, Freie Universitaet Berlin, Koenigsweg 67, D-14163 Berlin, Germany*
[c] *Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium*
[d] *International Livestock Research Institute, P.O. Box 30709, Nairobi, Kenya*
[e] *Department of Immunology, Erasmus University Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*

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Abstract

Using a novel multilocus DNA marker analysis method, we studied the population genetic structure of *Trypansoma brucei* stocks and derived clones isolated from animal and *rhodesiense* sleeping sickness patients during a national sleeping sickness control program in Mukono district, Uganda. We then performed a cladistic analysis to trace relationships and evolution, using stocks and clones recovered from geographically and temporally matched hosts, including inter-strain comparisons with *T. b. gambiense* stocks and clones. Our results show that while there was close genetic relatedness among parasite populations from the same geographical region, micro-heterogeneities exist between different stocks. Data are presented that indicate that not every human sleeping sickness focus may be associated with a particular human-infective trypanosome strain responsible for long-term stability of the reference focus. We provide evidence of genetic sub-structuring among type 1 *T. b. gambiense* stocks, which has potentially important implications for molecular epidemiology of *T. brucei*.
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Keywords: *Trypanosoma brucei*; Population genetic structure; Multilocus DNA marker analysis; Cladistic analysis

1. Introduction

The species *Trypanosoma brucei* consists of *T. b. brucei* (one of the causes of nagana in cattle), *T. b. rhodesiense*, and *T. b. gambiense* (the cause of human sleeping sickness in East and Central-West Africa, respectively). *T. b. brucei* is one of the trypanosomes that have severely limited livestock productivity in over one-third of sub-Saharan Africa. It does not cause disease in humans because this subspecies is lysed by normal human serum. The disease, collectively called trypanosomosis, has re-emerged as a major health threat in sub-Saharan Africa, with a conservative estimate of about 500,000 current human infections (WHO, 2001).

One of the fundamental tools that is required for a full understanding of the epidemiology of trypanosomosis caused by *T. brucei* is a reliable method for identifying and tracking strains. The complexity of the transmission cycles involved and the comparative dynamics of the transmission through parts of these cycles mean that systems for generating robust and reliable genetic markers for epidemiological studies are needed. It is now well established that *T. b. rhodesiense* and *T. b. brucei* genomes are more polymorphic than those of *T. b. gambiense* (Hide, 1999; Agbo et al., 2002). However, knowledge of the extent of genetic diversity of local parasite populations and the role of this diversity in the interactions of the parasite with both the animal and human hosts in related and distant populations remain poorly understood. The epidemiological analysis undertaken on stocks isolated during a human sleeping sickness epidemic in Tororo district of Uganda (Hide et al., 1994), as well as other studies (MacLeod et al., 1999, 2001a,b) provided unique opportunities to address these issues, because a number of parameters that may provide insight into population structure of natural populations of trypanosomes, were investigated.

In East Africa, certain geographically-related foci are characterised by periods of long-term endemicity inter-

* Corresponding author. Tel.: +31-320-238391; fax: +31-320-238050.
E-mail address: e.e.c.agbo@id.wag-ur.nl (E.C. Agbo).

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spersed with short epidemic episodes. To better understand the causes of these episodes, the molecular epidemiology and population structure of *T. brucei* within Busoga focus have been studied (Enyaru et al., 1993, 1997; Degen et al., 1995; MacLeod et al., 2000). A recently developed PCR system based on the serum-resistance-associated (*SRA*) gene (De Greef et al., 1989) has been shown to be specific for the identification of *T. b. rhodesiense* strains (Welburn et al., 2001; Gibson et al., 2002). However, the absence of the *SRA* gene from other *T. brucei* subspecies, even in the so-called *rhodesiense*-like, virulent or type 2 *T. b. gambiense* (Gibson, 1986) underscores the need for additional genetic markers.

We undertook a population genetic study to evaluate the population structure of parasite stocks isolated during endemic and epidemic periods within Mukono district in Busoga focus, using a recently described multilocus fine genotyping marker system (Agbo et al., 2003). An attractive feature of the approach is that multiple independent restriction enzyme-based polymorphisms can be genotyped in a single reaction and scored in a single lane of a gel on an automated sequencer. In this analysis, we looked for evidence of the uniqueness of circulating genotypes. Furthermore, we traced stock relationships and evolution by analysing the extent of genetic polymorphisms among human-infective stocks on the one hand, and between human- and animal-infective stocks on the other hand, from geographically and temporally matched populations, within the same and different foci. Our data suggest that this approach may offer a valuable tool for fine-scale epidemiological investigations of trypanosomosis and diseases caused by other agents.

2. Materials and methods

2.1. Trypanosome stocks and clones

The trypanosome populations listed in Table 1 were originally isolated between 1990 and 1992 from pigs (19), cattle (5) and *rhodesiense* sleeping sickness patients (4) during an evaluation of the National Sleeping Sickness Control Programme (NSSCP) in Bulutwe, Mukono district, South-eastern Uganda, a *rhodesiense* sleeping sickness endemic area. At that time 0.7% of villagers, 33.5% of cattle and 52.8% of domestic pigs harboured trypanosome infections (Nowak et al., 1992). Human serum response properties of the parasites were evaluated earlier (Von Dobschuetz, 2002; Mangeni, unpublished data) using the Blood Incubation Infectivity Test (BIIT) (Rickman and Robson, 1970) and the Human Serum Resistance Test (HSRT) (Jenni and Brun, 1982).

A collection of stocks and clones consisting of *T. b. brucei* (7), *T. b. gambiense* (10) and *T. b. rhodesiense* (8) derived during epidemic episodes from related and disparate locations (Table 2) was also analysed to compare the genotypic properties of the various populations, separated in space and time. Cloned parasite populations were generated following limited passage of cryostabilates in laboratory rodents, according to published protocols (Hawking, 1976; Brun et al., 1981). Genomic DNA from all samples was extracted according to Heath (1997). The DNA samples were screened by PCR for the presence of the *SRA* and *T. b. gambiense*-specific glycoprotein (*TgsGP*) genes, exactly as described by Gibson et al. (2002) and Radwanska et al. (2002), respectively.

2.2. Multiplex-endonuclease analysis

Multiplex genetic fingerprint patterns were generated for each sample according to the principle described by Agbo et al. (2003). The approach permits the simultaneous assessment of multiple independent polymorphic sites per genotyping analysis and ensures PCR stringency through the use of only one pair of adapters and primers. Briefly, 200–300 ng of genomic DNA was digested for 4 h with 10 U of each restriction enzyme, in combinations *Bgl*II–*Bcl*I–*Eco*RI–*Mfe*I and *Bgl*II–*Bcl*I–*Xho*II–*Eco*RI–*Mfe*I–*Acs*I, respectively.

The digests were precipitated and reconstituted in 10 μl distilled water. Ten microlitres of a buffer containing 660 mM Tris–HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM ATP, pH 7.5, and 20 pM of each adapter—*Bgl*II (5′-CGGACTAGAGTACACTGTC-3′; 5′-GATCGACAGTGTACTCTAGTC-3′) and *Mun*I (5′-AATTCCAAGAGCTCTCCAGTAC-3′; 5′-AGTACTGGAGAGCTCTTG-3′)—were added. One microlitre (400 U) of high concentration T4 DNA ligase (New England Biolabs) was added and the mixture incubated for 2 h at 25 °C. Pre-selective amplification was performed in a total volume of 20 μl containing 1 U of *Taq* polymerase (Roche Molecular Biochemicals, Almere, The Netherlands), 4 μl of 1:1-diluted ligation product, 2 μl of 10× PCR buffer (100 mM Tris–HCl pH 9.0, 50 mM KCl, 1% Triton X-100, 0.1% (w/v) gelatin), 2.5 mM $MgCl_2$, 200 μM of each dNTP and 5 pM of each primer—*Bgl*II (5′-GAGTACACTGTCGATCT-3′) and *Mun*I (5′-GAGAGCTCTTGGAATTG-3′). The reaction mixture was incubated for 2 min at 95 °C, and subjected to 20 cycles of PCR (30 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C).

Four microlitres of 1:20-diluted pre-selective products were used as template for selective primer combinations *Bgl*II-0/*Mfe*I-A (with ‘0’ and an ‘A’ selective nucleotide in the *Bgl*II and *Mfe*I primers, respectively), *Bgl*II-0/*Mfe*I-AA and *Bgl*II-0/*Mfe*I-AT. The PCR program was essentially the same as for pre-selective amplification, except that the last cycling step was followed by 30 min incubation at 60 °C. One microlitre of 1:4-diluted selective PCR products was mixed with Genescan-500 internal lane standard (PE Applied Biosystems). The mix resolved in a 7.3% denaturing polyacrylamide gel, using a model ABI 373A automated DNA sequencer. Gels were routinely prepared by using ABI protocols and electrophoresed for 5 h. Data collection

Table 1
Origins of trypanosome populations isolated during an endemic period and identity according to BIIT, HSRT, *SRA*- and *TgsGP19*-PCR

Stocks/clones	Origin	Isolation date	Host	BIIT[a]	HSRT[b]	*SRA*[c]	*TgsGP*[d]
1. SUS/BU 83/6	Bulutwe	March 1992	Pig	Sens[e]	Sens	−	−
2. SUS/BU 83/7	Bulutwe	April 1992	Pig	Sens[e]	Sens	−	−
3. SUS/BU 83/9	Bulutwe	July 1992	Pig	n.d.[f]	Sens	−	−
4. SUS/BU 83/9 Cl.1	Bulutwe	July 1992	Pig	Subres[g]	Sens	−	−
5. SUS/BU 83/9 Cl.2	Bulutwe	July 1992	Pig	Sens[g]	Sens	−	−
6. SUS/BU 83/9 Cl.4	Bulutwe	July 1992	Pig	Subres[g]	Sens	−	−
7. SUS/BU 83/9 Cl.5	Bulutwe	July 1992	Pig	Subres[g]	Sens	−	−
8. SUS/BU 132/2	Bulutwe	March 1991	Pig	n.d.[f]	Sens	−	−
9. SUS/BU 132/4	Bulutwe	August 1991	Pig	n.d.[f]	Subres	−	−
10. SUS/BU 139/2	Bulutwe	March 1991	Pig	Res[g]	Subres	−	−
11. SUS/BU 169/4	Bulutwe	August 1991	Pig	Sens[e]	Subres	−	−
12. SUS/BU 319/7	Bulutwe	April 1992	Pig	Subres[g]/sens[e]	Sens	−	−
13. SUS/BU 319/7 Cl.1	Bulutwe	April 1992	Pig	Sens[g]	Sens	−	−
14. SUS/BU 319/7 Cl.3	Bulutwe	April 1992	Pig	Subres[g]	Sens	−	−
15. SUS/BU 319/9	Bulutwe	July 1992	Pig	Sens[e]	Sens	−	−
16. SUS/BU 347/7	Bulutwe	April 1992	Pig	Res[e]	Res	−	−
17. SUS/BU 373/7	Bulutwe	April 1992	Pig	Res[e]	Res	−	−
18. SUS/BU 561/3	Bulutwe	June 1991	Pig	Subres[g]/sens[e]	Sens	−	−
19. SUS/BU 932/7	Bulutwe	April 1992	Pig	Subres[g]	Subres	−	−
20. BOT/BU 483/2	Bulutwe	March 1991	Cattle	Subres[g]	Sens	−	−
21. BOT/BU 492/2	Bulutwe	March 1991	Cattle	n.d.[f]	Sens	−	−
22. BOT/BU 602/7	Bulutwe	April 1992	Cattle	Subres[g]/res[e]	Res	−	−
23. BOT/BU 623/7	Bulutwe	April 1992	Cattle	Res[e]	Subres	−	−
24. BOT/BU 1845/7	Bulutwe	April 1992	Cattle	Res[e]	Subres	−	−
25. HOM/BU H1	Bulutwe	November 1990	Man	n.d.[f]	Res	+	−
26. HOM/BU H2	Bulutwe	November 1990	Man	n.d.[f]	Res	+	−
27. HOM/BU H5	Bulutwe	April 1991	Man	n.d.[f]	Res	+	−
28. HOM/IG 2602	Kapyanga	February 1990	Man	Res[e]	Res	+	−

[a] Blood Incubation Infectivity Test (Rickman and Robson, 1970).
[b] Human Serum Resistance Test (Jenni and Brun, 1982). The HSRT was performed twice per isolate, except for BOT/BU 623/7 and BOT/BU 602/7 where it was tested three and seven times, respectively (Rickman and Robson, 1970). The isolates were defined as resistant "Res" if they showed continuous growth in the presence of human serum for at least 10 days and sensitive "Sens" if they were lysed within 3 days. Isolates which showed non-continuous growth but remained alive in the presence of human serum for at least 3 days were classified as sub resistant "Subres" (Von Dobschuetz, 2002).
[c] Serum-resistance-associated (*SRA*) gene (+, present; −, not present), determined using primers defined by Gibson et al. (2002).
[d] *T. b. gambiense*-specific glycoprotein (*TgsGP*) gene (+, present; −, not present), established using primers defined by Radwanska et al. (2002).
[e] Tietjen (personal communication).
[f] Not done.
[g] Mangeni (unpublished data).

and analysis were conducted as previously described (Agbo et al., 2002).

2.3. Phylotyping by multiplex-endonuclease analysis

Genomic fingerprint patterns were generated in parallel for 13 reference populations (i.e. 5 *T. b. brucei*, 6 *T. b. gambiense* and 2 *T. b. rhodesiense*). Only fragments ranging from 35 to 500 bp were analysed from which a schematic representation of the fingerprint patterns was constructed. From these profiles, identified on the basis of their intensity and individuality, a numerical matrix of observations based on the presence (1) and absence (0) of bands was built. The data were compared using Pearson correlation product moment coefficient (Pearson, 1926), which determined the proportion of mismatched bands between samples. Based on the similarity matrix a dendrogram was generated using the unweighted pair-group method using arithmetic averages (UPGMA).

3. Results

3.1. Trypanosome identity

The results of the BIIT and HSRT (summarised in Table 1) permitted an early classification of the stocks or clones derived during the endemicity survey in Mukono district. Trypanosomes positive (resistant) in one of these tests were considered putative *T. b. rhodesiense*. All trypanosome DNAs were further screened by PCR for the 1.2 kb *SRA* and 308 bp *TgsGP* fragments, and scored as present or absent (+ or −) (Table 1). The *SRA* gene fragment was amplified only from endemic trypanosome populations of human origin, which were considered genuine *T. b. rhodesiense*.

Table 2
Origin and identity of trypanosome populations derived during epidemic outbreaks

Species subspecies	Trypanosome stocks and clones	Origin	Isolation year	Original host
1. *T. b. brucei*	AnTat1.8	Uganda	1966	Bushbuck
2. *T. b. brucei*	AnTat2.2	Nigeria	1970	Tsetse
3. *T. b. brucei*	AnTat5.2	Gambia	1975	Bovine
4. *T. b. brucei*	AnTat17.1	D.R. Congo	1978	Sheep
5. *T. b. brucei*	Ketri2494	Kenya	1980	Tsetse
6. *T. b. brucei*	J10	Zambia	1973	Hyena
7. *T. b. brucei*	TSW196	Côte d'Ivoire	1978	Pig
8. *T. b. gambiense*	AnTat9.1	Cameroon	1976	Man
9. *T. b. gambiense*	LiTat1.3	Côte d'Ivoire	1952	Man
10. *T. b. gambiense*	AnTat11.17	D.R. Congo	1974	Man
11. *T. b. gambiense*	AnTat22.1	Congo/Brazzville	1975	Man
12. *T. b. gambiense*	JUA	Cameroon	1979	Man
13. *T. b. gambiense*	BAGE	D.R. Congo	1995	Man
14. *T. b. gambiense*	NABE	D.R. Congo	1995	Man
15. *T. b. gambiense*	PAKWE	D.R. Congo	1995	Man
16. *T. b. gambiense*	SEKA	D.R. Congo	1995	Man
17. *T. b. gambiense*	PT312	Côte d'Ivoire	1992	Man
18. *T b. rhodesiense*	0404	Rwanda	1970	Man
19. *T. b. rhodesiense*	STIB847	Uganda (Busoga)	1990	Man
20. *T. b. rhodesiense*	STIB848	Uganda (Busoga)	1990	Man
21. *T. b. rhodesiense*	STIB849	Uganda (Busoga)	1991	Man
22. *T. b. rhodesiense*	STIB850	Uganda (Busoga)	1990	Man
23. *T. b. rhodesiense*	STIB851	Uganda (Tororo)	1990	Man
24. *T. b. rhodesiense*	STIB882	Uganda	1993	Man
25. *T. b. rhodesiense*	STIB883	Uganda	1994	Man

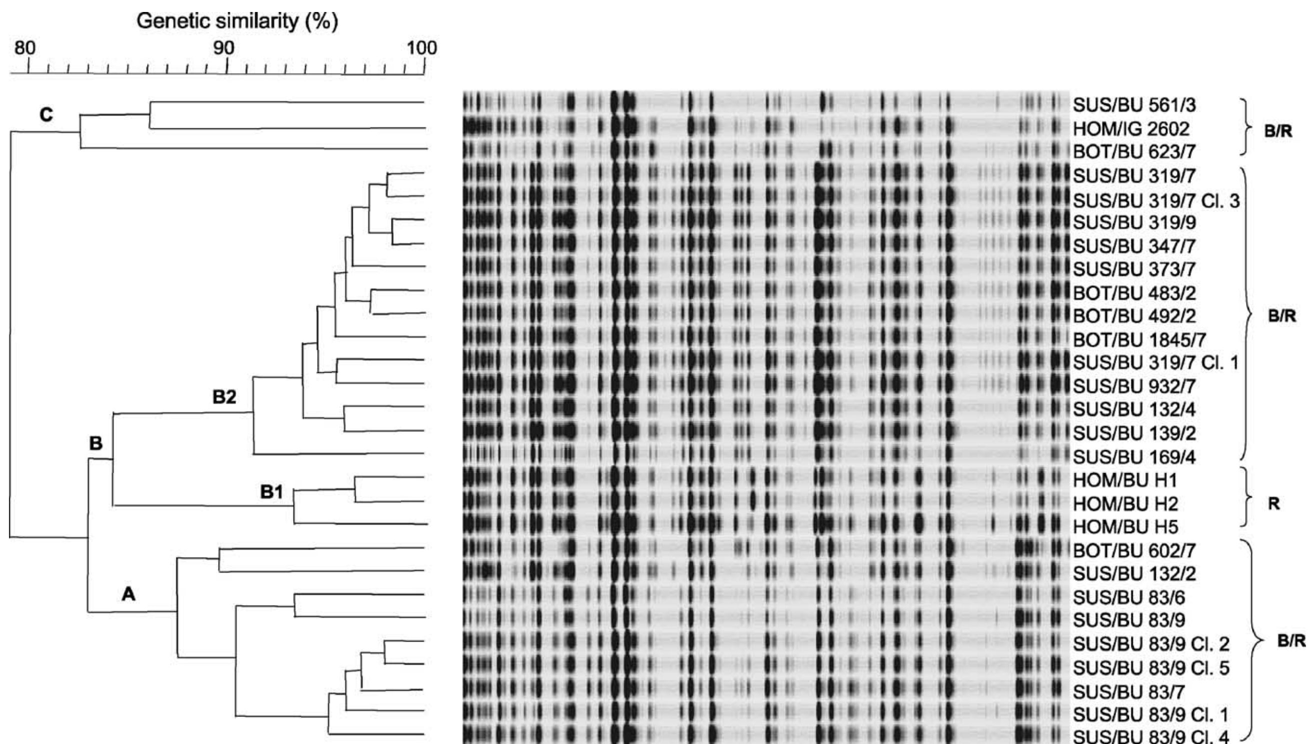

Fig. 1. Dendogram from four-endonuclease analysis of 28 *Trypanosoma brucei* populations isolated during endemic period (1990–1992), based on similarity relationships, according to composite genomic patterns. Cluster A corresponded with *T. b. brucei* populations, whereas clusters B and C each consisted of *T. b. brucei* and *T. b. rhodesiense* populations.

All the samples identified as 'sub-resistant' in the HSRT were negative for the *SRA*-specific PCR product. The specific *TgsGP19* fragment was generated from only the *T. b. gambiense* populations (data not shown).

3.2. Complexity of *T. brucei* populations during an endemic period

According to the composite *Bgl*II–*Bcl*I–*Eco*RI–*Mfe*I (four-endonuclease) analysis pattern for the samples from Mukono district (Table 1), *Bgl*II-0/*Mfe*I-A primer combination resulted in three phylogenetic clusters, i.e. A–C (Fig. 1). The pattern analysis of the control samples (data not shown), as well as human serum response trait and presence of *SRA* gene product, revealed that each cluster consisted of both *T. b. brucei* and *T. b. rhodesiense*. Also, the genetic relatedness and thus the distribution of the samples in the dendogram did not seem to correlate with serum response properties. Thus, samples with the same serum response trait are not necessarily more closely related than stocks and clones with a different trait (Fig. 1). For instance, human serum resistant SUS/BU 347/7 and SUS/BU 373/7 isolated from pig (by definition putative *T. b. rhodesiense*) share a genetic similarity level of 96%. On the other hand, *T. b. rhodesiense* HOM/BU H1 and HOM/BU H2 from different human hosts, which share 96.5% genetic similarity were 93% similar to HOM/BU H5 (cluster B). Furthermore, HOM/IG 2602 from human in another village was distantly outplaced from other *T. b. rhodesiense* (to cluster C). The similarity levels between different populations as determined by numerical analysis of fingerprint patterns were shown to span a continuous range of values between 79 and 98% (Fig. 1), with a dendogram (cophenetic) correlation of 88.4%. However, within each cluster, a highly similar genotype pattern was obtained. Overall, genotype of *T. b. rhodesiense* stocks from man share specific bands, but not the animal isolates (see boxes in Fig. 2).

Since the identity of populations is highly dependent upon the resolution power of the molecular tool employed, the samples were further processed using a combination of six endonucleases and a pair of adapters. The amplified representation fragments and generated fingerprint data were similarly analysed as described for the four-endonuclease procedure (Fig. 2). The approach consistently generated additional restriction fragments to permit for finer genome analysis. However, selection at both fragment ends seemed necessary for generating discrete fragments for further analysis. Numerical analysis of six-endonuclease fingerprint of the Mukono samples revealed two distinct clusters (Fig. 2). Cluster I comprised of a group of seven *T. b. brucei* stocks and clones (which share genetic similarity of more than 90%) and four *T. b. rhodesiense* stocks which are 77.8, 78.5, 80 and 85% related to other *T. b. brucei* stocks. Cluster II sub-divided into two sub-clusters comprising 3 *T. b. rhodesiense* stocks of human origin (A) and 13 *T. b. brucei* or *T. b. rhodesiense* stocks and clones (B), which share a correlation

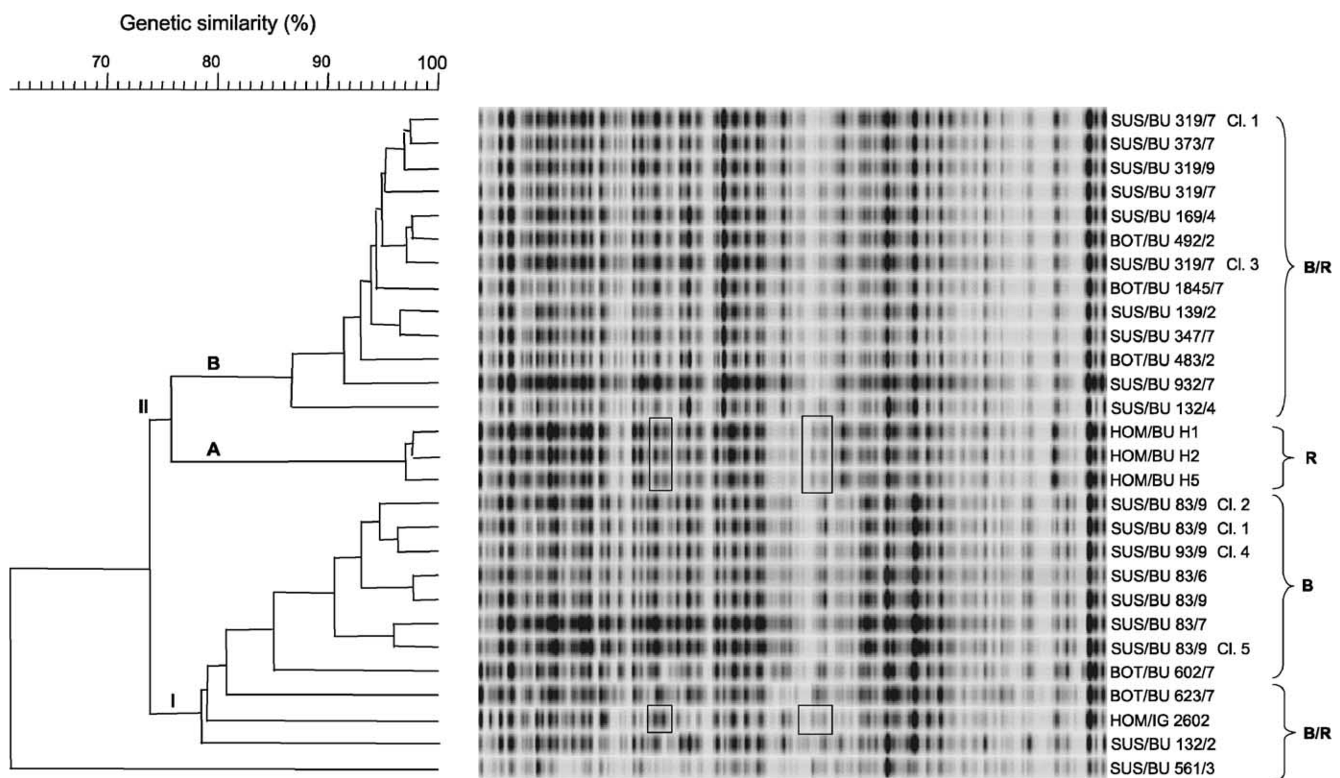

Fig. 2. Relationship between 28 *T. brucei* stocks or clones derived during endemic period survey between 1990 AND 1992, inferred by numerical analysis of fingerprint data generated by six-endonuclease analysis. Subspecies identification (B) and (R) represent *T. b. brucei* and *T. b. rhodesiense*, respectively.

coefficient of 75.5%. Both clusters share an overall similarity that ranged between 73.5 and 98%, with a dendogram (cophenetic) correlation of 95% (Fig. 2). Overall, comparing the dendograms (Figs. 1 and 2), analysis with six endonucleases revealed less genetic similarity between stocks or clones than with four enzymes.

3.3. Cladistic analysis

Genetic relatedness of the samples was evaluated on populations isolated during periods of low circulating parasitism (endemic) and upsurge in disease incidence (epidemic). Fingerprint patterns of the stocks isolated in Mukono district, Uganda between 1990 and 1992 were compared to *T. b. rhodesiense* strains isolated during 1990–1994 epidemic episodes in the same Busoga focus. Relationships between the sets of populations inferred by numerical analysis of fingerprint data were expressed as percentage values of the Pearson correlation product moment coefficient. From the dendogram (Fig. 3), the two groups comprising endemic and epidemic stocks and clones were clearly separated and showed a genetic similarity coefficient of 78%. On this basis, the dendogram was stratified into three groups. Window I represents a population with 79–89% genetic similarity and considered to be 'closely related'. Fingerprints in Window II comprise of stocks or clones that share 90–95% genetic similarity 'highly related', while Window III displays stocks or clones with more than 95% genetic similarity (considered 'identical'). On the basis of this classification, it can be seen that *T. brucei* populations circulating in Mukono district during the endemicity survey were closely related (at 82.8% for most samples, and in the range 79–82.8% for all samples). However, these were slightly different from those isolated during the epidemic within the same Busoga focus (which were 77.8% related). Although several of the stocks isolated during the endemic survey were identical (clonal), there were major genetic differences within the Mukono *T. b. rhodesiense* and *T. b. brucei* stocks, respectively. The genotype profiles indicate that most endemic stocks were genetically separated at a similarity coefficient of 82.8, and 79% for the stocks—SUS/BU 561/3, HOM/IG 2602 and BOT/BU 623/7 (Fig. 3).

Genotypic analysis of *T. b. brucei* stocks isolated from disparate geographical regions during various endemic and epidemic periods resulted in three broad groups, in which the samples isolated during the endemic survey are clustered together, with only 79% genetic similarity (Fig. 4). As expected, the *T. b. rhodesiense* stocks isolated during the 1990–1992 epidemic and the stocks from the endemicity survey (both in Busoga focus) were more genetically related than the disparate *T. b. brucei* populations isolated during epidemic periods. The genetic correlation coefficient

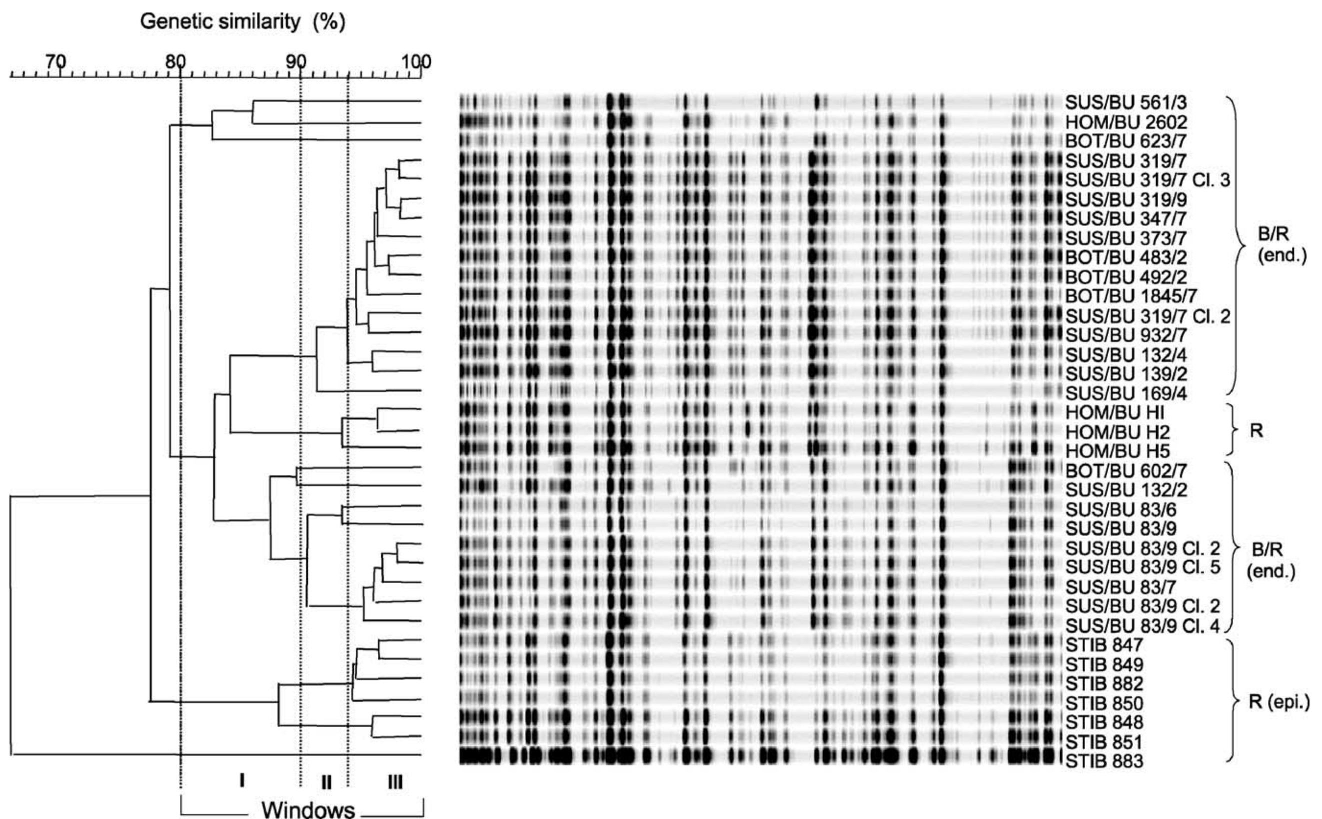

Fig. 3. Assessment of relationship between stocks isolated during endemic and epidemic periods in Busoga focus, Uganda. The extent relatedness of *T. b. brucei* (B) and *T. b. rhodesiense* (R) from epidemic (epi.) and endemic (end.) periods are grouped in Windows I, II and III.

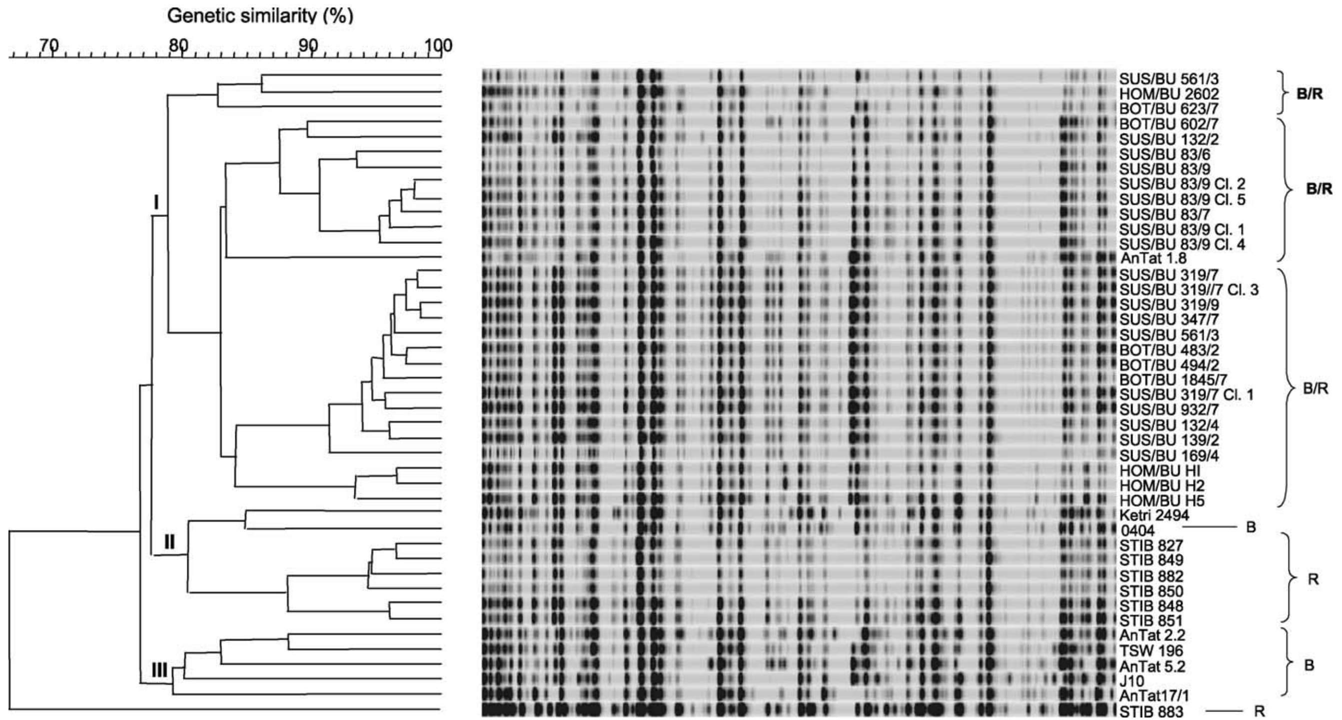

Fig. 4. Dendogram based on fingerprints from four-endonuclease analysis of *T. b. brucei* (B) and *T. b. rhodesiense* (R) populations isolated during endemic and epidemic periods in disparate geographical regions.

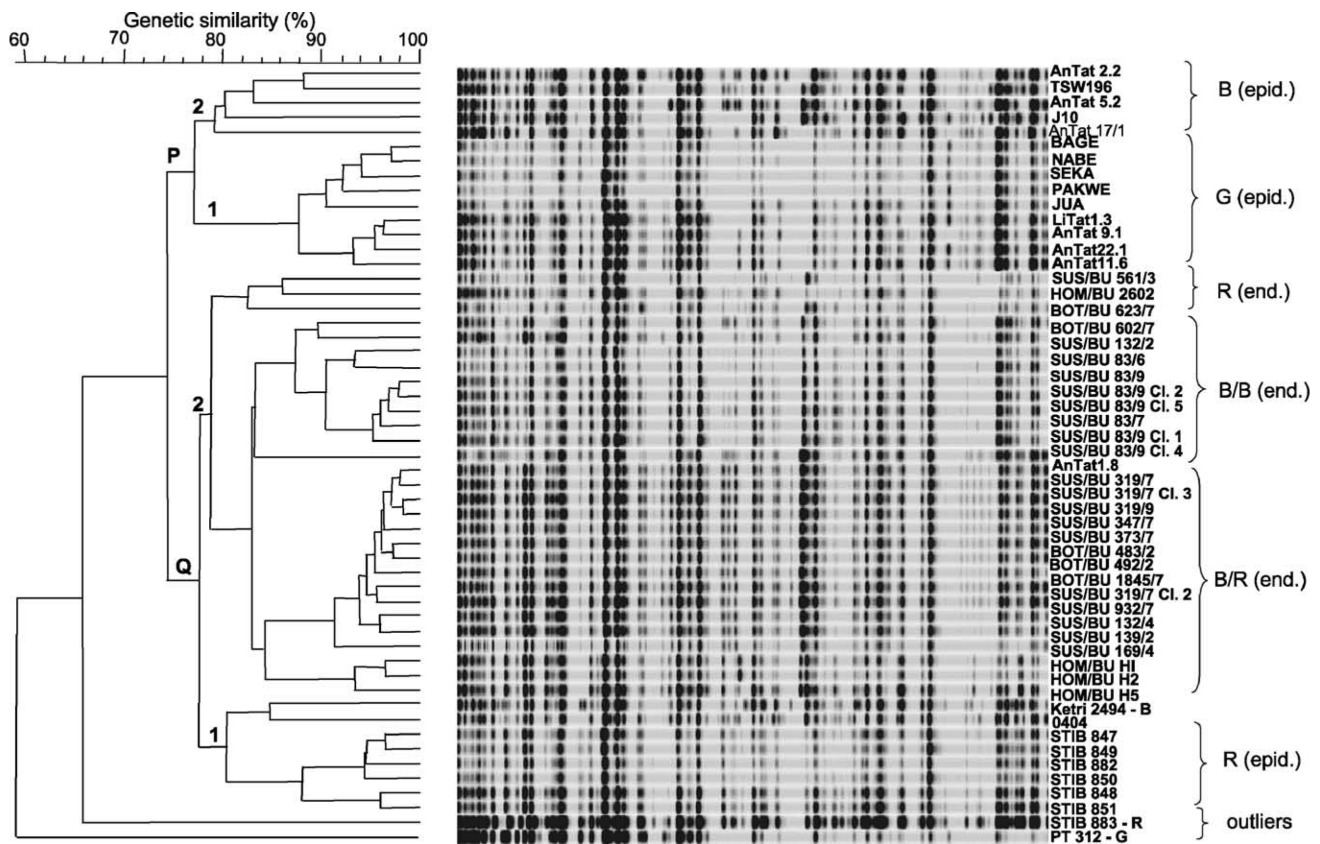

Fig. 5. Fingerprint types and relationship between *T. b. brucei* (B), *T. b. rhodesiense* (R) and *T. b. gambiense* (G) populations from epidemic (epid.) or endemic (end.) periods in Busoga focus and other disparate locations. The two main clusters P and Q are further sub-clustered.

of 77.8% indicated a remarkable difference between genotypes of the epidemic and endemic *T. b. brucei* populations.

When the Mukono samples were compared to all disparate stocks from epidemic episodes, the dendogram correlation showed essentially two clusters (Fig. 5). Cluster P comprises of disparate *T. b. brucei* and *T. b. gambiense* populations derived outside of Busoga focus during epidemic periods, while populations from Busoga focus form cluster Q. Each cluster is subgrouped into two sub-clusters differentiated on the basis of the subspecies identity of the samples. Sub-clusters P1 and P2 comprise of disparate *T. b. gambiense* and *T. b. brucei* stocks and clones, respectively. While the samples comprising the former are genotypically not homogeneous, the latter are most heterogeneous. In addition, the *T. b. gambiense* stocks and clones from Cameroon, Congo and Democratic Republic of Congo are sub-structured into two groups (including LiTat1.3 from Côte d'Ivoire) that share a genetic relatedness of 86.8%. This may also suggest that different genotypes were responsible for the 1995 outbreaks in Democratic Republic (D.R.) of Congo. On the other hand, Ketri2494, isolated in Kenya (1980) from tsetse fly, grouped within cluster Q. It is interesting that Ketri2494 (from Kenya) is sub-clustered in Q-1, while AnTat1.8 also a *T. b. brucei* isolated from Uganda (a closely related geographical area) did not fall within this group (Fig. 5). This indicates that there is close genetic relatedness of Ketri2494 to the epidemic *T. b. rhodesiense* stocks from Uganda. While the samples within cluster P share a coefficient genetic relatedness of only 76.5%, the subgroups comprising cluster Q share genetic similarity of 82.5%. Populations within both clusters share only 74% genetic relatedness. As expected, intra-subspecies relatedness of the disparate epidemic *T. b. brucei* stocks was less than for those derived during the endemic survey in Mukono district. As in previous clustering correlations (Figs. 3 and 4), STIB883 isolate (and now PT312) were classified as outliers in the dendograms. Their correlations to other populations were 64.8 and 59% respectively, indicating distant genetic relatedness. In addition, it shows that the STIB883 was completely different from other stocks circulating during the epidemic in Busoga focus and suggests that new parasite strains that upset host–parasite balance may have an important role in initiating an epidemic episode.

4. Discussion

Using restriction fragment length polymorphisms-based analysis, Hide et al. (1998) found little genetic differences between *T. b. brucei* populations isolated during an endemic period (Busia, Kenya, 1993–1994) and populations isolated during an epidemic period (Tororo, Kenya, 1988–1990), both within Busoga focus. Further, no major differences were found between the genotypes of circulating populations in the two regions. In our study, we expanded the number of genomic sites accessed per analysis using a multiplex set of four and six different endonucleases respectively, to genotype samples from the same focus in order to evaluate the complexity of parasite populations in an area in which trypanosomosis is holoendemic.

In general, there was congruence between clustering based on fingerprint type and parasite subspecies identification of these stocks isolated during an endemic survey (Mukono, Uganda, 1990–1992) and during an epidemic period (Busoga, Uganda, 1990–1994), but remarkable differences were also revealed. As expected, the average genetic correlation coefficient between samples in the dendogram was less when six endonucleases were used for the analysis than when four were used. In contrast to Hide et al. (1998), we found micro-heterogeneities between genomes of populations from both regions. At the time of sampling in Mukono district, 0.7% of villagers, 33.5% of cattle and 52.8% of pigs were harbouring trypanosome infection (Nowak et al., 1992), and it was remarkable to find putative *T. b. rhodesiense* in the pigs, as well as in the cattle (Fig. 1). Thus, besides the acknowledged role of cattle as reservoir for *T. b. rhodesiense* (Welburn et al., 2001; Fevre et al., 2001), pigs may also constitute an important consideration in targeting human disease control strategies, such as molecular epidemiology and chemotherapy.

The importance of identifying human-infective strains in the field to determine the relevance of animal reservoirs and the prevalence of human-infective trypanosomes has previously been highlighted (Hide, 1999). The use of isoenzyme analysis on to characterise *T. brucei* populations permitted early attempts to study genetic diversity and population dynamics of field strains (Godfrey and Kilgour, 1976; Gibson et al., 1980; Tait, 1980). Further isoenzyme studies on populations isolated from the same locations at the same time clearly implicated cattle as potential reservoirs (Gibson and Gashumba, 1983; Gibson and Wellde, 1985). Its application to distinguish *T. b. brucei* and *T. b. rhodesiense* populations failed to establish unequivocal criteria for distinction (Tait et al., 1985; Godfrey et al., 1990). This may also have been so because of the reliance on human serum response trait as a basis for strain characterisation.

In our studies, the *SRA*-PCR offered a good tool for correlating serum response properties of samples to BIIT and HSRT results. However, it is interesting to note that two populations isolated from pig (SUS/BU 347/7, SUS/BU 373/7) and one isolated from cattle (BOT/BU 602/7) shown to be resistant to lysis to human serum in both BIIT and HSRT were negative for the *SRA*-based PCR gene fragment. This suggests possible variations in the sequence of the *SRA* gene leading to the failure to amplify the target during PCR. It may also indicate that BIIT and HSRT assays are not sufficient to unequivocally prove infectivity to humans (i.e. genuine *T. b. rhodesiense*) or underscore that the *SRA* gene is not the only genetic factor conferring resistance to normal human serum. The epidemiological significance of such differences among trypanosome populations has been previously highlighted (Gibson et al., 2002). As shown by our data, the use

of human infectivity as a basis for the characterisation of *T. brucei* populations, especially of those from East Africa, needs to be fully complemented by other fine-scale analysis method. Using this multi-locus genotyping approach, we show that finer genetic differences exist among *T. brucei* stocks derived from the same focus during endemic and epidemic periods, which could be complemented with phenotypic traits in order to characterise strains.

Why human and animal trypanosomosis in East Africa occur in cyclical periods of epidemics, interspersed with long periods of low-level endemicity in only a small number of foci, is unclear. It would be desirable to study the nature of these mechanisms in the overall context of possible inter-subspecies genetic interactions with strains from related and distant epidemiological areas and time. Therefore, as a pilot study, we correlated genotypes of stocks isolated during an endemic survey from related geographical regions and those of disparate stocks from epidemic episodes in distant regions. Our data show that *T. brucei* stocks isolated during 1990–1994 epidemic in Busoga focus were closely related to stocks isolated during a 1990–1992 endemicity survey in Mukono district of Uganda. Further, our data suggest that both groups are sufficiently heterogeneous to exclude clonality. As expected, the heterogeneity coefficient between stocks increased in space and time, and inter-stock differences exist on the basis of geographical origin as well as subspecies identity.

When we compared genotype profiles of the 'type 1' *T. b. gambiense* populations, according to the classification of Gibson (1986), the dendogram correlations gave evidence of genetic sub-structuring within the subspecies (Fig. 5). The 'type 2' strains have mostly been isolated from Côte d'Ivoire and, together with the clustering pattern of the fine-scale genotyping data, might confirm the identity of PT312 as belonging to 'type 2'. This is in contrast to previous isoenzyme study which classified the same strain as 'type 1' (Truc et al., 1997). In addition, STIB883 and PT312 were classified as outliers in the dendogram (Fig. 5) and correlate at 64.8 and 59% respectively, in relation to other populations, indicating distant genetic relatedness to other isolates. This also shows that STIB883 was completely different from other stocks isolated during the epidemic in Busoga focus. Such observation indicates that new parasite strains that upset host–parasite balance may have an important role in initiating an epidemic episode. The introduction of such new strains and subsequent outbreaks of *T. b. rhodesiense* might have been in direct coincidence with trans-humane movement of cattle in the area.

To summarise, we studied within- and between-stock variations using a fine genotyping method to develop an understanding of the levels of variation within and between geographical foci and to describe genetic relatedness of epidemiologically-defined *T. brucei* populations. Genome composition of the stocks seemed sufficiently stable in space and time, in agreement with the findings of Godfrey and Kilgour (1976). However, significant genetic differences exist among stocks even from within a disease focus, while *T. b. brucei* and *T. b. rhodesiense* appear to be sufficiently genetically separated to merit their retention as separate subspecies. Taken together, the data indicate that considerable genetic differences exist among *T. brucei* populations within the same disease focus. These differences are more evident in cladistic analysis of stocks separated in space and time of isolation.

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