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Genetic characterisation of *Trypanosoma brucei* s.l. using microsatellite typing: New perspectives for the molecular epidemiology of human African trypanosomosis

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Abstract

The pathogenic agent of human African trypanosomiasis (HAT) is a trypanosome belonging to the species *Trypanosoma brucei* s.l. Molecular methods developed for typing *T. brucei* s.l. stocks are for the most part not polymorphic enough to study genetic diversity within *T. brucei gambiense* (*T. b. gambiense*) group 1, the main agent of HAT in West and Central Africa. Furthermore, these methods require high quantities of parasite material and consequently are hampered by a selection bias of the isolation and cultivation techniques. In this study, we evaluated the potential value of microsatellite markers (eight loci) in the genetic characterisation of *T. brucei* s.l. compared to the multi-locus enzyme electrophoresis reference technique. Stocks isolated in Ivory Coast and reference stocks were used for this purpose. Microsatellite markers were shown to be polymorphic enough to evidence the existence of genetic diversity within *T. b. gambiense* group 1 and to show the existence of mixed infections. Furthermore, they were able to amplify trypanosome DNA directly from field samples without the usual culturing stages. While the ability of microsatellite markers to detect mixed infections in such field samples is currently being discussed, they appear to be useful to study the parasite population's geographical structure and may provide new insight into their reproductive mode, a topic that is still under debate. Thus, use of microsatellite markers will contribute to the study of the influence of parasite genetics in the diversity of responses to HAT and may contribute to the improvement of HAT molecular diagnosis.

Keywords: *Trypanosoma brucei*; Trypanosomosis; Microsatellite; PCR; Molecular epidemiology; Diagnosis

1. Introduction

The pathogenic agent of human African trypanosomiasis (HAT) or sleeping sickness is a trypanosome belonging to the species *Trypanosoma brucei* (*T. brucei*) transmitted by the bite of a tsetse fly. Classically, *T. brucei* s.l. is subdivided into three subspecies (Hoare, 1972) on the basis of extrinsic criteria (host, clinical features and geographical distribution), since the trypanosomes of this species are morphologically identical: *T. brucei gambiense* (*T. b. gambiense*) is responsible for the chronic form of HAT in Western and Central Africa, *T. b. rhodesiense* is the agent causing the acute form of HAT in East Africa and *T. b. brucei* is not infectious to humans but is infectious to cattle, causing nagana. However, many exceptions to these criteria have been observed. For example, a progression in clinical patterns has been reported for *T. b. gambiense* from asymptomatic forms (Sternberg, 2004) to acute forms (Truc et al., 1997; Garcia et al., 2000). In East Africa, there is a

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long-standing belief that the disease is chronic in southern countries and increases in severity towards the north. In addition, chronic infections with *T. b. rhodesiense* have been described (see MacLean et al., 2004; Checkley et al., 2007).

During the past few decades, molecular methods have been developed for typing *T. brucei* s.l. stocks, partly to study the role that parasite genotypic diversity plays in the range of responses to HAT infections (see Garcia et al., 2006 for review). The reference gold standard technique was and remains multi-locus enzyme electrophoresis (MLEE, see Gibson et al., 1999). In West and Central Africa, only one group has been clearly identified by MLEE as a distinct genetic entity: *T. b. gambiense* group 1 (Gibson, 1986). The remaining heterogeneous stocks have been named *T. b. gambiense* group 2 (Godfrey et al., 1990). Some animal stocks of *T. brucei* s.l. were shown to be closely related to this latter group. In East Africa, using the same MLEE technique, five strain groups were identified within *T. b. rhodesiense* – busoga, kakumbi, kiboko, sindo and zambezi – containing human and animal stocks (reviewed in Gibson et al., 1999). Since then, other molecular methods have been developed for *T. brucei* s.l. strain typing such as restriction fragment length polymorphism (RFLP, Hide et al., 1990), random amplified polymorphism DNA (RAPD, Kanmogne et al., 1996) and amplified fragment length polymorphism (AFLP, Agbo et al., 2002), giving the same type of results. These techniques require high quantities of parasite material that must be isolated and cultured in several stages, resulting in selection bias (Jamonneau et al., 2003) that may have important implications in terms of the population genetics of trypanosomes. This sampling problem could be solved by directly characterising trypanosomes from biological fluids using PCR-based methods (Jamonneau et al., 2004a,b). Recently, in East Africa, using PCR on the serum resistance-associated gene (SRA gene, Gibson et al., 2002) made it possible to distinguish between human infective (i.e. *T. b. rhodesiense*) and non-human infective (i.e. *T. b. brucei*) trypanosomes and proved useful for tracking associations between parasite genotype and the severity of the disease (MacLean et al., 2004).

These PCR-based methods have been developed over the past few years for detecting variation at very polymorphic loci (with a higher mutation rate) to detect genetic differences at a higher resolution. Some very promising results were obtained mainly in East Africa with PCR of minisatellite sequences (Barret et al., 1997; MacLeod et al., 1999, 2000) and of mobile genetic elements (MGE-PCR, Tilley et al., 2003), since these two techniques offer potential as methods for differentiating human infective and non-human infective trypanosomes. Minisatellites were also shown to detect mixed infections (at least in tsetse flies, MacLeod et al., 1999), but the primers designed seem to be unusable for typing *T. b. gambiense* group 1. MGE-PCR could be an effective tool for investigating the genetic diversity of *T. brucei* s.l. (Tilley et al., 2003; Simo et al., 2005) but gives some very complex multi-band patterns, making the interpretation of results difficult, particularly in cases of mixed infections.

The study of microsatellite-length polymorphisms using PCR (microsatellite loci) has been recently and widely used for molecular typing of genetically distinct parasite populations such as *Plasmodium* spp. (Anderson et al., 1999; Greenhouse et al., 2006), *Theileria parva* (Oura et al., 2003), *Cryptosporidium parvum* (Widmer et al., 2004), *Toxoplasma gondii* (Ajzenberg et al., 2002; Dumètre et al., 2006), *Leishmania* spp. (Ochsenreither et al., 2006; Schwenkenbecher et al., 2006) and *Trypanosoma cruzi* (Oliveira et al., 1998; Macedo et al., 2004). These studies were initiated for *T. brucei* s.l. and the preliminary results were promising since the technique, beyond showing a high degree of genetic polymorphism, appeared very specific (Biteau et al., 2000). Furthermore, PCR of microsatellite sequences should be sensitive enough to detect trypanosome DNA in patients' biological fluids but also to detect mixed infections, as already shown in *Plasmodium* studies (Greenhouse et al., 2006). Microsatellite markers were recently widely used for *T. brucei* genetic mapping and allelic segregation studies (MacLeod et al., 2005).

In this study, we evaluated the potential value of microsatellite loci for the genetic characterisation of *T. brucei* s.l. and investigated its potential application in population studies. We used eight microsatellite loci: three selected from previous studies (Biteau et al., 2000; Truc et al., 2002) and five new ones designed in our laboratory. Specifically, we compared this technique with MLEE on 20 reference stocks and 55 field stocks isolated in Ivory Coast using two isolation methods: the kit for *in vitro* isolation of trypanosomes (KIVI, Aerts et al., 1992) and rodent inoculation (RI). In addition, we evaluated the technique for *T. brucei* s.l. profiling directly from blood samples of HAT patients diagnosed in Ivory Coast and tested its ability to detect mixed infection.

2. Materials and methods

2.1. Field sampling

Field sampling was undertaken in 2000, 2002 and 2004 in the Bonon HAT focus in the pre-forest zone of Central-Western Ivory Coast during medical surveys conducted by the HAT National Control Programme (NCP) in collaboration with Institut Pierre Richet, Bouaké, Ivory Coast and the Institut de Recherche pour le Développement (IRD). The HAT diagnostic procedure in the field was carried out according to WHO and NCP recommendations.

In 2000, 38 HAT cases (T+) were bled twice – the first time during the field medical survey (ms) and the second time at the hospital just before treatment (tt) – for KIVI inoculation. There was a minimum interval of 1 week and a maximum interval of 1 month between the two inoculations. Only the 17 T+ for which parasite stocks were isolated twice (labelled ''patient number KIVI ms'' and ''patient number KIVI tt''; for example, for patient S24/7/9, the two isolated stocks were labelled S24/7/9 KIVI ms and S24/7/9 KIVI tt) were included in this study (see Table 1).

In 2002, blood was taken from 33 T+ only during the field medical survey (ms) and inoculated in KIVI and rodent (rodent inoculation, RI) for trypanosome isolation, as described in Jamonneau et al. (2003). Only the seven T+

Table 1
Description of the sampling in the HAT focus of Bonon (Ivory Coast)

Patient	Year	KIVI ms	KIVI tt	RI ms	BS ms
402/1	2000	+	+	ND	ND
B12/2/8	2000	+	+	ND	ND
B3/1/3	2000	+	+	ND	ND
DF1/4	2000	+	+	ND	ND
F41/7/2	2000	+	+	ND	ND
F5/1 OM	2000	+	+	ND	ND
F7/1/2	2000	+	+	ND	ND
G10/6/2	2000	+	+	ND	ND
G11/6/4	2000	+	+	ND	ND
G11/8/2	2000	+	+	ND	ND
G17/6/1	2000	+	+	ND	ND
G3/10/25	2000	+	+	ND	ND
S24/7/9	2000	+	+	ND	ND
S27/16/13	2000	+	+	ND	ND
S27/2/6	2000	+	+	ND	ND
S3/4/1	2000	+	+	ND	ND
T66/4/2	2000	+	+	ND	ND
S12/9/5	2002	+	ND	+	ND
S14/5/1	2002	+	ND	+	ND
S1/1/6	2002	+	ND	+	ND
S7/2/2	2002	+	ND	+	ND
T41/4/14	2002	+	ND	+	ND
TT2/4	2002	+	ND	+	ND
TT22/1	2002	+	ND	+	ND
B4/F303	2004	+	ND	+	+
B4/G27	2004	+	ND	+	+
B4/I314	2004	+	ND	−	+
B4/U163	2004	+	ND	−	+
B4/I315	2004	−	ND	+	+
B4/E120	2004	−	ND	−	+
B4/E427	2004	−	ND	−	+
B4/G13	2004	−	ND	−	+
B4/I36	2004	−	ND	−	+
B4/I245	2004	−	ND	−	+

KIVI ms = KIVI performed during medical survey, KIVI tt = KIVI performed at the time of treatment, RI ms = rodent inoculation performed during medical survey, BS ms = blood sample taken during medical survey, ND = not done.

for which stocks were isolated using both techniques (labelled ''patient number KIVI ms'' and ''patient number RI ms''; for example for patient S1/1/6, the two isolated stocks were called S1/1/6 KIVI ms and S1/1/6 RI ms) were included in this study (see Table 1).

In 2004, blood was also taken from 10 T+ during the medical field survey (ms) for trypanosome isolation. Isolation results are given in Table 1. It is noteworthy that only four and three stocks could be isolated from these patients by KIVI and RI, respectively. For these 10 T+, 1 ml of blood was directly aliquoted in a 1.5-ml microcentrifuge tube and stored at −20 °C with no isolation step. These blood samples (BS) were labelled ''patient number BS ms''. For example, for patient B4/F303, two stocks were isolated: B4/F303 KIVI ms and B4/F303 RI ms, and one blood sample was taken: B4/F303 BS ms.

For each isolated stock, two pellets of parasite were harvested for subsequent MLEE and microsatellite loci analysis. Patients included in this study gave their consent after receiving an explanation of the study's objective and rationale.

2.2. Multi-locus enzyme electrophoresis

MLEE was only performed on the reference and the isolated stocks. Proteins were extracted from one pellet according to Truc et al. (1991). Stocks were characterised by MLEE; seven enzymatic systems (the most polymorphic ones from previous studies) representing 10 loci were revealed: ALAT (EC. 2.6.1.2), GOT (EC. 2.6.1.1), Nhi (EC. 3.2.2.1), ME (EC. 1.1.1.40), PEP-2 (EC. 3.4.11), IDH (1.1.1.42) from Truc et al. (1991) and SOD (EC. 1.15.1.1) from Stevens et al. (1989). As inconsistent results were obtained with PEP-2 and GOT (reproducibility problems), probably caused by differences in metabolic or enzyme activity between trypanosome life-cycle stages, as previously reported (Kaukas et al., 1990; Jamonneau et al., 2003), these two enzymatic systems were excluded from the MLEE analysis.

2.3. Microsatellite loci

The microsatellite loci procedure was carried out on the DNA extracted from the reference stocks, the isolated stocks and the blood samples. All DNA extractions were performed using the DNeasy® Tissue kit (Qiagen). For the blood samples, 500 μl were transferred to a 1.5-ml microcentrifuge tube containing 500 μl of pure water. The mixture was vortexed every other 2 min at room temperature for 10 min. The mixture was then centrifuged at 13,000 × *g* for 4 min, the supernatant gently removed and discarded and the pellet resuspended in 200 μl of PBS. These prepared samples were processed according to the instructions provided by the manufacturer. The last step of the protocol consisted in the elution of DNA in 400 μl AE buffer. For the isolated stocks, a pellet of parasite was resuspended in 500 μl of pure water. The mixture was then processed as described above. DNAs were stored at −20 °C until use.

Microsatellite loci were amplified using eight primer pairs, three of which were selected from previous studies: M6C8-CA (M6C8) and MT30/33-AC/TC (MT30/33) (Biteau et al., 2000) and TRBPA1/2 (Truc et al., 2002). Five new primer pairs were designed in our lab: Micbg1, Micbg5, Micbg6, Misatg4 and Misatg9. Microsatellite sequences and corresponding primers (Table 2) were selected by screening for dinucleotide repeats in the *T. brucei* s.l. genomes (http://www.sanger.ac.uk). The chromosomal location of the microsatellite loci is given in Table 2. Specificity of selected primers was then tested using a BLASTN search (www.ncbi.nlm.nih.gov/blast) against human and mouse genomes but also against *Plasmodium falciparum* and *Trypanosoma congolense*, which are very widespread in our study areas.

All PCR amplifications were carried out in a final volume of 50 μl containing 10 pmol of each primer, 0.2 mM of each desoxyribonucleotide, 1× incubation buffer with 1.5 mM $MgCl_2$, 0.5 units of Taq polymerase (QBIOgene, Ilkirch, France) and the 10-μl DNA sample. For primer pairs designed in our lab, amplification conditions were as follows: 95 °C for 3 min, then two cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, followed by 38 cycles lasting 30 s at 95 °C, 30 s at

Table 2
Description of the eight primer pairs used for microsatellite loci of *T. brucei* samples and number and size of alleles obtained

Designation	Repeat sequence	Sequences (5′–3′)	Chromosome	Total number of alleles	Number of alleles among field-isolated stocks	Allele size (bp)
Micbg1	(CA)n	TAACCCCAAATGCTCTCCCCC CACACCACAGCCAATTCCGCC	II	19	3	136–310
Micbg5	(AT)n	CAAAGCAAAGGAGAAGCGTGA CCCAGTTGGACTTGTTAACGGT	X	15	3	166–238
Micbg6	(AC)n(AT)n	CGGTGTAGTGGCATTCGGGT GCTCGCCGCCTCATGAGGTG	XI	12	2	162–266
Misatg4	(CA)n	GAAGTAACTCTATACGGGTGG GTTTTCCCCTTGTTGTAATC	XI	20	7	087–309
Misatg9	(AC)n	GAGCTCTTACTTATTGCCCGTCT GTGGGGAAGAACAAAGCAGA	V	23	8	116–256
M6C8[a]	(CA)n	CTTTCAACCGCCTTATCAGC GGCTAGTTACACTGTAGTTCTC	XI	28	8	075–275
MT3033[a]	(AC/TC)n	GAGTGACAATGGTGAAGATCG TTTTTCTTTGGTGCTTGTGAG	X	17	4	122–196
TRBPA1/2[b]	(AACCTG)n	GCGCCGACGATACCAATGG AACGGATTTCAGCGTTGCAG	X	19	4	129–275

[a] Biteau et al. (2000).
[b] Truc et al. (2002).

60 °C (Micbg1, Micbg5 and Micbg6 primers) or 56 °C (Misatg4 and Misatg9 primers) and 1 min at 72 °C, and a final extension for 5 min at 72 °C. The amplification conditions of M6C8, MT30/33 and TRBPA1/2 were identical to those previously described (Biteau et al., 2000; Truc et al., 2002). PCR amplification was checked by electrophoresis on 2% agarose gel. Allele bands were then resolved and band size determined in 10% non-denaturing acrylamide gels. A large panel of approximately 30 size markers was run in the same polyacrylamide gel as the samples, allowing the allele size of the samples to be determined accurately (see details in Ravel et al., 2007). For agarose and acrylamide gels, bands were visualized by ethidium bromide staining and UV illumination.

2.4. Microsatellite loci and mixed infections, experimental study

In this study, we also wanted to test experimentally the ability of the primers used to detect mixed infections. Two stocks with different genotypes at each microsatellite locus were selected for this purpose: Biyamina reference stock (*T. b. gambiense* group 2), called A, and S12/9/5 RI ms isolated during this study (*T. b. gambiense* group 1), called B. A 10-ng/μl DNA solution was prepared for each stock and mixed as follows, A/B (1000/1, 100/1, 10/1, 1/1, 1/10, 1/100 and 1/1000) prior to microsatellite amplification.

2.5. UPGMA analysis

An unweighted pair-group method with the arithmetic average (UPGMA) dendrogram was built using the Jaccard genetic distances (*d*, Jaccard, 1908) calculated for visualizing the similarities between the stocks isolated in this study (Sneath and Sokal, 1973). Reference stocks of *T. b. brucei, T. b. rhodesiense* and *T. b. gambiense* groups 1 and 2 were included for the UPGMA comparison between MLEE and microsatellite loci (Table 3). These reference stocks were processed as described above.

3. Results

3.1. Comparison of MLEE and microsatellite loci typing

The isoenzyme patterns obtained for the isolated and reference stocks are given in a Supplementary Table 1 available on the Infection, Genetics and Evolution (IGE) journal's website. The eight loci were polymorphic with two to eight different phenotypes observed with Nhi2 and SOBb, respectively. Stocks giving the same electrophoretic pattern for each locus were grouped by zymodeme. A total of 17 zymodemes (Z1–Z17) were identified. All the stocks from the field belonged to Z1, except TT2/4 RI ms, which belonged to Z2. These two zymodemes only differed on the SODa locus (3-6-7-12-14 for Z1 and 3-6-7-11-12 for Z2). Six of the eight *T. b. gambiense* group 1 reference stocks also belonged to Z1. The two remaining ones, Dal972 and 2561, belonged to Z3 and Z4, respectively, and only differed from Z1 by one locus (IDH and ME2, respectively). These MLEE results showed no differences between stocks isolated by KIVI during the medical survey (KIVI ms) and stocks isolated by KIVI at the time of treatment (KIVI tt) from the same patient. Only one difference was found (SODa) between KIVI and RI stocks isolated from the same patient (TT2/4 KIVI ms = Z1, TT2/4 RI ms = Z2), confirming a

Table 3
Reference stocks used for MLEE and microsatellite loci characterisation, taxonomic identification

Stocks	Parasite form	Host	Origin	Year	Species	Ref.
Jua	Procyclic	Human	Cameroon	1979	Tbg1	Stevens et al. (1992)
Peya	Procyclic	Human	Congo	1980	Tbg1	Stevens et al. (1992)
A005	Procyclic	Human	Cameroon	1988	Tbg1	Stevens et al. (1992)
Dal972	Procyclic	Human	IC	1986	Tbg1	Stevens et al. (1992)
OK	Bloodstream	Human	Congo	1974	Tbg1	Stevens et al. (1992)
2561	Procyclic	Human	IC	1997	Tbg1	Jamonneau et al. (2000)
D12K	Bloodstream	Sheep	Congo	1980	Tbg1	Godfrey et al. (1990)
TH113	Procyclic	Human	IC	1978	Tbg2	Mathieu-Daudé et al. (1995)
TSW65	Procyclic	Pig	IC	1982	Tbg2	Stevens et al. (1992)
TSW53	Bloodstream	Pig	IC	1982	Tbg2	Stevens et al. (1992)
Ligo	Bloodstream	Human	IC	1984	Tbg2	Hide et al. (1990)
Abba	Bloodstream	Human	IC	1983	Tbg2	Hide et al. (1990)
TH2	Procyclic	Human	IC	1978	Tbg2	Mehlitz et al. (1982)
Biyamina	Bloodstream	Human	Sudan	1982	Tbg2	Godfrey et al. (1990)
Feo	Bloodstream	Human	Togo	1961	Tbb	Tait et al. (1984)
Eatro 1125	Procyclic	Bushbuck	Uganda	1966	Tbb	Hide et al. (1990)
Stib 215	Procyclic	Lion	Tanzania	1971	Tbb	Gibson (1986)
TRPZ166	Procyclic	Zebu	Zambia	1982	Tbrh	Mathieu-Daudé et al. (1995)
LVH143	Bloodstream	Human	Kenya	1982	Tbrh	Mathieu-Daudé et al. (1995)

IC, Ivory coast; Tbg1, *Trypanosoma brucei gambiense* group 1; Tbg2, *Trypanosoma brucei gambiense* group 2; Tbb, *Trypanosoma brucei brucei*; Tbrh, *Trypanosoma brucei rhodesiense*.

selection bias of the isolation technique and suggesting a mixed infection for this patient.

Complete microsatellite loci results obtained for the isolated and reference stocks are given in a Supplementary Table 2 available on the IGE website. Nearly all stocks were heterozygous at each microsatellite locus and the number of alleles observed at each locus ranged between a minimum of 12 for Micbg6 to a maximum of 28 for M6C8 (Table 2). While this level of polymorphism was observed in the reference stocks, it was also found in the stocks isolated from the field (two alleles with Micbg6 to eight alleles with Misatg9 and M6C8), thus displaying greater polymorphism than what was observed with MLEE. The allelic patterns observed for the stocks not belonging to *T. b. gambiense* group 1, besides showing a very high diversity, were all different from those observed for the *T. b. gambiense* group 1 stocks. We observed some patterns diagnosing *T. b. gambiense* group 1 from the field and reference stocks with Micbg6, M6C8 and MT30/33 primers. For the field stocks, we observed some conserved patterns within the *T. b. gambiense* group 1 subdivision with Micbg1, Micbg5 and TRBPA1/2 primers. Details are given in a Supplementary Table 2 available on the IGE website.

Stocks giving the same microsatellite multi-locus genotype (M) were grouped. A total of 50 multi-locus genotypes (M1–M50) were identified, 31 of which involved the 55 field isolated stocks (the 19 remaining ones correspond to the 19 reference stocks). While M1 was overrepresented with 14 stocks, the other multi-locus genotypes were represented by one to four stocks.

3.2. Comparison MLEE–microsatellite loci, UPGMA analysis

Jaccard's distances were used to build UPGMA trees, as stated in Section 2, but all the distance data cannot be presented for reasons of space. The UPGMA dendrograms built for visualising the similarities between zymodemes (for MLEE, Fig. 1a) and multi-locus genotypes (for microsatellite loci, Fig. 1b) confirm the highest diversity observed with microsatellite loci. However, the two techniques confirm the homogeneity of *T. b. gambiense* group 1 (grouping the isolated stocks and the *T. b. gambiense* group 1 reference stocks with significant bootstrap values: 90 for MLEE and 85 for microsatellite loci, data not shown), which supports the hypothesis of a monophyletic genetic entity. All the other reference stocks were heterogeneous since no single cluster appeared within these references.

The polymorphism observed with microsatellite loci within the field-isolated stocks made it possible to analyse the genotype distribution within this group and to compare stocks isolated from the same patient (KIVI ms/KIVI tt and KIVI ms/RI ms).

3.3. Microsatellite loci and mixed infections

For 9 patients out of 17 (53%), differences were observed between KIVI ms and KIVI tt stocks isolated from the same patient (Table 4). In four cases, differences were observed at only one locus (Misatg9 or M6C8). For the five other cases, differences were observed at several independent loci. For example, different patterns were observed with Misatg4, Misatg9, M6C8 and MT30/33 primers (located on different chromosomes; see Table 2) between G11/6/4 KIVI ms and G11/6/4 KIVI tt. From these patients, at least two different genotypes were isolated after an interval of several days or weeks. For six cases out of nine (67%), differences were also observed between KIVI ms and RI ms stocks isolated from the same patient at the same time (ms): TT2/4, as already observed with MLEE, and five new ones (Table 4). These results show that mixed infections of at least two stocks circulating in a

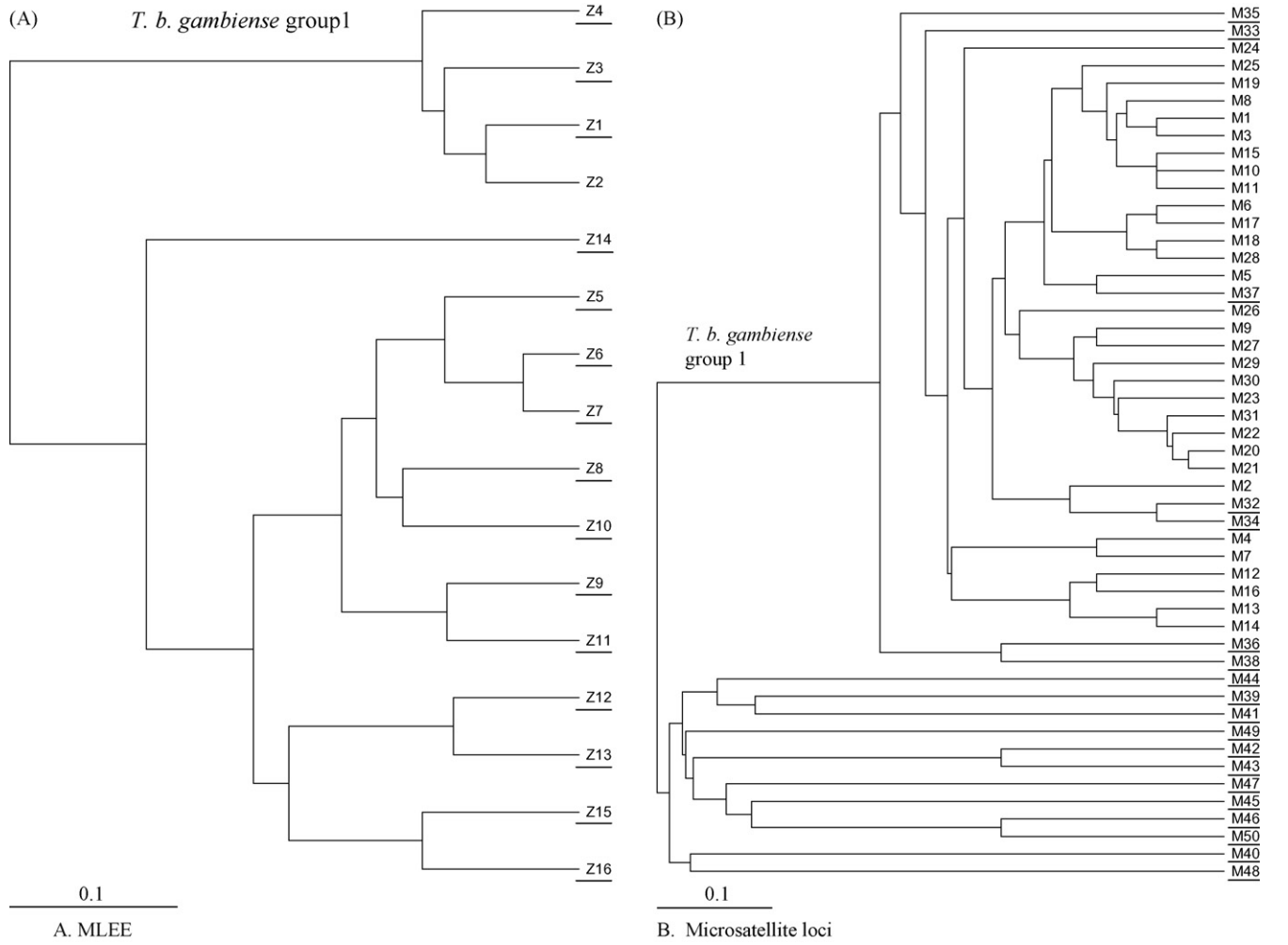

Fig. 1. UPGMA dendrograms based on the matrix of Jaccard genetic distance calculated on MLEE results (A) and microsatellite loci results (B) obtained with the isolated and reference stocks. In (A), all field-isolated stocks under study belonged to Z1 and Z2. Reference stocks are underlined.

patient simultaneously isolated by KIVI and RI may be quite common. The ability to detect mixed infections (KIVI ms/KIVI tt and KIVI/RI) was higher for Misatg9 and M6C8.

3.4. Microsatellite loci typing on blood samples

Our primers were confirmed to be specific since no homology was evidenced using a BLASTN search against *P. falciparum* and *T. congolense* genomes, nor against the human and mouse genomes. This confirms that microsatellite loci can be applied in presence of the host DNA or other parasites, i.e. on blood samples (BSs). For the 10 BSs being studied, the results obtained with the eight primer pairs are detailed in Table 4. Amplification products were obtained for all BSs, with at least six loci confirming that the technique is sensitive enough to detect and amplify trypanosome DNA in human blood. The six amplification failures mainly concern Misatg4 (three cases), but also M6C8 (one case), Micbg6 (one case) and Mistag9 (one case). In general, we observed the same alleles for these BSs as those obtained for the field-isolated stocks. Six BSs belonged to multi-locus genotypes already observed with the isolated stocks (M1, M8, M22, M27). Three new multi-locus genotypes (M51, M52 and M54) differed only at one locus compared to the other field-isolated stocks (M1–M31). The fourth one (M53), corresponding to B4/E427 BS ms, seems to differ more, with 9 specific alleles, but fits into *T. b. gambiense* group 1 in the UPGMA dendrogram including the 10 BSs (Fig. 2).

Surprisingly, no patterns of mixed infection were observed (more than two amplification products), although it was expected at least for B4/F303 and B4/G27, for which mixed infections had been observed with the isolation methods (see above). For instance, in B4/F303, the 121/143 and 085/157 patterns were observed with Misatg4 and M6C8, respectively, for the KIVI stocks (B4/F303 KIVI ms), whereas both the RI stocks (B4/F303 RI ms) and the BSs (B4/F303 BS ms) gave the 115/145 and 085/165 patterns with the same primers (Table 4). In B4/G27, the BS and KIVI stock gave an identical pattern, different from the RI stock pattern. So at least two genotypes circulated in these patients at the sampling time, giving two different isolated stocks using KIVI and RI, but only one was detectable by microsatellite loci in the BS. This prompted us to test the ability of the primers to detect mixed infections experimentally.

Table 4
Microsatellite multi-locus genotypes of: (i) isolated stocks showing the existence of mixed infections and (ii) blood samples

Stock	Microsatellite primers								
	Micbg1	Micbg5	Micbg6	Misatg4	Misatg9	M6C8	MT3033	TRBPA1/2	M
Comparison KIVI ms/KIVI tt									
B3/1/3 KIVI ms	162/192	170/226	182/266	115/145	128/184	**085/157**	**154/170**	149/203	2
B3/1/3 KIVI tt	162/192	170/226	182/266	115/145	128/184	**085/165**	**154/190**	149/203	3
F7/1/2 KIVI ms	162/194	170/226	182/266	117/149	**128/190**	**085/157**	154/190	**149/185**	4
F7/1/2 KIVI tt	162/194	170/226	182/266	117/149	**128/184**	**085/165**	154/190	**149/203**	5
G11/6/4 KIVI ms	162/194	170/226	182/266	**117/149**	**130/190**	**085/157**	**154/178**	149/185	7
G11/6/4 KIVI tt	162/194	170/226	182/266	**115/145**	**128/184**	**085/165**	**154/190**	149/185	8
G11/8/2 KIVI ms	162/194	170/226	182/266	115/145	**128/184**	**085/165**	**154/190**	**149/203**	1
G11/8/2 KIVI tt	162/194	170/226	182/266	115/145	**130/190**	**085/157**	**154/178**	**149/185**	9
G17/6/1 KIVI ms	162/194	170/226	182/266	115/145	**128/184**	085/165	154/190	149/203	1
G17/6/1 KIVI tt	162/194	170/226	182/266	115/145	**128/186**	085/165	154/190	149/203	10
G3/10/25 KIVI ms	162/194	170/226	182/266	115/145	**128/186**	085/165	154/190	149/203	10
G3/10/25 KIVI tt	162/194	170/226	182/266	115/145	**128/184**	085/165	154/190	149/203	1
S24/7/9 KIVI pm	162/194	170/226	182/266	115/145	**128/184**	085/165	154/190	149/203	1
S24/7/9 KIVI tt	162/194	170/226	182/266	115/145	**128/182**	085/165	154/190	149/203	11
S27/2/6 KIVI ms	162/194	170/226	182/266	121/145	130/184	**085/125**	154/190	149/185	13
S27/2/6 KIVI tt	162/194	170/226	182/266	121/145	130/184	**085/149**	154/190	149/185	14
S3/4/1 KIVI ms	162/194	170/226	182/266	**115/145**	**128/176**	**085/165**	154/190	**149/203**	15
S3/4/1 KIVI tt	162/194	170/226	182/266	**121/147**	**130/186**	**085/149**	154/190	**149/185**	16
Comparison KIVI ms/RI ms									
S1/1/6 KIVI ms	162/194	170/226	182/266	115/145	128/184	**085/169**	154/178	149/185	20
S1/1/6 RI ms	162/194	170/226	182/266	115/145	128/184	**085/085**	154/178	149/185	21
S12/9/5 KIVI ms	162/194	170/226	182/266	115/143	128/184	**085/165**	154/178	149/203	17
S12/9/5 IR ms	162/194	170/226	182/266	115/143	128/184	**085/175**	154/178	149/203	18
S7/2/2 KIVI ms	162/194	170/226	182/266	115/145	128/184	**085/165**	154/178	149/185	22
S7/2/2 RI ms	162/194	170/226	182/266	115/145	128/184	**085/085**	154/178	149/185	21
TT2/4 KIVI ms	162/194	170/226	182/266	**115/145**	**128/184**	**085/157**	154/170	**149/185**	23
TT2/4 RI ms	162/194	174/226	182/266	**121/143**	**128/176**	**085/165**	154/170	**149/203**	24
B4/F303 KIVI ms	162/194	170/226	182/266	**121/143**	130/184	**085/157**	154/178	149/185	26
B4/F303 RI ms	162/194	170/226	182/266	**115/145**	130/184	**085/165**	154/178	149/185	27
B4/G27 KIVI ms	162/194	170/226	182/266	**115/143**	128/184	**085/165**	**154/190**	149/203	1
B4/G27 RI ms	162/194	170/226	182/266	**115/145**	128/184	**085/175**	**154/178**	149/203	28
Blood samples									
B4/F303 BS ms	162/194	170/226	182/266	115/145	130/184	085/165	154/178	149/185	27
B4/G27 BS ms	162/19 4	170/226	182/266	115/143	128/184	085/165	154/190	149/203	1
B4/I314 BS ms	162/194	170/226	182/266	115/145	128/184	085/165	154/178	149/185	22
B4/U163 BS ms	162/194	170/226	182/266	115/145	128/184	*0000*	154/190	149/185	8
B4/I315 BS ms	164/200	170/226	182/266	115/145	128/184	085/165	154/190	149/185	52
B4/E120 BS ms	162/194	170/226	*0000*	115/145	*0000*	085/085	154/178	149/203	53
B4/E427 BS ms	162/194	176/210	182/266	115/145	122/256	097/195	126/170	185/215	54
B4/G13 BS ms	162/194	170/226	182/266	101/143	128/184	085/175	154/190	149/185	55
B4/I36 BS ms	162/194	170/226	182/266	115/145	*0000*	085/165	154/178	149/185	8
B4/I245 BS ms	162/194	170/226	182/266	*0000*	*0000*	085/165	154/178	149/185	8

Results are given as follows: XXX/YYY where XXX is the size (band pair) of the smallest allele and YYY is the size of the largest allele. Allelic differences are in bold. 0000 = Amplification failure (in italics).

3.5. Microsatellite loci and mixed infections, experimental study

Each mix of A and B stocks (1000/1, 100/1, 10/1, 1/1, 1/10, 1/100 and 1/1000) was processed with microsatellite loci. The two stocks were only detected (four band patterns) with the following proportions: 1/1 and 1/10 (A/B). For the other proportions, only the stock with highest DNA concentration was detected. Therefore, it seems that the technique is only able to detect mixed infections that are present in nearly equal proportions. As for B4/F303 and B4/G27, mentioned above, at least two genotypes were circulating in these patients, but at the sampling time one was probably over-represented and was the only one detectable by microsatellite loci in the BS.

4. Discussion

In this study, we evaluated the potential value of microsatellite loci for the genetic characterisation of *T. brucei* s.l. compared to the MLEE reference technique, taking into account the selection bias found with isolation techniques. Stocks isolated in Ivory Coast and reference stocks were used for this purpose.

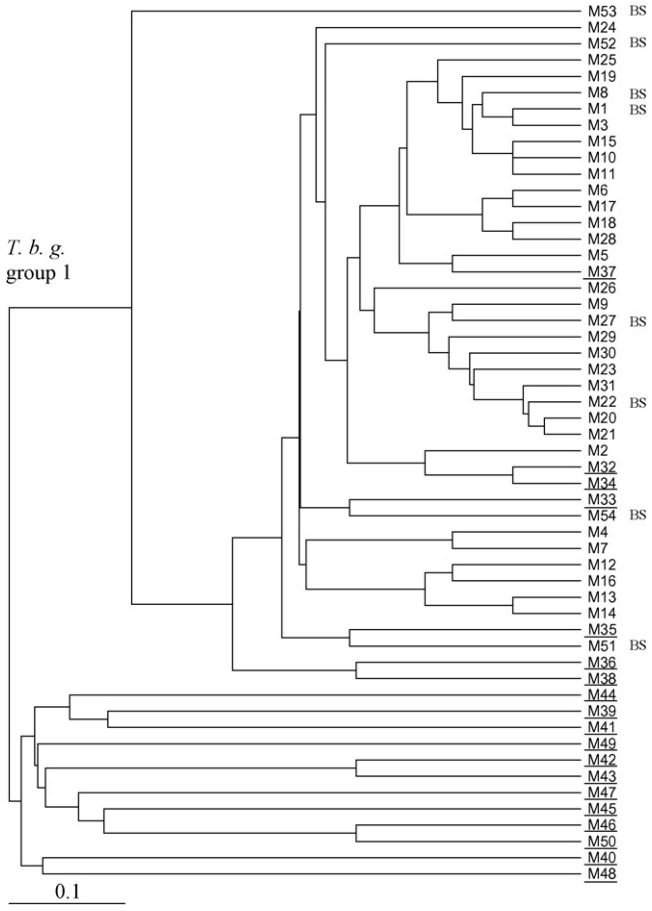

Fig. 2. UPGMA dendrogram based on the matrix of Jaccard genetic distance calculated on microsatellite loci results obtained with the isolated and reference stocks and the blood samples. The blood samples are noted as follows: Mx BS, the reference stocks are underlined.

An important monomorphism was observed with MLEE in the stocks isolated during this study. All the stocks (except TT2/4 RI ms) belonged to the same zymodeme, which has actually been the major zymodeme characterised in West and Central Africa over the past few years, called Z3 in studies conducted in Ivory Coast, Guinea and Equatorial Guinea (Truc et al., 1997; Jamonneau et al., 2000) or Z1 in studies conducted in Cameroon (Njiokou et al., 2004). According to Tibayrenc, "There are no good and bad genetic markers. There are only genetic markers that are better adapted to answer given questions" (Tibayrenc, 1998). These results confirm that MLEE is not well suited for genetic studies aimed at unraveling the genetic diversity of African trypanosomes isolated from humans, thereby justifying the use of other markers such as microsatellites. As expected, microsatellite loci displayed greater polymorphism than MLEE, not only in reference stocks but also in stocks isolated during this study. According to the UPGMA analysis, the microsatellite loci gave the same pattern of results as those obtained by MLEE, demonstrating a monophyletic genetic taxonomic unit, i.e. *T. b. gambiense* group 1, and a very heterogeneous group gathering *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* group 2 reference stocks. These results, already observed in previous studies (Gibson et al., 2002), reinforce the hypothesis describing *T. b. gambiense* group 1 as the species *T. b. gambiense*, and *T. b. rhodesiense* and *T. b. gambiense* group 2 as variants of *T. b. brucei* that could acquire a resistance to human serum (e.g. expression of the serum resistance-associated gene, SRA, in *T. b. rhodesiense*).

It should be noted that no new *T. b. gambiense* group 2 stock was observed during this study, whereas this group had been first and mainly described from genotypes coming from Ivory Coast. To our knowledge, no stock from this group has been isolated from either humans or animals for at least 15 years. The exhaustive use of KIVI (i.e. selection bias) since 1992 was suspected as being responsible for this observation (Jamonneau et al., 2003), but the problem seems to remain genotyping both stocks obtained by rodent inoculation (RI) and trypanosomes from blood samples (BS). This hypothesis of the possible disappearance of *T. b. gambiense* group 2 has to be confirmed on a larger sample size of RI stocks and BSs.

The polymorphism observed within *T. b. gambiense* group 1 (31 microsatellite multi-locus genotypes) evidenced within the 55 isolated stocks (versus only two zymodemes with MLEE) made it possible to analyse the genotype distribution within this group. Differences mainly evidenced with Misatg9 and M6C8 between two stocks isolated by KIVI from the same patient at different times (KIVI ms/KIVI tt) or between two stocks isolated by KIVI and RI from the same patient at the same time (KIVI ms/RI ms) were observed for 53% and 67% of the patients, respectively. Differences between KIVI ms and RI ms *T. b. brucei* stocks from pigs have already been described using MLEE (Jamonneau et al., 2003) and then confirmed with microsatellite markers (unpublished data). These new results not only confirm the existence of mixed infections of at least two genotypes in humans, but also show that such infections are widespread. The consequences of this sort of phenomenon on the diversity of responses to infection must be investigated. Recently, it was suggested that mixed infections could be associated with very low and even undetectable parasitemia in asymptomatic patients, though the mechanisms involved remain to be elucidated (Jamonneau et al., 2004b). Such patterns have already been described for *T. cruzi* infections (Franco et al., 2003). These results show that microsatellite markers could offer new perspectives in studying the role of *T. brucei* s.l. genetic polymorphism in the diversity of responses to HAT infections.

In this study, we confirmed that microsatellite markers are specific and sensitive enough to be applied to *T. brucei* s.l. profiling directly in HAT patient blood samples. All 10 of these samples fit within the homogeneous *T. b. gambiense* group 1, although 1 appeared to be genetically distant (B4/E427 BS ms). It should be noted that no stocks could be isolated from this patient. Further investigations should be conducted on a larger sample of blood samples to confirm these observations. Indeed, such findings may change the epidemiological picture of HAT. No mixed infections were observed within the blood samples and experimental tests confirmed that microsatellite loci PCR cannot detect different genotypes unless they are in equal quantities in the blood sample. This is a major drawback of microsatellite loci and probably of PCR techniques in general: over-represented genotypes may be preferentially amplified

compared to the others. The only solution making it possible to study mixed infections, their prevalence and their epidemiological significance will therefore probably be multiplying isolation attempts using various isolation techniques, as done during the present study. It is worth noting that mixed infections have recently been observed using microsatellite markers in blood samples of asymptomatic patients in Ivory Coast (Jamonneau et al., 2004b). The two genotypes evidenced in these particular cases were probably present in more or less balanced proportions at the time of bleeding. This is also probably the case when detecting mixed infections in tsetse using minisatellite markers (MacLeod et al., 1999) or when detecting mixed infections in *Plasmodium* studies using microsatellite markers (Greenhouse et al., 2006).

The results obtained in this study show that one sampling technique, i.e. isolation techniques (KIVI, rodent inoculation) or blood samples (BS), did not select for specific genotypes. As mentioned above, the selection for a genotype instead results from its aptitude to develop in a given medium at a given time. Genotypes observed within the BSs seem to be potentially representative of those circulating in the natural populations. In this context, despite their inability to detect mixed infection, microsatellite markers seem worth using for molecular epidemiology studies of HAT.

Some patterns obtained with three markers (Micbg1, Micbg5 and TRBPA1/2) seemed to be specific to the HAT Bonon focus, suggesting a geographical structuring within *T. b. gambiense* group 1. In addition, three loci (Micbg6, M6C8 and MT30/33) showed diagnostic patterns specific to *T. b. gambiense* group 1 (reference and isolated stocks). These sensitive markers could be used for the molecular diagnosis of this group (the main causative agent of HAT in West and Central Africa). Microsatellite markers could be used to detect *T. b. gambiense* group 1 in parasitologically unconfirmed seropositive individuals (Garcia et al., 2006) and contribute to studying the role of such subjects, potentially infected by trypanosome but not treated, in HAT transmission sites (Koffi et al., 2006). These markers may also be useful to study tsetse infections, i.e. for epidemiological studies and risk assessment.

In conclusion, the use of microsatellite markers offers new perspectives for the genetic characterisation of *T. brucei* s.l. for several reasons: in addition to being codominant, abundant throughout the genome and easy to score, they allow working from small amounts of biological material (e.g. trypanosomes in BS) and, above all, they seem to be polymorphic enough to study population genetics within *T. b. gambiense* group 1. These markers will make it possible to study the role of the genetic polymorphism of these parasites on the diversity of responses to HAT. Furthermore, they appear to be useful to study the geographical population structure of these parasites and may provide new insight on their reproductive mode, a topic still under debate (clonality versus sexuality). This study is underway in our laboratory on a larger sampling of isolated stocks and blood samples from Ivory Coast and Guinea. As microsatellite typing of parasites were shown to be efficient directly from biological sample, they could also contribute to the improvement of HAT molecular diagnosis since some alleles at various microsatellite loci appeared to be specific to *T. b. gambiense* group 1. This study, based on eight microsatellite markers, shows that microsatellite loci are a promising tool for studying the epidemiology of HAT. It should be noted that numerous other microsatellite markers are now easily available as a result of the diverse genomic projects investigating trypanosome taxa worldwide (see, for example, MacLeod et al., 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2007.07.001.

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