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Research paper

Ribosomal DNA analysis of tsetse and non-tsetse transmitted Ethiopian *Trypanosoma vivax* strains in view of improved molecular diagnosis

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ABSTRACT

Animal trypanosomosis caused by *Trypanosoma vivax* (*T. vivax*) is a devastating disease causing serious economic losses. Most molecular diagnostics for *T. vivax* infection target the ribosomal DNA locus (rDNA) but are challenged by the heterogeneity among *T. vivax* strains. In this study, we investigated the rDNA heterogeneity of Ethiopian *T. vivax* strains in relation to their presence in tsetse-infested and tsetse-free areas and its effect on molecular diagnosis.

We sequenced the rDNA loci of six Ethiopian (three from tsetse-infested and three from tsetse-free areas) and one Nigerian *T. vivax* strain. We analysed the obtained sequences *in silico* for primer-mismatches of some commonly used diagnostic PCR assays and for GC content. With these data, we selected some rDNA diagnostic PCR assays for evaluation of their diagnostic accuracy. Furthermore we constructed two phylogenetic networks based on sequences within the smaller subunit (SSU) of 18S and within the 5.8S and internal transcribed spacer 2 (ITS2) to assess the relatedness of Ethiopian *T. vivax* strains to strains from other African countries and from South America.

In silico analysis of the rDNA sequence showed important mismatches of some published diagnostic PCR primers and high GC content of *T. vivax* rDNA. The evaluation of selected diagnostic PCR assays with specimens from cattle under natural *T. vivax* challenge showed that this high GC content interferes with the diagnostic accuracy of PCR, especially in cases of mixed infections with *T. congolense*. Adding betain to the PCR reaction mixture can enhance the amplification of *T. vivax* rDNA but decreases the sensitivity for *T. congolense* and *Trypanozoon*. The networks illustrated that Ethiopian *T. vivax* strains are considerably heterogeneous and two strains (one from tsetse-infested and one from tsetse-free area) are more related to the West African and South American strains than to the East African strains.

The rDNA locus sequence of six Ethiopian *T. vivax* strains showed important differences and higher GC content compared to other animal trypanosomes but could not be related to their origin from tsetse-infested or tsetse-free area. The high GC content of *T. vivax* DNA renders accurate diagnosis of all pathogenic animal trypanosomes with one single PCR problematic.

1. Introduction

In almost half of the African countries, 30% of the population is primarily dependent on livestock for their survival. Among the devastating animal diseases, African animal trypanosomosis (AAT) holds the lion share in reducing the benefit to be obtained from livestock agriculture (Perry and Grace, 2009). The problem of trypanosomosis, affecting both human and animal health, is significant and eradication of the disease figures among the millennium development goals of African countries (Programme Against African Trypanosomiasis, 2008; Shaw, 2009).

Abbreviations: AAT, animal African trypanosomosis; Indels, inserstion-deletion polymorphisms; ITS1, internal transcribed spacer 1; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; rDNA, ribosomal deoxyribonucleic acid; SSU, small subunit; TvPRAC, *Trypanosoma vivax* proline racemase.

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AAT is a parasitic disease caused by different pathogenic species of Salivarian trypanosomes of which several affect livestock by inducing anaemia, loss of condition and emaciation and many untreated cases are fatal (Batista et al., 2011). Among the Salivarian trypanosomes, *Trypanosoma vivax* (*T. vivax*) is transmitted both cyclically and mechanically and the latter transmission route has enabled it to spread beyond the tsetse-infested areas of Africa and even into South America (Birhanu et al., 2015; Fikru et al., 2012; Osório et al., 2008; Sinshaw et al., 2006). Several reports describe that West African *T. vivax* strains are more pathogenic than East African strains and that they are genetically more closely related to South American strains (Cortez et al., 2006; Gardiner and Mahmoud, 1992; Ventura et al., 2001). A recent study based on the cathepsin L-like gene confirms the relatedness of West African and South American isolates (Nakayima et al., 2013), but in another study, phylogenetic analysis on smaller subunit (SSU) variable (V7-V8 region) region of 18S sequences showed that *T. vivax* strains from Tanzania and Mozambique differ from the East African and West African strains, whilst they still cluster within the *T. vivax* clade (Rodrigues et al., 2008). Similarly, among *T. vivax* strains from wild animals in the Serengeti and Luangwa valley ecosystems, three variants were observed based on ITS1, 5.8S and ITS2 sequences and even a new genotype of *T. vivax* was detected in tsetse flies captured in Tanzania (Adams et al., 2010a,b; Auty et al., 2012).

The degree of genetic heterogeneity was reported to be higher in East African *T. vivax* as compared to West African and South American *T. vivax* (Adams et al., 2010b). In South America, *T. vivax* is transmitted only mechanically, favoring clonal expansion, whereas in Africa both mechanical and cyclical transmission occur, the latter allowing genetic exchange between strains. Clonal expansion resulting from tsetse independent transmission is well documented in *T. evansi* and *T. equiperdum* and has been suggested to occur as well in *T. vivax* populations in The Gambia (Duffy et al., 2009; Tait et al., 2011). However, to confirm that genetic diversity of African *T. vivax* strains is related to cyclical transmission by tsetse flies thus explaining the high homogeneity of South American isolates, analysis of more isolates from tsetse-infested and tsetse-free areas is needed (Rodrigues et al., 2008). In studies on AAT, the genetic heterogeneity of *T. vivax* hampers unequivocal identification of the infecting species because most *T. vivax*-specific diagnostic PCR tests are based on West African *T. vivax* DNA sequences and some fail to recognise many *T. vivax* infections in livestock and tsetse in East Africa (Adams et al., 2010b; Fikru et al., 2014; Gonzatti et al., 2014).

The ribosomal DNA (rDNA) locus is a preferential target to develop species-specific molecular diagnostics because of its multicopy nature (Desquesnes et al., 2001; Geysen et al., 2003; Njiru et al., 2005). The 18S and 28S rRNA genes are ideal targets to design simple generic diagnostic PCR tests because they are composed of alternating conserved and variable domains, allowing the variable domains to be amplified using primers in the flanking conserved regions. Therefore, sequence analysis of the rDNA locus from several representative *T. vivax* strains may help to solve problems associated with accurate identification of all *T. vivax* strains regardless of their geographic origin and/or means of transmission. In this study we sequenced the rDNA locus of seven *T. vivax* strains to assess whether sequence heterogeneity can be related to their geographical origin and whether this has an effect on the accuracy of molecular diagnostics, in particular for detecting multiple-species infections.

2. Methods

T. vivax DNA was extracted from the Nigerian strain Y486 (ILRAD 700) grown in mice, from 4 Ethiopian strains (4337, 4338, Di, Fc) grown in calves and from the blood of two Ethiopian bovines (306, 310) that were naturally mono-infected with *T. vivax* (Fikru et al., 2012) (Table 1). All strains were confirmed as *T. vivax* by *Tv*PRAC PCR and ITS1-PCR (Fikru et al., 2014). All strains are available on request.

The full sequence of the rDNA locus of the Nigerian strain *T. vivax* Y486 has been published in GenBank (U22316). The nucleotide basic local alignment search tool (BLASTn) revealed a 99% sequence identity with another full sequence of the rDNA locus on chromosome 3 of the same *T. vivax* strain (HE573019). Based on these two sequences, we designed with Primer3 software two diagnostic primer pairs (A-B, E-F) to amplify 2 overlapping fragments of about 2350 bp in order to get the full sequence of the rDNA locus (Fig. 1) (Koressaar and Remm, 2007). These primers were designed in compliance with the In-Fusion cloning protocol (Clontech) with addition of 15 nucleotides that are complementary to the cloning site on the plasmid. Two more primer pairs (C–D, G–H) were designed to sequence the inner part of each fragment to get sufficient overlap for sequence assembly. The primer sequences, their binding sites and expected amplicon sizes based on the *T. vivax* rDNA GenBank sequence U22316 are shown in Table 2.

The two overlapping fragments (fragment AB and EF) were PCR amplified with proofreading Phusion® Hot Start Flex DNA polymerase (New England Biolabs). The reaction was carried out in a PCR cocktail containing in 1× Phusion GC buffer: 1 mM dNTPs, 0.5 μM forward and 0.5 μM reverse primer, 5% DMSO and 1 unit Phusion Hot Start Flex DNA polymerase. A template DNA concentration of about 50 ng was used per reaction. The cycling conditions for the amplification of fragment AB were: initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 61 °C for 30 s and polymerisation reaction at 72 °C for 90 s, and a final extension at 72 °C for 7 min. For fragment EF, the cycling conditions were similar but with a primer annealing temperature of 64 °C. The presence of an amplification product was confirmed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV transilluminescence.

The amplicons were cloned into *Bam*HI linearised pUC19 vector (In-Fusion® HD Cloning kit; Clontech) and transformed into chemically competent Stellar *E. coli* cells. To construct the insert, 5 μL of PCR product was mixed with 2 μL of cloning enhancer and incubated for 15 min at 37 °C followed by 15 min at 80 °C. Two μL of this mixture were added to 5× In-Fusion HD enzyme premix, 1 μL of linearised pUC19 vector and 5 μL of distilled water and incubated for 15 min at 50 °C. Transformation was done by mixing 2.5 μL of the plasmid construct with 100 μL of chemically competent Stellar *E. coli* cells, incubating on ice for 30 min followed by a heat shock at 42 °C for 45 s. The cells were recuperated in SOC medium (Clontech) after incubating at 37 °C under shaking at 300 rpm for 1 h. Hundred μL of the cells were plated on selective agar plates, Lysogeny Broth-carbenicilin, containing 40 mg/mL X-gal to allow blue/white selection.

Colony-PCR was carried out on the white bacterial colonies to ascertain the presence of the required fragments. The following primers that anneal on the plasmid and on the insert were used: fragment 1 forward (18SColF, 5′-CTCTTCGCTATTACGCCAGC-3′) and reverse (18SColR, 5′-GTGATTGCGGCAGATTACGT-3′) and fragment 2 forward (5.8SColBF, 5′-CTCTTCGCTATTACGCCAGC-3′) and reverse (5.8SColBR, 5′-TGTTCGACACTGAGACTGCG-3′). Plasmids containing the insert were purified using Qiagen Miniprep plasmid extraction kit. Two clones were sequenced for every stain except for fragment 2 of Y486, 4338 and Di, where only one clone was sequenced. Sequencing was carried out at the VIB gene sequencing facility of the University of Antwerp. The obtained sequences were assembled using DNAMAN and aligned to each other using CLC Sequence Viewer 6.8.2.

Table 1
International code, alias name and origin of *T. vivax* strains used in this study. na = not applicable.

International code for trypanosome primary isolates	Alias name	Origin	Reference
na	Y486[a]	Nigeria	(Leeflang et al., 1976)
MBOV/ET/2012/AAU-VMA/001	4337	Ethiopia (tsetse infested)	(Fikru et al., 2014)
MBOV/ET/2012/AAU-VMA/002	4338	Ethiopia (tsetse infested)	(Fikru et al., 2014)
MBOV/ET/2012/AAU-VMA/003	Di	Ethiopia (tsetse infested)	(Fikru et al., 2014)
MBOV/ET/2012/AAU-VMA/004	Fc	Ethiopia(tsetse free)	(Fikru et al., 2014)
na	306	Ethiopia (tsetse free)	Unpublished
na	310	Ethiopia(tsetse free)	Unpublished

[a] Also known as ILRAD 700 and represented by the clone ILDat1.2 (Cortez et al., 2006).

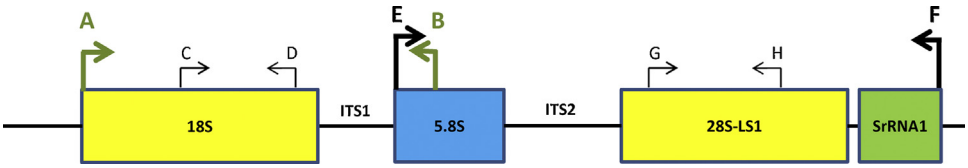


Fig. 1. Schematic representation of the rDNA locus and the primer binding sites for the amplification of the desired fragments. Primer pairs A–B and E–F were used to amplify the first and the second fragment of the rDNA locus respectively, while C–D and G–H were used as internal primers to sequence the first and second fragments.

Table 2
Primers used for PCR amplification and sequencing of the rDNA locus. The letters between brackets behind the names refer to the primers represented in Fig. 1.

Target	Name	Sequence 5′ → 3′ on plasmid	Sequence 5′ → 3′ on rDNA	Binding site	Amplicon size
Fragment AB	Tv18SF-IF (A)	CGGTACCCGGGGATC	CTCTGGTTGATTCTGCCAGTAGT	18S at 3–24 bp	2341 bp
	Tv18SR-IF (B)	CGACTCTAGAGGATC	CCCGAGTGGTTCTGCAGGTCAT	5.8S at 2343–2324 bp	
	Tv18sF (C)		GCGCCCGCGAGTCTTTA	18S at 821–838 bp	1000 bp
	Tv18sR (D)		CTCCACCGACCAAAAGCG	18S at 1575–1593 bp	
Fragment EF	Tv5.8SF-IF (E)	CGGTACCCGGGGATC	CAGCAAAGCGCGATAGTTGG	5.8S at 2303–2332 bp	2294 bp
	Tv5.8SR-IF (F)	CGACTCTAGAGGATC	CGCGCAGAACTCACAACAAGG	srRNA1 at 4596–4577 bp	
	Tv5.8s F (G)		GTAGCGTGAGCGAAAGTTTG	ITS2 at 3022–3042 bp	1000 bp
	Tv5.8s R (H)		GCGCGCCCTTGCG	28S at 3756–3769 bp	

Accuracy of diagnostic PCR assays was assessed on DNA samples extracted from the blood of 46 bovines (23 positive and 23 negative in *Tv*PRAC PCR) collected during an epidemiological survey in Ethiopia. Details on these samples have been published previously (Fikru et al., 2012). The following diagnostic PCR assays were tested without and with the replacement of 5 μL H_2O by 5 μL of 5x Q-Solution (Qiagen) in the reaction mixture in order to increase specificity and sensitivity: ITS1 Touchdown PCR, ITS1 PCR and nested ITS PCR (Cox et al., 2005; Desquesnes et al., 2001; Tran et al., 2014).

The reaction conditions for ITS1 PCR were as follows: 25 μL reaction volume contained 12.5 μL Taq PCR Master Mix (Qiagen) (containing a final concentration of 1.5 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U Taq DNA polymerase and 1× buffer), 0.4 μM of ITS-1 forward primer (5′-TGTAGGTGAACCTGCAGCTGGATC-3′) and 0.4 μM of ITS-1 reverse primer (5′-CCAAGTCATCCATCGCGACACGTT-3′), 0.1 mg/mL acetylated BSA (Promega) in H_2O and 2.5 μL target DNA. The cycling conditions were as described in Fikru et al. (Fikru et al., 2012).

For the ITS1 Touchdown PCR (ITS1 TD PCR) the reaction conditions were: 25 μL reaction volume containing 12.5 μL Taq PCR Master Mix (Qiagen), 0.2 μM of ITS-1 forward primer (5′-TGTAGGTGAACCTGCAGCTGGATC-3′) and 0.2 μM of ITS-1 reverse primer (5′-CCAAGTCATCCATCGCGACACGTT-3′), 0.1 mg/mL acetylated BSA (Promega) in H_2O and 2.5 μL target DNA. The cycling conditions were as described in Tran et al. (Tran et al., 2014).

For the nested ITS PCR the reaction conditions were: 25 μL reaction volume containing 12.5 μL Taq PCR Master Mix (Qiagen), 1 μM ITS1 (5′-GATTACGTCCCTGCCATTTG-3′) and 1 μM ITS2 (5′-TTGTTCGCTATCGGTCTTCC-3′) primer and 2.5 μL target DNA. The cycling conditions were as described in Cox et al. (Cox et al., 2005).

To infer the relationships of the *T. vivax* sequences obtained in this study with existing *T. vivax* sequences, phylogenetic networks were constructed. Full or partial sequences within the rDNA locus of different *T. vivax* were obtained from GenBank. Isolate names, origin and sequence accession numbers are presented in Table 3. All the sequences were subjected to multiple alignment using MUSCLE with default settings and edited to the length of available sequences (Edgar, 2004). The SSU network was built with NETWORK v. 4.6 (fluxus-engineering.com) using the median joining algorithm (Bandelt et al., 1999). The 5.8S-ITS2 region neighbor-net analysis network was built with SplitsTree v.4.14.2 with uncorrected *p*-distance and gaps included (Huson and Bryant, 2006). The support for each branch was evaluated by bootstrap with 1000 replicates. GC content was calculated by MEGA 6 software (Tamura et al., 2013).

3. Ethics statement:

For the collection of blood specimens from bovines, ethical approval was obtained from the Ethics Committee for Veterinary Medicine of the Institute of Tropical Medicine Antwerp (EXT 2012-1, EXT 2012-2 and BM-2013-1). All protocols adhere to the European Commission Recommendation on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (18 June, 2007, 2007/526/EG) and the Belgian National law on the protection of animals under experiment.

4. Results

The sequence of fragment AB (18S, ITS1 and part of the 5.8S) was obtained from six Ethiopian strains, three from tsetse-infested and three from tsetse-free areas. In addition, we also sequenced this fragment from the Nigerian *T. vivax* strain Y486, also known as ILRAD 700 (Table 4). The sequence of fragment EF (parts of 5.8S, ITS2, 28S-LS1 and SrRNA1) was obtained for four isolates from

Table 3
T. vivax isolate names, origin of the organism and rDNA locus complete and partial sequence accession numbers.

Isolate	Origin	SSU	ITS region
ILDat1.2	Nigeria	U22316	U22316
TS06009	Tanzania		JN673394
TviMzNy	Mozambique	EU477537	EU482078, EU482080, EU482081, EU482082
IL3905	Kenya	DQ317414	DQ316037, DQ316038, DQ316039, DQ316040, DQ316041, DQ316042, DQ316043, DQ316044
TviBrMi	Brazil	DQ317415	DQ316047, DQ316048
TviBrCa	Brazil	DQ317413	DQ316045, DQ316046
TviBrPo	Brazil		DQ316049, DQ316050
TviVeMe	Venezuela	DQ317416	DQ316051, DQ316052

Table 4
GenBank accession numbers of rDNA sequences of the different *T. vivax* strains obtained in this study. na = not applicable.

		Accession number	
T. vivax strain	Clone	rDNA fragment AB	rDNA fragment EF
306	1	KM391816	na
	2	KM391817	na
310	1	KM391818	na
	2	KM391819	na
MBOV/ET/2012/AAU-VMA/001	1	KM391820	KM391830
	2	KM391821	KM391831
MBOV/ET/2012/AAU-VMA/002	1	KM391822	KM391832
	2	KM391823	na
MBOV/ET/2012/AAU-VMA/003	1	KM391824	KM391833
	2	KM391825	na
MBOV/ET/2012/AAU-VMA/004	1	KM391826	KM391834
	2	KM391827	KM391835
Y486[a]	1	KM391828	KM391836
	2	KM391829	na

[a] Also known as ILRAD 700 and represented by the clone ILDat1.2 (Cortez et al., 2006).

Ethiopia (three from tsetse-infested area and one from tsetse-free area) and the Nigerian *T. vivax* strain. Fragment EF could not be obtained from two strains from tsetse-free area due to insufficient template DNA extracted from the buffy coat of infected animals (Table 4).

Nucleotide composition analysis of the obtained sequences revealed rather high GC contents with an average of 58.4% in rDNA fragment AB, 62.0% in rDNA fragment EF and even 68.6% in the ITS1 region.

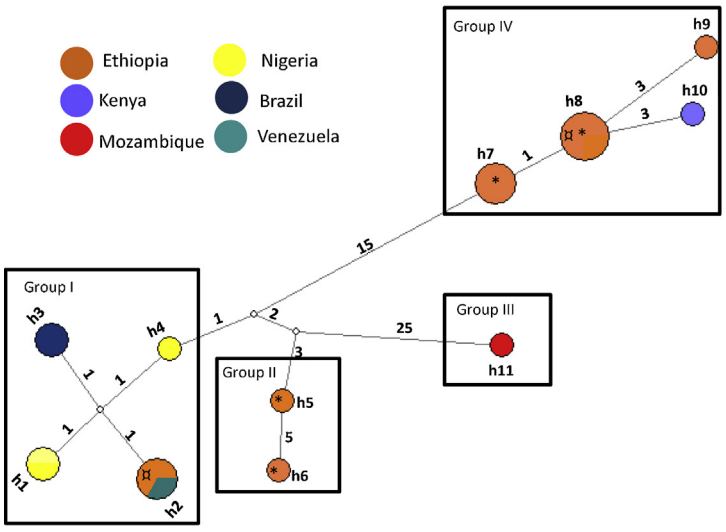
In silico analysis indicated that the ITS1 primers designed by Desquesnes et al. which are also used in the ITS1 TD PCR and that the nested ITS PCR primers designed by Cox et al., which are the same as used by Thumbi et al., perfectly match with all the sequences obtained in our study (Cox et al., 2005; Desquesnes et al., 2001; Thumbi et al., 2008; Tran et al., 2014). On the other hand, the ITS1 forward primer 5′-CCGGAAGTTCACCGATATTG-3′ used by Njiru et al. shows one nucleotide mismatch (as indicated by the underscore) (Njiru et al., 2005). Also, the primers used by Geysen et al. in a 18S PCR-RFLP showed some mismatches with the corresponding *T. vivax* sequences (Geysen et al., 2003) (18ST nF2 5′-CAACGATGACACCCATGAATTGGGGA-3′, 18ST nR2 5′-GTGTCTTGTTCTCACTGACATTGTAGTG-3′). In addition, both primers show mismatches with known *T. congolense* and *Trypanozoon* sequences, mostly at their 5′ end.

Given the high GC content of the rDNA region of *T. vivax*, we analysed whether adding betaine (Q-solution) to the PCR reaction mixture had an influence on the accuracy of diagnostic PCR assays for detection of *T. vivax* infection. We performed three diagnostic PCR assays for which no primer mismatches were observed on 46 DNA samples (23 *Tv*PRAC positive and 23 *Tv*PRAC negative) extracted from the blood of cattle under natural AAT challenge comprising mixed and single infections of *T. vivax*, *T. congolense*, *T. theileri* and *Trypanozoon* (Fikru et al., 2012). The results are represented in Table 5. With ITS1 PCR, irrespective of the addition of Q-solution, we observed a higher number of positives for all the trypanosome species compared to ITS1 TD PCR and nested ITS PCR. Adding Q-solution in the PCR mixture of ITS1 TD PCR profoundly decreases the number of positive reactions from 44 to 12. In contrast, adding Q-solution to the ITS1 PCR results in stronger *T. vivax*-specific bands thus revealing more (25) *T. vivax* infections than *Tv*PRAC (23). These two additional *T. vivax* positives can be explained by the fact that *Tv*PRAC PCR targets a single copy gene whereas ITS1 occurs in multiple copies. Moreover, the addition of Q-solution to the ITS1 PCR reduced the number of nonspecific amplicons that can be wrongly interpreted as *T. vivax* positive (29 without Q-solution compared to 25 with Q-solution). However, the increased accuracy in *T. vivax* detection was accompanied by weaker *T. congolense*-specific and *T. theileri*-specific bands. Also in the nested ITS PCR, amplification of *T. vivax*-specific sequences is enhanced by adding Q-solution, while for *T. congolense* and *T. theileri* amplification is reduced.

For the network analysis of the 18S rRNA, a twenty *T. vivax* sequences (Tables 3 and 4) alignment (681 bp) was generated (Supplementary file S1). The 62 polymorphic sites identified in the data set, lead to the definition of eleven haplotypes (Fig. 2). Of the eleven haplotypes six of them were singleton haplotypes. The haplotype relationships indicated four groups. The first group contains the West African strains Y486 and ILDat1.2,

Table 5
Number of positives among 23 *Tv*PRAC PCR positive and 23 *Tv*PRAC PCR negative bovine samples tested in ITS1, ITS1-TD and nested ITS PCR with and without addition of Q-solution in the reaction mixture. QS+ = with Q-solution, QS− = without Q-solution.

	ITS1 PCR		ITS1-TD PCR		nested ITS PCR	
	QS+	QS−	QS+	QS−	QS+	QS−
T. vivax	25	29	7	20	20	14
T. congolense	17	22	5	19	14	19
Trypanozoon	0	1	0	0	0	0
T. theileri	12	12	0	5	1	6



Hap	Sequences
h1	ILDat1.2; Y486_cl1
h2	TviVeMe; MBOV/ET/2012/AAU-VMA/004_cl1; MBOV/ET/2012/AAU-VMA/004_cl2
h3	TviBrMi; TviBrCa
h4	Y486_cl2
h5	MBOV/ET/2012/AAU-VMA/002_cl1
h6	MBOV/ET/2012/AAU-VMA/002_cl2
h7	306_cl1; 306_cl2; MBOV/ET/2012/AAU-VMA/001_cl2
h8	MBOV/ET/2012/AAU-VMA/001_cl1; MBOV/ET/2012/AAU-VMA/003_cl1; 310_cl1; 310_cl2
h9	MBOV/ET/2012/AAU-VMA/003_cl2
h10	IL3905
h11	TviMzNy

Fig. 2. Network of the 18S haplotypes of *T. vivax* isolates from Ethiopia with other known *T. vivax* strains. The pie size is proportional to the haplotype frequencies. Colours correspond to the different countries. The numbers displayed on branches refer to mutation changes separating the haplotypes. The white pies represent missing haplotypes. The symbols inside the pies indicate sequences obtained in this study of Ethiopian *T. vivax* isolates * – from tsetse-infested areas and ¤ – from tsetse-free areas.

the South American strains from Brazil and Venezuela and one Ethiopian isolate from a tsetse-free area (MBOV/ET/2012/AAU-CVMA/004_cl1 and MBOV/ET/2012/AAU-CVMA/004_cl2) (Fig. 2, h1–h4). The second group contains only one Ethiopian isolate from a tsetse-infested area (MBOV/ET/2012/AAU-CVMA/002_cl1) and MBOV/ET/2012/AAU-CVMA/002_cl2) (Fig. 2, h5–h6). The third group contains only the *T. vivax*-like strain from Mozambique (Fig. 2, h11). The fourth group contains the Kenyan strain and four Ethiopian strains, two from tsetse-infested area (MBOV/ET/2012/AAU-CVMA/001 and MBOV/ET/2012/AAU-CVMA/003_cl1 and MBOV/ET/2012/AAU-CVMA/003_cl2) and two from tsetse-free area (306_cl1, 306_cl2, 310_cl1 and 310_cl2) (Fig. 2, h7–h10).

For the analysis on the 5.8S-ITS2 region, a twenty-nine sequences alignment (260 bp) from different *T. vivax* strains (Tables 3 and 4) was generated (Supplementary file S2). The median joining algorithm implemented in NETWORK v. 4.6 software created a complicated structure with many reticulations mainly due to the excessive amounts of indels in the alignment. Therefore, for the 5.8S-ITS2 a distance matrix based network was constructed. The Neighbour-net analyses integrated in SplitsTree revealed three main groups (Fig. 3), with a similar grouping pattern as the one observed using the SSU haplotype network approach. The first group contains the West African strains Y486 and ILDat1.2, the South American strains from Brazil and Venezuela and two Ethiopian isolates, one from a tsetse-infested area (MBOV/ET/2012/AAU-CVMA/002) and one from a tsetse-free area (MBOV/ET/2012/AAU-CVMA/004). The second group contains all four sequences derived from the *T. vivax*-like strain from Mozambique. The third clade group contains the Kenyan and the Tanzanian *T. vivax* and two Ethiopian strains from tsetse-infested area (MBOV/ET/2012/AAU-CVMA/001 and MBOV/ET/2012/AAU-CVMA/003).

5. Discussion

The genes encoding the rRNA are very conserved but contain sufficient differences that allow for genetic characterisation of organisms (Urakawa et al., 1998). The rDNA locus of trypanosomes consists of 18S, ITS1, 5.8S, ITS2 and 28S (Dlugosz and Wisniewski, 2006; Urakawa et al., 1998). In this study we sequenced a fragment AB (18S, ITS1 and part of the 5.8S region) and a fragment EF (part of 5.8S, ITS2, 28S-LS1 and SrRNA1) of the rDNA locus of four Ethiopian *T. vivax* isolates that grow in calves and of one Nigerian *T. vivax* strain (Y486) that easily grows in mice. For two more Ethiopian strains, only the fragment AB was successfully sequenced starting from DNA extracted from the blood of two bovines naturally infected with *T. vivax*.

Due to its multiple copy nature, the rDNA locus is a commonly used target for PCR-based molecular diagnosis of trypanosome infections at subgenus and species level (Desquesnes and Dávila, 2002; Lukes et al., 1997; Thumbi et al., 2008). However, in previous studies we encountered inconsistent results obtained with ITS1 PCR and 18S PCR-RFLP, though both tests are based on rDNA. Furthermore, we observed a lower analytical and diagnostic sensitivity of the 18S PCR-RFLP as compared to ITS1 PCR and *Tv*PRAC PCR and we recommended the combination of *Tv*PRAC PCR and ITS1 PCR for species specific diagnosis of *T. vivax* (Fikru et al., 2014). These inconsistencies can now be explained by the primer sequence mismatches in the 18S PCR-RFLP and by the high GC content of the *T. vivax* rDNA. It has been described that template DNA with high GC content has secondary structures that hinder denaturation and primer annealing thus frequently giving rise to very weak amplification of the target sequence and non-specific amplification of non-target sequences under standard PCR conditions (Sahdev et al., 2007; Strien et al., 2013). Weak amplification of DNA with high GC content is particularly problematic in the case of mixed infections which are quite common in tsetse-infested areas. Thus, in mixed infections of *T. congolense* or *Trypanozoon* with *T. vivax*, the latter species may remain undetected since the PCR will preferentially amplify *T. congolense* and *Trypanozoon* sequences. By adding Q-solution to the PCR reaction mixture, we could enhance the amplification of *T. vivax* rDNA and simultaneously reduce aspecific reactions that can be misinterpreted as *T. vivax* amplicons. This is however linked to a decreased sensitivity for other trypanosome species. For accurate molecular diagnosis of the three pathogenic African trypanosomes, the combination of ITS1 PCR and *Tv*PRAC PCR (Fikru et al., 2014) or of ITS1 PCR with and without betaine looks the most appropriate.

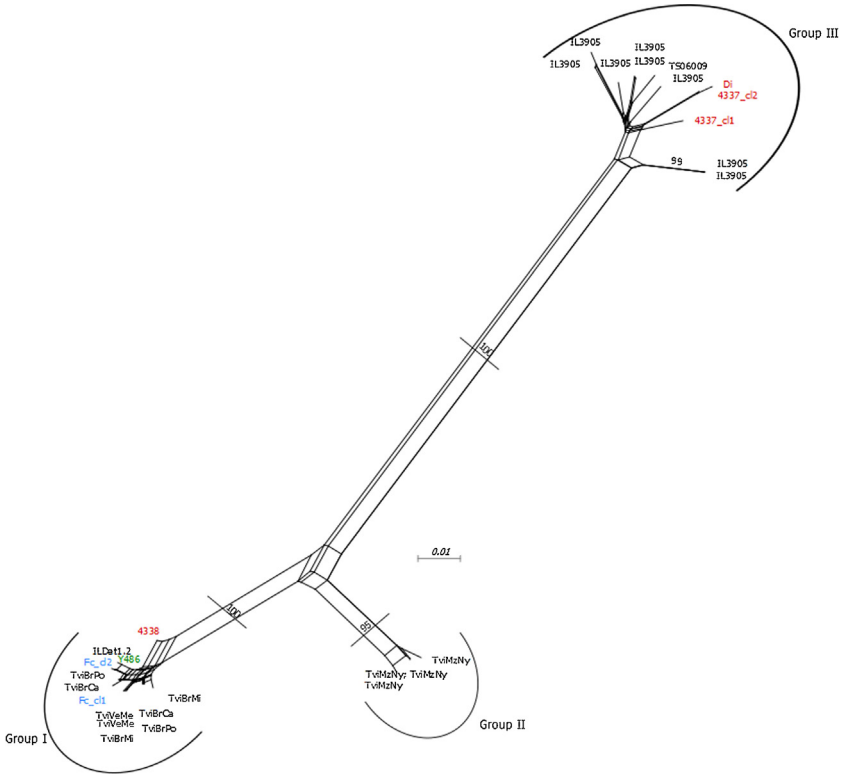


Fig. 3. Neighbour-net analysis of 5.8S-ITS2 sequences of different *T. vivax* strains based on uncorrected *p*-distances. Numbers on the main branches are indicating significant bootstrapping values, derived from 1000 replicates. Sequences obtained in this study of Ethiopian *T. vivax* isolates from tsetse-infested area are labelled in red, from tsetse-free areas are labelled in blue and of the Nigerian isolate are labelled in green. The other sequences that were retrieved from GenBank, are labelled in black.

Both the SSU and ITS2 analyses show for the first time that the so-called West African *T. vivax* genotype also occurs in Ethiopia. The occurrence of the West African genotype in East Africa has been suggested in previous studies. For example, we observed that within the partial sequences of the *Tv*PRAC gene of five Ethiopian strains, three of them are almost identical to the sequence of the Nigerian Y486 strain (Fikru et al., 2014). Also based on *Tv*PRAC sequences, Garcia et al. suggested a close relationship between two strains from Mozambique (East Africa) and the West African and South American genotypes (Garcia et al., 2014). In addition, isoenzyme analysis revealed that five Kenyan *T. vivax* strains were more heterogeneous and were divergent from three Ugandan *T. vivax* strains that, on their turn, were more related to the West African strain (Fasogbon et al., 1990). Moreover, based on DNA fingerprinting and 180 bp satellite DNA repeat hybridisation, Dirie et al. (1993) reported about two groups of *T. vivax*, one group containing strains from Colombia, The Gambia, Nigeria and Uganda and another group containing Kenyan isolates (Dirie et al., 1993).

In the phylogenetic networks constructed with the 18S and 5.8S-ITS2 sequences, two similar *T. vivax* groups are present. One group consists of the Tanzanian, the Kenyan and four Ethiopian strains (group IV in Fig. 2 and group III in Fig. 3). Another group contains the Mozambican *T. vivax*-like strain isolated from wild antelope (group III in Fig. 2 and group II in Fig. 3). The other strains form two distinct groups in the 18S network (group I and II in Fig. 2) and only one in the 5.8S-ITS2 network (group I in Fig. 3) as was observed in other studies (Adams et al., 2010b; Auty et al., 2012). The discordant topology between the two networks is due to the MBOV/ET/2012/AAU-VMA/002 strain. In the 5.8S-ITS2 network alignment the MBOV/ET/2012/AAU-VMA/002 sequence contains three indels, with size varying from 4 to 30 bp (Supplementary file S2), placing the strain in a separate split in group I. More samples or a concatenated network could help understand better the subgroups division of *T. vivax* strains.

Interestingly, our study shows that the similarity of the Ethiopian *T. vivax* strains to other *T. vivax* strains is not related to their origin from tsetse-infested or tsetse-free areas. Whether *T. vivax* strains circulating in tsetse-infested and tsetse-free areas in Ethiopia are genetically separated or not remains an open question that might be answered by other technologies. For instance, the results from microsatellite analysis on *T. vivax* strains from The Gambia showed the absence of recombinant genotypes and a limited number of unique genotypes suggesting a clonal expansion of *T. vivax* as the main means of propagation (Duffy et al., 2009). However earlier studies on the genome of *T. brucei* have identified eight meiosis-associated genes which are also conserved in *T. vivax* thus implicating the possibility of mating in *T. vivax* (El-Sayed et al., 2005).

6. Conclusions

We conclude that among the Ethiopian *T. vivax* strains, the rDNA locus sequence shows considerable heterogeneity which is not consistent with the classification of these strains according to their geographical origin neither according to their origin from tsetse-infested and tsetse-free areas within Ethiopia. The low sensitivity of some ITS1 and 18S diagnostic PCR assays for *T. vivax* can be attributed to the high GC content of *T. vivax* rDNA and/or, to a lesser extent, primer mismatches. Addition of betaine to the PCR reaction mix only improved detection of *T. vivax*. Therefore, accurate detection of all pathogenic animal trypanosomes in one single diagnostic PCR assay remains problematic.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

RF and PB conceived the study, generated, analysed and interpreted the data and prepared the manuscript. SR and NVR participated in the laboratory work, IM prepared the cladogram, BM and BMG contributed in the conception of the study. All authors revised and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2016.02.013.

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