The taxonomic and phylogenetic relationships of *Trypanosoma vivax* from South America and Africa

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SUMMARY

The taxonomic and phylogenetic relationships of *Trypanosoma vivax* are controversial. It is generally suggested that South American, and East and West African isolates could be classified as subspecies or species allied to *T. vivax*. This is the first phylogenetic study to compare South American isolates (Brazil and Venezuela) with West/East African *T. vivax* isolates. Phylogeny using ribosomal sequences positioned all *T. vivax* isolates tightly together on the periphery of the clade containing all Salivarian trypanosomes. The same branching of isolates within *T. vivax* clade was observed in all inferred phylogenies using different data sets of sequences (SSU, SSU plus 5.8S or whole ITS rDNA). *T. vivax* from Brazil, Venezuela and West Africa (Nigeria) were closely related corroborating the West African origin of South American *T. vivax*, whereas a large genetic distance separated these isolates from the East African isolate (Kenya) analysed. Brazilian isolates from cattle asymptomatic or showing distinct pathology were highly homogeneous. This study did not disclose significant polymorphism to separate West African and South American isolates into different species/subspecies and indicate that the complexity of *T. vivax* in Africa and of the whole subgenus *Trypanosoma* (*Duttonella*) might be higher than previously believed.

Key words: *Trypanosoma vivax*, taxonomy, phylogeny, evolution, ribosomal genes, Brazil, genetic diversity, South America, West Africa, East Africa.

INTRODUCTION

Trypanosoma (*Duttonella*) *vivax* is a major livestock pathogen, which is cyclically transmitted between domestic and wild ruminants by tsetse flies over most of its range in Africa. However, it can also be mechanically transmitted by other biting flies, and has therefore been able to spread beyond the African tsetse belt to Central and South America in recent centuries (Gardiner, 1989; Gardiner and Mahmoud, 1992).

Trypanosoma vivax was first reported in the New World in cattle in French Guiana and named as *T. guyanense* (Leger and Vienne, 1919, cited by Hoare, 1972). Later, renamed *T. vivax viennei*, it was reported in other parts of Central and South America (Hoare, 1972; Shaw and Lainson, 1972). In South America this species has an overlapping distribution with *T. evansi* (Ventura *et al*. 2000, 2001). There are confirmed reports of its presence in 10 South American countries, including Colombia, Venezuela, French Guiana, Bolivia, Peru and Brazil (Jones and Davila, 2001). Shaw and Lainson (1972) reported the first occurrence of *T. vivax* in Brazil, in water buffalo in the Pará State of the Amazon Region. *T. vivax* outbreaks causing wasting and haematological changes were reported in cattle in Pantanal, a wetland region in Central Brazil and in Bolivia (Silva *et al*. 1999), but asymptomatic cattle have also been commonly found in Pantanal (Paiva *et al*. 2000; Ventura *et al*. 2001). A *T. vivax* outbreak with severe disease was recently reported in the Paraiba State, a semi-arid region of Northeastern Brazil (Batista *et al*., manuscript in preparation). Nowadays, *T. vivax* is commonly found in enzootic equilibrium in the Brazilian Pantanal and surroundings (Ventura *et al*. 2001; Davila *et al*. 2003). Whether outbreaks and different disease syndromes are associated with particular *T. vivax* isolates, or with host factors such as poor health condition or breed remains to be elucidated. Similarly, in Africa *T. vivax* shows variable levels of virulence and distinct pathogenicity. In West Africa, *T. vivax* infection in cattle is often acute and accompanied by weight loss, reduced milk yields, abortions and

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mortality. In contrast, with the exception of sporadic haemorrhagic syndrome in cattle, East African isolates of *T. vivax* tend to produce mild infections, which are self-limiting in healthy animals (Gardiner and Mahmoud, 1992).

Besides pathogenicity and virulence, *T. vivax* and related taxa have also been reported to differ in morphology and molecular features. Short trypomastigote forms were associated with acute disease in cattle in West Africa and long forms with chronic infection in East Africa. Isolates from Central Africa (Uganda and Congo) classified as *T. uniforme* presented the smallest forms of the subgenus *T.* (*Duttonella*). A caprine trypanosome from East Africa was described as a separated species (*T. caprae*) and later reclassified as *T. vivax ellipsiprymni* due its morphological peculiarities. South American and West African isolates, although morphologically indistinguishable, were separated in 2 subspecies, *T. vivax vivax* and *T. vivax viennei*, according to cyclical or mechanical transmission, respectively (Hoare, 1972; Shaw and Lainson, 1972; Gardiner and Mahmoud, 1992).

Although relatively few isolates of *T. vivax* have been compared by molecular techniques, all studies revealed differences according to geographical origin. Isoenzyme, satellite DNA, kDNA minicircles and karyotype patterns grouped West African and South American (Colombian) isolates together, and apart from East African (Kenya) isolates (Fasogbon *et al.* 1990; Dirie *et al.* 1993*a*, *b*), corroborating the hypothesis that *T. vivax* was introduced into South America with bovines imported from West Africa (Hoare, 1972; Gardiner and Mahmoud, 1992; Dirie *et al.* 1993*a*, *b*). *T. vivax* from Central Africa shared molecular features with both the East and West African isolates (Fosogbon *et al.* 1990; Gardiner, 1989).

Studies based on SSU ribosomal RNA (SSU rRNA) have addressed the phylogeny of *T. vivax* and its peculiarities within the genus *Trypanosoma* (Haag *et al.* 1998; Stevens and Rambaut, 2001; Stevens *et al.* 2001). Most phylogenies supported the monophyly of *Trypanosoma* and positioned *T. vivax* on the clade containing all Salivarian trypanosomes (Stevens and Rambaut, 2001; Stevens *et al.* 2001; Hamilton *et al.* 2004; Piontkivska and Hughes, 2005; Suzuki *et al.* 2005). However, Hughes and Piontkivska (2003) based on phylogenetic analysis done with a larger data set of both outgroup and ingroup taxa than used in previous studies, demonstrated that Salivarian trypanosomes, except for *T. vivax*, formed a highly supported clade outside the clade formed by Stercorarian trypanosomes and other trypanosomatid genera, thus providing strong evidence against the monophyly of *Trypanosoma*. According to their analysis, the position of *T. vivax* was not considered resolved and this species clustered apart from all other trypanosomatids outside even bodonids. Nevertheless, all published phylogenies of *T. vivax* to date were based on a single isolate (Y486 from Nigeria), and analyses of more isolates using different genes are urgently required to clear up this question. To assess the genetic diversity and taxonomic position of *T. vivax* in this study we compared SSU, 5.8S and ITS ribosomal sequences aiming (a) to infer phylogenetic relationships among South American (Brazil and Venezuela), West and East African isolates, (b) to compare *T. vivax* isolates of different regions and from cattle showing distinct clinical and pathological features and (c) to re-examine the taxonomy of the subgenus *Trypanosoma* (*Duttonella*) and the validity of the South American subspecies *T. vivax viennei*.

MATERIALS AND METHODS

Origin, identification, and clinical features of trypanosomes

In this study we compared 6 *T. vivax* isolates, four from South America, from different outbreaks with cattle showing different clinical and pathological features, and 2 from Africa (Table 1). South American isolates of *T. vivax* were obtained from the blood of naturally infected cattle or from experimentally infected sheep as before (Ventura *et al.* 2001). Giemsa-stained blood smears from cattle and sheep infected by these isolates were analysed. All these isolates were submitted to a *T. vivax*-specific PCR assay based on spliced-leader gene sequence (Ventura *et al.* 2001). Details of the African isolates used in this study are as follows: West African *T. vivax* Y486 from Nigeria (Leeflang *et al.* 1976) grown in mice and donated by Dr Théo Baltz (University of Bordeaux, France) and clone ILDat 1.2 derived from *T. vivax* Y486; East African isolate IL3905 from Kenya (Rebeski *et al.* 1999), grown in cell culture and donated by Dr Dierk E. Rebeski (FAO, Austria).

DNA templates, PCR amplification, sequencing and alignment of ribosomal sequences

Genomic DNA of trypanosomes from blood of cattle or sheep, preserved at $-20\,^{\circ}C$ or dried on filter papers, were extracted by phenol-chloroform according to the method reported by Ventura *et al.* (2001). The oligonucleotides employed for PCR amplifications of ribosomal sequences were described previously (Maia da Silva *et al.* 2004; Rodrigues *et al.* 2006). Due to poor quality of DNA templates only the region corresponding to V7-V8 SSU sequences could be amplified for most samples. The amplified products of SSU and whole ITS (ITS1 + 5.8S + ITS2) genes were cloned and at least 3 clones from each gene/isolate were sequenced.

Table 1. Isolates of *Trypanosoma vivax* used in this study, geographical origin and health condition of naturally infected cattle

Trypanosoma vivax isolates	Geographical origin		Infected cattle	GenBank Accession number ribosomal sequences	
				SSU	ITS
TviBrMi[a]	South America	Brazil	Center (Miranda South Pantanal)	Chronic Asymptomatic	DQ317415	DQ316047, DQ316048
TviBrPo[b]	South America	Brazil	Center (Poconé North Pantanal)	Chronic Symptomatic Haematological changes	—	DQ316049, DQ316050
TviBrCa[c]	South America	Brazil	Northeast (Catolé)	Acute Symptomatic Nervous signs	DQ317413	DQ316045, DQ316046
TviVeMe	South America	Venezuela	West (Mérida)	Chronic Asymptomatic	DQ317416	DQ316051, DQ316052
IL3905[d]	Africa	Kenya	East	Chronic	DQ317414	DQ316039–DQ316044
Y486[e,f]	Africa	Nigeria	West	Chronic	U22316	U22316
ILDat 1.2[g]						

a, Paiva *et al*. 2000; b, Silva *et al*. 1999; c, Batista *et al*., manuscript in preparation; d, Rebeski *et al*. 1999; e, Leeflang *et al*. 1976; f, Sanger Centre Genome project; g, clone derived from the isolate Y486.

The ribosomal sequences of the SSU, 5.8S and ITS (ITS1 and ITS2) genes of *T. vivax* isolates determined in this study were aligned with sequences from several other trypanosome species using the BioEdit program followed by visual optimization. SSU rRNA sequences from other species of trypanosomes were retrieved from GenBank (Accession number): *T. b. brucei* (M12676); *T. b. gambiense* (AJ009141); *T. b. rhodesiense* (AJ009142); *T. congolense* Kilifi (AJ009144); *T. congolense* savannah (AJ009146); *T. congolense* forest (AJ009145); *T. simiae* (AJ009162); *T. godfreyi* (AJ009155); *T. equiperdum* (AJ009153); *T. evansi* (D89527); *T.* sp. D30 from deer (AJ009165); *T. theileri* Tthc3 from cattle (AY773681); *T. theileri* Tthb12 from buffalo (AY773678); *T. pestanai* (AJ009159), *T.* sp. H26 (AJ009169); *T. cruzi* Sylvio X10 (AJ009147), *T. cruzi* CL (AF245383); *T. rangeli* San Agustin (AJ012417); *T. rangeli* legeri (AY491769); *T. cyclops* (AJ250743). Sequences from *Bodo caudatus* (X53910) and *Bodo designis* (AF209856) were used as outgroup for *Trypanosoma*, and sequence of the Parabodonida AT1-3 (AF50051) as outgroup for Trypanosomatidae. We also aligned SSU and ITS sequences retrieved from genome data banks of *T. b. brucei*, *T. congolense* and *T. vivax* Y486 (http://www.genedb.org). We also analysed an SSU rRNA gene fragment from putative Tanzanian *T. vivax* (AJ563916) (Malele *et al*. 2003).

Phylogenetic inferences and analysis of GC contents

Different alignments from distinct data sets were employed in this study. (1) Alignment of SSU ribosomal sequences corresponding to V7-V8 variable region plus conserved flanking region of different species of trypanosomes using bodonids as outgroups (1162 characters). (2) Alignment of these SSU sequences plus 5.8S sequences of Salivarian trypanosomes (1169 characters). (3) Alignment of whole ITS (ITS1 + 5.8S + ITS2 sequences) of *T. vivax* isolates (554 characters). (4) Alignment of different copies of ITS1 and ITS2 sequences from *T. vivax*, *T. b. brucei* and *T. congolense* done separately for each species due to unreliable alignments of these sequences from different species. Maximum parsimony (MP) and maximum-likelihood (ML) analysis were inferred based on V7-V8 and V7-V8 plus 5.8S alignments. The ML model and parameters were estimated using the hierarchical likelihood test implemented in the Modeltest, 3.06 (Posada and Crandall, 1998). MP and ML bootstrapping with 100 replicates were done as before (Hamilton *et al*. 2004). A dendrogram based on whole ITS sequences (alignment 3) was done using MP. Similarity matrixes were calculated as before (Maia da Silva *et al*. 2004). Alignments used in this study are available from the authors upon request. Analyses of GC contents were

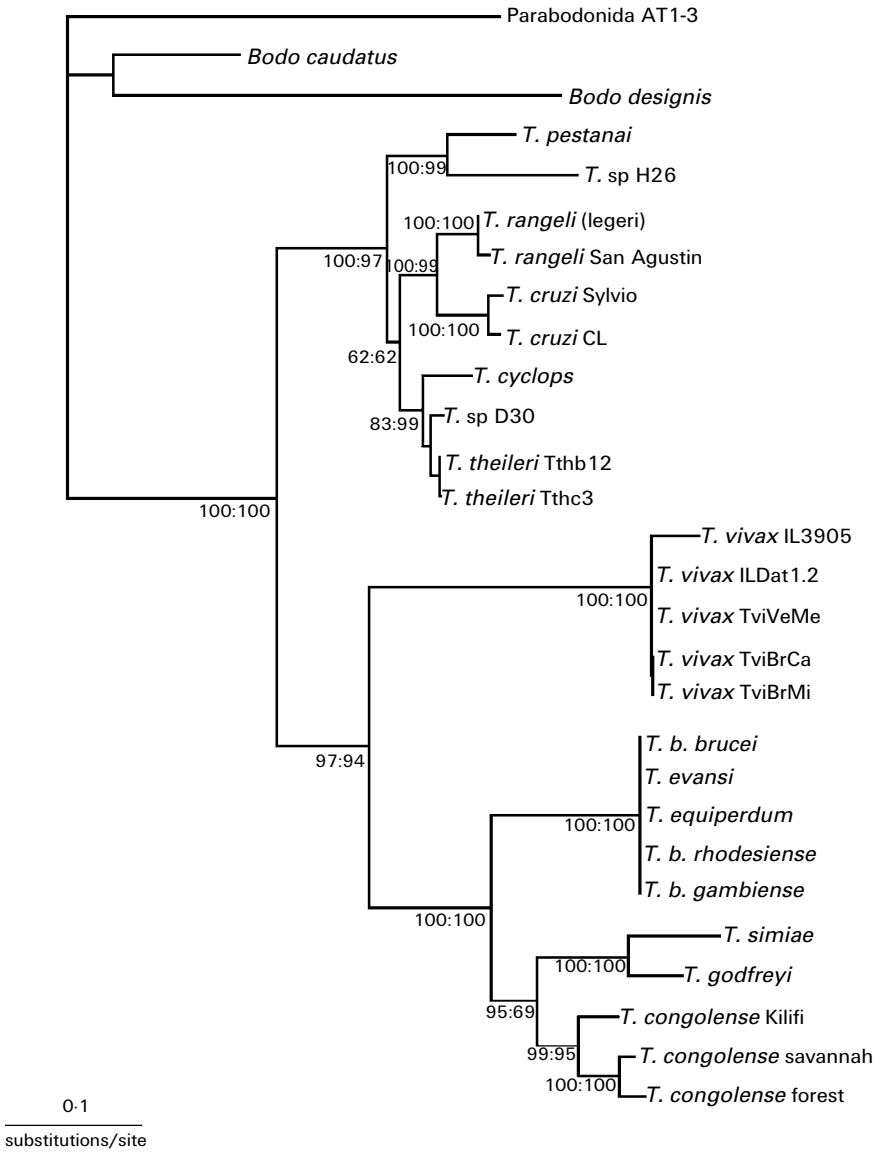

Fig. 1. Phylogenetic tree based on Maximum Likelihood analysis of SSU (V7-V8) rRNA sequences from *Trypanosoma vivax* isolates and other trypanosome species. Bodonids (Parabodonida AT1-3, *Bodo caudatus*, *B. designis*) were used as outgroup for *Trypanosoma*. The best-fit evolutionary model for the likelihood analysis (as determined by Modeltest) was Tamura and Nei with gamma and invariant parameters. The numbers at nodes correspond to percentage of bootstrap values (Maximum Parsimony : Maximum Likelihood) derived from 100 replicates.

done using the program MEGA 2.1 (Kumar *et al*. 2001).

RESULTS

Identity of T. vivax *isolates using morphological and molecular diagnosis*

Identification of the South American *T. vivax* isolates (TviBrMi, TviBrPo, TviBrCa and TviVeMe) was originally based on morphology of the trypomastigotes in Giemsa-stained smears of field-collected blood samples from cattle, and subsequently of blood trypanosomes from experimentally infected sheep or calves. All isolates presented similar blood trypomastigotes (Ventura *et al*. 2001), without significant variability in shape or length, with morphometrical features typical for *T. vivax* from West Africa according to Hoare (1972). Identification of South American isolates was confirmed using a *T. vivax* specific TviSL-PCR assay (Ventura *et al*. 2001). This method amplified DNA of all *T. vivax* isolates from South America and West Africa, whereas DNA from the isolate IL3905 (East Africa) could not be amplified (data not shown).

Phylogeny of T. vivax *isolates based on SSU and 5.8S ribosomal sequences*

Both ML (Fig. 1) and MP (not shown) trees inferred using SSU rRNA sequences of a diverse range of

Salivarian and other trypanosome species showed very similar topologies and the same position for all *T. vivax* isolates. *T. vivax* was always positioned marginally in the clade containing all Salivarian trypanosomes within the genus *Trypanosoma*. In agreement with this positioning, *T. vivax* divergence was high (~30%) when compared with Salivarian species belonging to subgenera *T.* (*Trypanozoon*) (*T. brucei*, *T. evansi* and *T. equiperdum*) and *T.* (*Nannomonas*) (*T. congolense*, *T. godfreyi* and *T. simiae*). In this study we employed as outgroups bodonids and a deep-sea kinetoplastid (Parabodonida) considered to be closer to bodonids than to euglenids (Piontkivska and Hughes, 2005). In analysis including more distantly related euglenid species, the *T. vivax* clade also clustered with Salivarian trypanosomes (99% bootstrap), and this clade with all other trypanosomes (not shown), although supported by a lower bootstrap value (76%). In addition to this study, we previously showed that trees based on V7-V8 SSU rRNA sequences generated a similar branching pattern and all major clades (Maia da Silva *et al*. 2004; Rodrigues *et al*. 2006) compared to trees generated using larger SSU rRNA sequences (Stevens *et al*. 2001; Hamilton *et al*. 2004).

At least 3 independent SSU rRNA sequences from each South American isolate and from the East African isolate were obtained and compared with sequences from the West African Y486 (genome data bank) and Y486 clone ILDat (GenBank). Brazilian isolates did not show significant sequence polymorphism (average ~0·15%) and divergence was only ~0·4% between these isolates and the Venezuelan isolate. While there was little divergence between West African and South American isolates (~0·34%), sequences of the East African isolate were highly divergent compared with all other *T. vivax* isolates (~3·2%).

A partial SSU rRNA sequence corresponding to variable V7 region amplified directly from tsetse collected in East Africa (Tanzania) and attributed to a *T. vivax*-like trypanosome (Malele *et al*. 2003) was aligned with corresponding sequences analysed in this study. This sequence was highly divergent from other *T. vivax* sequences, including that of the Kenyan isolate sequenced here (~11% sequence divergence). Despite this, phylogenetic analysis clearly clustered this sequence in the *T. vivax* clade (data not shown).

To evaluate the relationships among *T. vivax* isolates and the positioning of these isolates within the clade comprising the Salivarian trypanosomes, we decided to use a combined data set formed by variable (V7-V8) and conserved (5.8S) sequences for phylogenetic analysis. V7-V8 regions are variable sequences flanked and interspersed by conserved regions, and are thus good targets for comparison of related organisms. The 5.8S sequences are also good targets allowing very consistent alignments due to conservation among different species of the same subgenus, and significant variability among species belonging to distinct subgenera. We restricted this analysis to Salivarian trypanosomes (all 5.8S sequences obtained in this study plus all available sequences from GenBank), because the alignment of closely related trypanosomes allowed characters in more variable regions to be aligned with higher confidence and included in the ML analysis. The topology of the inferred Salivarian trypanosome tree (Fig. 2) revealed the same branching pattern obtained for these trypanosomes in the phylogeny of *Trypanosoma* using only V7-V8 SSU rRNA sequences (Fig. 1). The position of the *T. vivax* clade as a marginal group of the Salivarian clade was confirmed. South American and West African isolates were clustered tightly together, whereas the isolate from East Africa was clearly separated, although closer to other *T. vivax* isolates than to any other trypanosome species (Fig. 2).

Analysis of genetic relatedness using only the 5.8S sequences also separated the East African *T. vivax* isolate from the group formed by South American and West African isolates. Comparison of aligned 5.8S sequences from different clones of South American *T. vivax* isolates showed a divergence varying from ~0·6%, among Brazilian isolates, to ~1·2%, between Brazilian and Venezuelan isolates. Sequence divergence between South American and African isolates ranged from ~0·7% to ~3·2% for West and East African isolates, respectively. Contrasting with the highly conserved 5.8S sequences of American and West African isolates (~0·4% divergence), there is a large polymorphism (~1·0%) among sequences of 8 clones of 5.8S from the East African isolate IL3905. Analysis of 5.8S gene sequences from data banks disclosed high sequence conservation (minimum 99·8%) in *T. brucei, T. congolense* and *T. vivax* Y486 ILDat 1.2. Divergence separating 5.8S sequences of *T. vivax* from *T. congolense* or *T. brucei* was ~15% and ~16%, respectively.

Polymorphism and genetic relatedness among T. vivax *isolates based on ITS rDNA sequences*

To demonstrate the genetic diversity within the *T. vivax* clade, we also evaluated polymorphisms among 6 isolates examined in this study using the more divergent ITS region of the rRNA gene array. For analysis of ITS polymorphism we compared Brazilian isolates from distant geographical regions (Central and Northeast), recovered from cattle showing different clinical and pathological profiles (Table 1) with isolates from Venezuela and Africa. We investigated the polymorphism by comparing length and sequence of the PCR-amplified DNA fragments containing the whole ITS rDNA (ITS1,

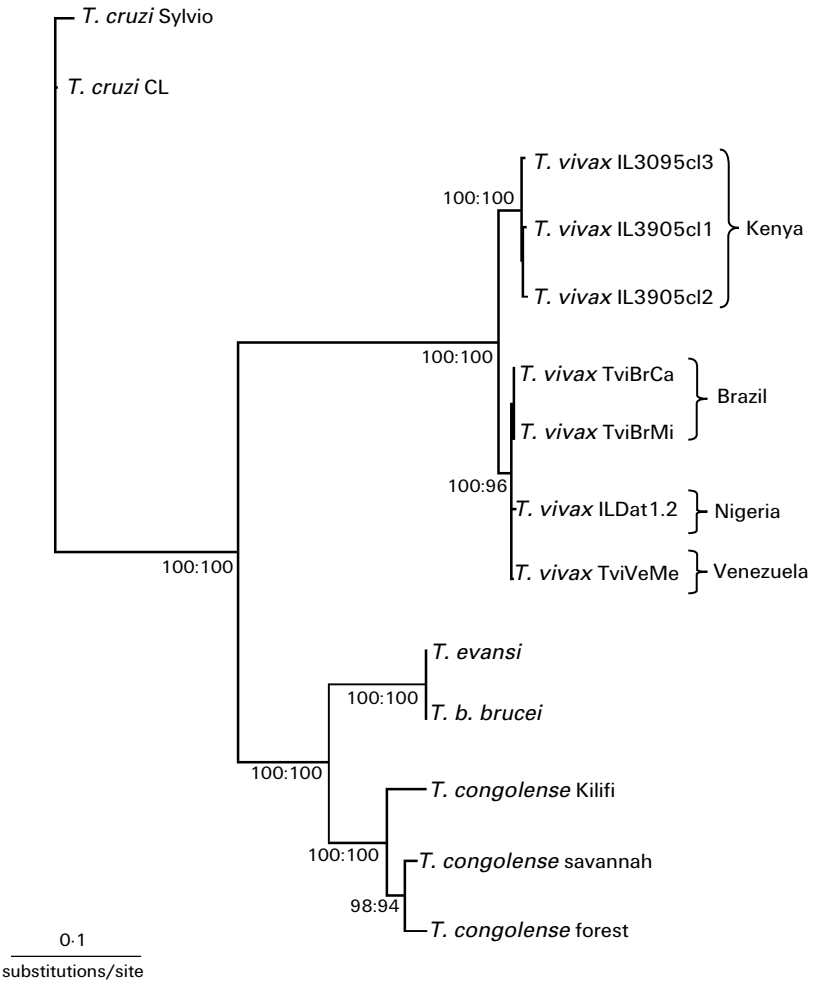

Fig. 2. Phylogenetic tree based on Maximum Likelihood analysis of SSU and ITS (V7-V8 + 5.8S) rDNA sequences from *Trypanosoma vivax* isolates and other salivarian trypanosome species. The best-fit evolutionary model for the likelihood analysis (as determined by Modeltest) was Tamura and Nei with gamma. The numbers at nodes correspond to percentage of bootstrap values (Maximum Parsimony : Maximum Likelihood) derived from 100 replicates.

5.8S and ITS2) sequences. All South American/West African isolates had sequences of similar length (490 bp). However, the East African isolate IL3905 had different ITS lengths, varying from 525 to 534 bp. Small ITS sequence divergence separated American and West African isolates (~0·8%). However, a high polymorphism separated *T. vivax* of South America/West Africa from the East African IL3905 isolate, with the average divergence index increasing drastically to ~33%. We selected the 2 most polymorphic cloned sequences of ITS (ITS1 + 5.8S + ITS2) from each isolate to be included in the alignment used to assess genetic relatedness among *T. vivax* isolates, with the exception of the isolate IL3905, for which ITS sequences of 8 clones were included to represent the high degree of polymorphism within this isolate (Fig. 3). Other trypanosome species were not included in the alignment, because their ITS sequences could not be aligned with confidence. Despite the low degree of genetic variability, all ITS sequences from South American isolates were always grouped with those from West African isolates in a relatively homogeneous cluster, segregated in unsupported subclusters (Fig. 3). This cluster was clearly separated from that formed by the heterogeneous sequences from the East African isolate (Fig. 3), corroborating the segregation pattern of *T. vivax* isolates based on more conserved SSU and 5.8S ribosomal sequences (Figs 1 and 2).

The genetic polymorphism detected among all *T. vivax* isolates, in both ITS1 (~27%) and ITS2 (~25%) sequences, comprises several large blocks of deletions and insertions, in addition to numerous substitutions. Alignment of ITS2 sequences of all *T. vivax* isolates illustrates the polymorphism within the same isolate and among isolates of this species (Fig. 4). Analyses of sequence polymorphism among 3–4 clones from each *T. vivax* isolate showed low ITS1 and ITS2 divergence among sequences from Brazilian and Venezuelan isolates (~0·6% for both ITS1 and ITS2). However, analysis of ITS sequences of *T. vivax* Y486 revealed a significant polymorphism (~2·7% and 1·6% for ITS1 and

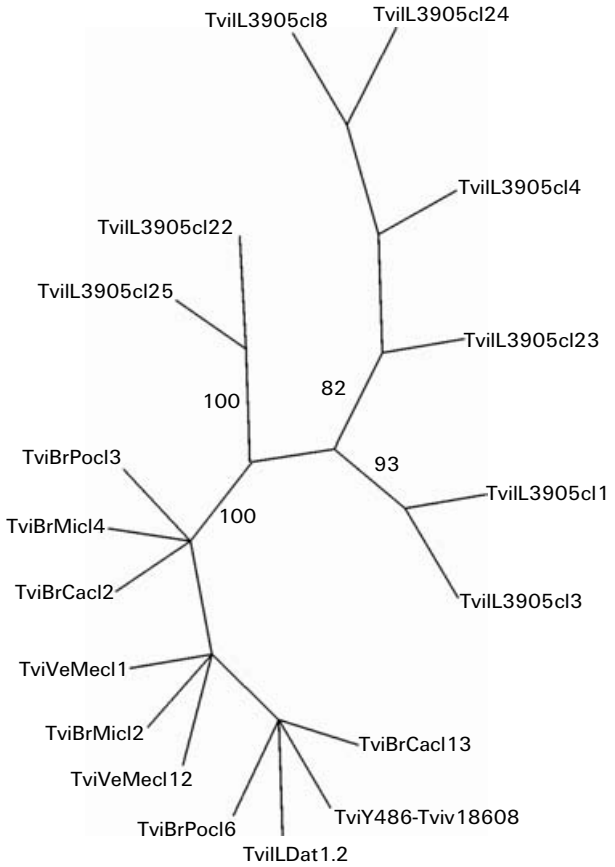

Fig. 3. Unrooted tree based on Parsimony analysis of whole ribosomal ITS sequences (ITS1 + 5.8S + ITS2) from *Trypanosoma vivax* isolates. The numbers at nodes correspond to percentage of bootstrap values derived from 100 replicates.

ITS2, respectively). The highest polymorphism was observed among the ITS sequences of IL3905, varying from ~1·0 to 8·7% of divergence (average of ~7·0% and 6·8% for ITS1 and ITS2, respectively). Analysis of ITS sequences from data banks disclosed a high polymorphism within *T. congolense* savannah (maximum ~9·7% for ITS1 and ~8·6% for ITS2), whereas *T. b. brucei* showed more conserved sequences, although still significantly polymorphic (~2·8% for ITS1 and 1·2% for ITS2).

Analysis of GC contents in the ribosomal genes of T. vivax *isolates*

Previous studies showed that the whole SSU rDNA of *T. vivax* Y486 has a high GC content when compared with several other trypanosome species, with ~55·4% GC, which is ~3·0% higher than any other trypanosomatid (Haag *et al.* 1998; Stevens and Rambaut, 2001). To verify if the high GC content is shared by all *T. vivax* isolates analysed in this study, we compared different regions of rRNA genes of these organisms and other trypanosome species. Results showed that the GC contents of sequences from V7-V8 SSU rDNA are quite similar among American and West African isolates of *T. vivax* (~63%). The GC content of sequences of the East African isolate was in the same range (~64%), despite the high genetic distance separating these isolates. Comparison with other Salivarian trypanosomes showed that the average GC content of the V7-V8 SSU rDNA region of *T. vivax* isolates was significantly higher than other subgenera (subgenus *T.* (*Trypanozoon*) ~52%, subgenus *T.* (*Nannomonas*) ~56%). Similarly, non-salivarian trypanosomes e.g. *T. cruzi, T. rangeli, T. theileri* and other species included in Fig. 1 also showed lower GC values (~52%). High GC contents were also observed in the 5.8S sequences of *T. vivax* isolates compared to other trypanosomes: *T. vivax* 57–58%; subgenus *T.* (*Trypanozoon*) ~49%; subgenus *T.* (*Nannomonas*) ~50%. Thus, so far, high GC content in ribosomal sequences of mammalian trypanosomes appears to be unique to *T. vivax*.

DISCUSSION

In this study we compared South American and African isolates of *T. vivax* by phylogenetic analysis based on a data set consisting of conserved (5.8S) and variable (V7-V8 regions of SSU rRNA) ribosomal sequences. All *T. vivax* isolates clustered tightly together on the margin of the clade containing all Salivarian trypanosomes (clade *T. brucei*), in agreement with previous studies (Stevens *et al.* 2001; Stevens and Rambaut, 2001; Hamilton *et al.* 2004; Piontkivska and Hughes, 2005). Based on SSU rDNA phylogenies, the Salivarian trypanosomes seem to be the most rapidly evolving group of trypanosomes, with *T. vivax* evolving faster than all other Salivarian species and the entire range of trypanosomatids (Stevens and Rambaut, 2001). In this study we demonstrated that all *T. vivax* isolates analysed share this high evolutionary rate and have higher GC contents of ribosomal sequences than all other trypanosomatids, as previously shown for *T. vivax* Y486 (Haag *et al.* 1998; Stevens and Rambaut, 2001).

The position of *T. vivax* in the phylogenetic trees suggests that it was the first taxon to diverge, thus representing the most ancient species of Salivarian trypanosome (Haag *et al.* 1998; Stevens *et al.* 2001; Stevens and Rambaut, 2001; Hamilton *et al.* 2004). Although all Salivarian trypanosomes are transmitted via tsetse saliva, the life-cycle of *T. vivax* is distinct in that it does not undergo development in the fly hindgut like the other subgenera, but completes its development wholly within the mouthparts (Hoare, 1972). In common with the other Salivarian trypanosomes, *T. vivax* undergoes antigenic variation, but shows distinct features, e.g. biochemical and antigenic properties of the variant surface glycoproteins (VSGs) and smaller and more heterogeneous VAT (variable antigen type)

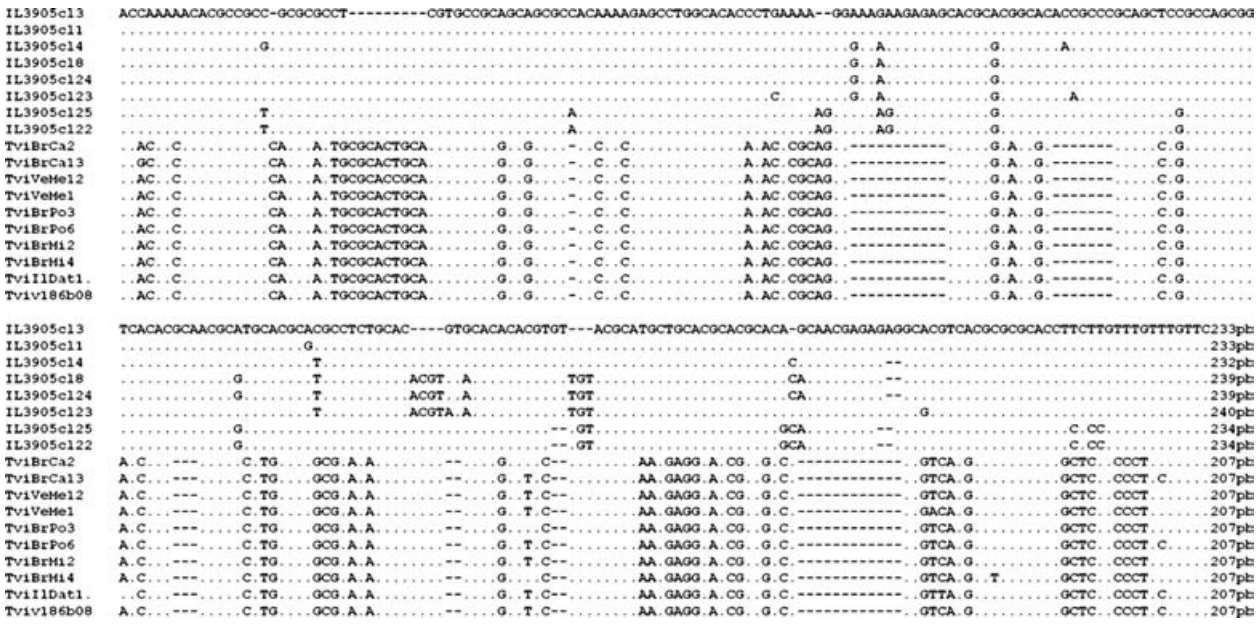

Fig. 4. Alignment of selected ITS2 rDNA cloned sequences of *Trypanosoma vivax* isolates from different geographical regions.

repertoire (Gardiner *et al*. 1996). The chromosomal organization in *T. vivax* is also distinct, since it has only 1–2 minichromosomes contrasting with 50–100 in the other Salivarian trypanosomes (Dickin and Gibson, 1989). *T. vivax* is predominantly a parasite of bovids, presenting a smaller range of mammalian hosts compared to *T. congolense* or *T. brucei* (Hoare, 1972; Gibson, 2002). Thus the position of *T. vivax* on the periphery of the *T. brucei* clade is compatible with its distinct biological and molecular features compared to the rest of the Salivarian trypanosomes.

This study confirmed the close genetic relatedness of *T. vivax* isolates from South America and West Africa (Fasogbon *et al*. 1990; Dirie *et al*. 1993*a*, *b*; Ventura *et al*. 2001). A large genetic distance separated these isolates from the only East African isolate analysed in this study. These data are in agreement with previous studies that distinguished these two populations of *T. vivax* by morphology (Hoare, 1972); development in tsetse (Moloo *et al*. 1987); susceptibility of host species and pathology (Leeflang *et al*. 1976; Gathuo *et al*. 1987; Williams *et al*. 1992), besides immunological (Vos and Gardiner, 1990) and molecular (Fasogbon *et al*. 1990; Dirie *et al*. 1993*a*, *b*) features.

Contrasting with the great genetic distance separating East African *T. vivax* from all other isolates of this species, a high homogeneity was observed among South American isolates. However, the Brazilian isolates were more closely related to each other than to the Venezuelan isolate. This genetically homogeneous group of Brazilian isolates of *T. vivax* originated from cattle of distinct outbreaks of disease and was also recovered from asymptomatic cattle. For example, isolate TviBrPo came from an outbreak in Poconé, Northern Pantanal, Central Brazil, with cattle showing severe haematological changes, mainly anaemia and leucopenia, progressive weakness, marked weight loss and abortions (Silva *et al*. 1999), while isolate TviBrCa came from an outbreak in Catolé da Rocha, Northeast Brazil, where cattle showed similar symptoms but also presented nervous signs (meningoencephalitis and malacia) with an invariably fatal outcome irrespective of drug treatment (Batista *et al*. manuscript in preparation). The isolates TviBrMi from Brazil (Paiva *et al*. 2000) and TviVeMe from Venezuela (Añez *et al*. manuscript in preparation) were both from chronic and asymptomatic infected cattle. Thus, this study demonstrated that cattle infected with the same or very similar isolates of *T. vivax* can show distinct diseases or be totally asymptomatic.

Very few isolates of *T. vivax* have been analysed from East Africa and thus little is known about genetic variation of isolates from this region. Lack of detection of Kenyan *T. vivax* using PCR primers derived from a West African isolate indicates the widespread occurrence of genetic variants of this species (Njiru *et al*. 2004), a fact corroborated in this study by the failure of the PCR based on SL gene sequences of South American/West African *T. vivax* (Ventura *et al*. 2001) to detect the East African isolate examined in this study. Other distinct genetic variants of *T. vivax* may well exist, for example that represented by a partial SSU rRNA sequence amplified directly from tsetse collected in Tanzania, East Africa (Malele *et al*. 2003). The SSU rRNA sequence fragment shows this trypanosome to be more related to *T. vivax* than to any other

trypanosome species, although significantly divergent from all other *T. vivax* isolates, including the isolate from Kenya included in this study. Previous studies showed that *T. vivax* populations in East Africa differed in morphology, host susceptibility, isoenzyme patterns and virulence (Hoare, 1972; Murray and Clarkson, 1982; Gathuo *et al*. 1987; Fasogbon *et al*. 1990).

The ability to develop in tsetse flies would not appear to be a major evolutionary factor in this segregation pattern, since West African and South American *T. vivax* are very closely genetically related. Despite considerable divergence among copies of ITS ribosomal genes, especially within the East African isolate, sequences from this isolate were always clustered together in all analyses, clearly separated from sequences of West African and South American isolates, which were also always clustered together. Interestingly, South American isolates had highly homogeneous copies of the ITS sequence compared to the tsetse-transmitted *T. vivax* from Africa. We also detected highly polymorphic ITS regions in *T. congolense* savannah and significant divergence among ITS copies of *T. b brucei*, which are cyclically transmitted by tsetse, whereas polymorphism was not observed in a mechanically transmitted Brazilian isolate of *T. evansi* (data not shown). Comparison of a larger number of trypanosome isolates from tsetse-infested and tsetse-free regions is required to clarify this evolutionary pattern of ribosomal genes. Our previous studies disclosed small polymorphisms among cloned copies of SSU and ITS sequences among *T. rangeli* (Maia da Silva *et al*. 2004) and *T. theileri* (Rodrigues *et al*. 2006) isolates, which are Stercorarian trypanosomes cyclically transmitted by triatomine bugs or tabanids, respectively.

Although *T. vivax* populations from South America and West Africa have different modes of transmission (cyclical or mechanical), they have similar pathology and, as shown by this study, cannot be distinguished clearly by molecular phylogenetic analysis using ribosomal sequences. These data corroborated our previous study based on SL gene sequences (Ventura *et al*. 2001). South American isolates of *T. vivax* have previously been classified as a distinct subspecies, *T. vivax viennei*, the validity of which can now be seen to rest on mode of transmission. Since there is widespread suspicion that *T. vivax* can be mechanically transmitted in areas of Africa where tsetse are sparse (Gardiner, 1989; Gardiner and Mahmoud, 1992), a definition of *T. vivax viennei* based only on mode of transmission is clearly problematic. So far, West African and South American (Colombia) isolates of *T. vivax* have only been found to differ in antigenic profiles (Murray and Clarkson, 1982; Dirie *et al*. 1993*b*). In contrast, there would appear to be far greater justification for recognition of the East African forms of *T. vivax* as separate species or subspecies of the subgenus *T.* (*Duttonella*). However, this must await the molecular and phylogenetic analysis of greater numbers of *T. vivax* isolates from East Africa.

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