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Phylogenetic analysis reveals the presence of the *Trypanosoma cruzi* clade in African terrestrial mammals☆

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ABSTRACT

Despite the impact of some trypanosome species on human and livestock health, the full diversity of trypanosomes in Africa is poorly understood. A recent study examined the prevalence of trypanosomes among a wide variety of wild vertebrates in Cameroon using species-specific PCR tests, but six trypanosome isolates remained unidentified. Here they have been re-examined using fluorescent fragment length barcoding (FFLB) and phylogenetic analysis of glycosomal glyceraldehyde phosphate dehydrogenase *gGAPDH* and 18S ribosomal RNA (rDNA) genes. Isolates from a monkey (*Cercopithecus nictitans*) and a palm civet (*Nandinia binotata*) belonged to the *Trypanosoma cruzi* clade, known previously only from New World and Australian terrestrial mammals, and bats from Africa, Europe and South America. Of the four other isolates, three from antelope were identified as *Trypanosoma theileri*, and one from a crocodile as *T. grayi*. This is the first report of trypanosomes of the *T. cruzi* clade in African terrestrial mammals and expands the clade's known global distribution in terrestrial mammals. Previously it has been hypothesized that African and New World trypanosomes diverged after continental separation, dating the divergence to around 100 million years ago. The new evidence instead suggests that intercontinental transfer occurred well after this, possibly via bats or rodents, allowing these trypanosomes to establish and evolve in African terrestrial mammals, and questioning the validity of calibrating trypanosome molecular trees using continental separation.

1. Introduction

Trypanosomes are single-celled (protozoan), obligate parasitic eukaryotes found worldwide and in all vertebrate classes. They have digenetic (two-host) life cycles, which alternate between vertebrate and blood-sucking invertebrate hosts, such as aquatic or terrestrial leeches, tsetse flies, ticks and triatomine bugs. Most trypanosomes are believed to cause little harm to the host, but in tropical Africa tsetse-transmitted trypanosomes are responsible for Human African Trypanosomiasis (HAT; Sleeping Sickness) and Animal African Trypanosomiasis (AAT; Nagana), while in the New World *Trypanosoma cruzi* is the causative agent of Chagas disease.

In Africa, the main focus has always been on the tsetse-transmitted, pathogenic trypanosome species. Comparatively little is known about the range and diversity of these ubiquitous parasites in the huge variety of indigenous terrestrial vertebrates. For example, recent studies have used PCR to identify trypanosomes in tsetse flies (e.g. Morlais et al., 1998; Lehane et al., 2000; Jamonneau et al., 2004; Njiru et al., 2004), livestock (Jamonneau et al., 2004; Ng'ayo et al., 2005) or wild vertebrates (Njiokou et al., 2004), but the primer sets used were specific for the known pathogenic species. New PCR-based, generic identification techniques (e.g. based on length variation of the internal transcribed spacer regions of the ribosomal RNA (rRNA) locus) have the potential to recognize all trypanosomes and therefore reveal the full diversity of trypanosome species, but so far these methods have been applied only to the identification of trypanosomes in tsetse or livestock (Desquesnes et al., 2001; Cox et al., 2005; Njiru et al., 2005; Adams et al., 2006). In particular, fluorescent fragment length barcoding (FFLB) can provide extremely fine discrimination of different species (Adams et al., 2008; Hamilton et al., 2008). In

☆ Accession numbers: GAPDH sequences: FM164792–FM164795, 18S rDNA sequences: FM202489–FM202493.

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this technique, short sections of the variable regions of the 18S and 28S ribosomal RNA genes are PCR amplified using fluorescently labeled primers, allowing accurate sizing of the amplified fragments on an automated DNA sequencer. Since primers are targeted to relatively conserved sequences flanking the variable length regions, it is possible to design genus-specific primers capable of distinguishing individual species, subspecies or strains within the genus by fragment length polymorphisms. For genus *Trypanosoma*, where more than 472 species have already been described (Noyes, 1998), FFLB has the potential to be a very useful tool and has already identified a putative new species within the tsetse-transmitted group (Hamilton et al., 2008). Here, we have applied FFLB to identify trypanosomes collected from indigenous African vertebrates.

In 2004, an extensive survey of 1141 wild vertebrates from Cameroon revealed an infection rate of 18.7% using specific PCR tests for *Trypanosoma vivax*, *T. brucei*, *T. congolense* "forest" and *T simiae*. However, presumably many infections with other trypanosome species remained unidentified and, in particular, six trypanosomes isolated into culture tested negative with this range of species-specific tests (Njiokou et al., 2004). In this study, we characterize these isolates using a combination of FFLB and phylogenetic analysis of 18S rRNA and glyceraldehyde phosphate dehydrogenase (*gGAPDH*) genes.

2. Materials and methods

2.1. DNA origins

The six isolates used in this study originated from a study in Cameroon in which 1141 wild vertebrates were examined for trypanosomes (Njiokou et al., 2004). These isolates were grown *in vitro* with 'kit for *in vitro* isolation' (KIVI) kits, yet could not be identified and tested negative using the species-specific PCR tests for *Trypanosoma brucei* s.l., *T. vivax*, *T. congolense* "forest" and *T. simiae* (Njiokou et al., 2004). Details of these isolates are given in Table 1. DNA was prepared from cultured procyclic forms using DNeasy tissue kit (Qiagen®) and stored at −20 °C.

2.2. Fluorescent fragment length barcoding and DNA sequencing

All six DNA isolates were analyzed by FFLB as described previously using a total of four primer sets: two sets each for 18S and 28S rRNA genes (Hamilton et al., 2008). The profile of each isolate was compared to those of other African trypanosomes obtained in the previous study (see Table 1) (Hamilton et al., 2008). For all isolates, partial or complete sequences of the 18S rRNA and *gGAPDH* genes were obtained by direct sequencing of PCR fragments amplified from genomic DNA, using Phusion (New England BioLabs) as described previously (Maslov et al., 1996; Stevens et al., 1999b; Hamilton et al., 2004, 2005).

2.3. DNA sequence alignments

Four alignments of the *gGAPDH* and/or 18S rDNA sequences were created and analysed.

2.3.1. Alignment1

The four >800 bp *gGAPDH* sequences obtained from trypanosomes from a monkey (FM164794), palm civet (FM164793) crocodile (FM164795) and sitatunga (FM164792) were aligned using our previous alignment (Accession ALIGN_001079) (Hamilton et al., 2007). In the final alignment, 56 trypanosome *gGAPDH* sequences, representing all major clades, and 25 other trypanosomatid sequences for use as outgroups, were selected for analysis.

2.3.2. Alignment 2

Full length (>2189 bp) 18S rDNA sequences were obtained from trypanosomes from a monkey (FM202493) and palm civet (FM202492) and were aligned using our previous alignment (Accession ALIGN_001077) (Hamilton et al., 2007). In the final alignment, 74 trypanosome sequences, representing all known trypanosome clades, and 14 other trypanosomatid sequences for use as outgroups, were selected for analysis. 1822 characters (322 parsimony informative) were included in analysis; excluded characters are given in Hamilton et al. (2007).

Table 1
Origin of trypanosomes from this study, and their FFLB profiles.

Isolate/species	Host	FFLB profile				Gene Accessions 18S rDNA *gGAPDH*
		18S1	18S3	28S1	28S2	
CepCamp4	Blue Duiker, *Cephalophus monticola*	234	217	250	182	FM202491
CepCamp5	Blue Duiker, *Cephalophus monticola*	235	216	250	183	FM202490
SitaBip1	Sitatunga, *Tragelaphus spekei*	236	216	250	183	FM202489, FM164792
CroCamp1	Crocodile, *Crocodylus niloticus*	233	216	246	181	FM164795
HochNdi1	Greater white-nosed monkey, *Cercopithecus nictitans*	274	221	294	206	FM202493, FM164794
NanDoum1	Palm civet, *Nandinia binotata*	265	224	279	188	FM202492, FM164793
Reference isolates						
T. brucei sspp, *T. evansi*, *T. equiperdum*		208–209	234	291–294	198–199	
T. congolense forest		234–239	233–241	281–285	217–220	
T. congolense kilifi		228, 233	259	288	208	
T. congolense savannah		239	233	284–288	206–207	
T. godfreyi		160	212, 215–216	279, 281	202–204	
T. grayi		233	216	246	181	
T. simiae		159	213	271, 273	199–200	
T. simiae Tsavo		162	211	275–278	195–196	
T. sp. F4		241	217	259	186	
T. sp. (*T. brucei*-related trypanosome)		224	237	332	193–194	
T. theileri		233–234	216	250–251	183	
T. vivax		182	199	–	173	

For origins of trypanosomes, see Njiokou et al. (2004). Genotypes of reference isolates are from Hamilton et al. (2008).

2.3.3. Alignment 3

In order to determine the position of the trypanosomes from the palm civet and the monkey within the *T. cruzi* clade with greater precision, the sequences were added to a combined alignment of *gGAPDH* and 18S rDNA sequences (Accession ALIGN_001079). Analysis was then restricted to 10 trypanosomes in the *T. cruzi* clade; this increased the number of characters in the more variable regions of 18S rDNA that could be included in analysis by 69, as they were judged to be well aligned (Accession ALIGN_001288). A total of 3022 (210 parsimony informative) characters were included in analysis. The 18S rDNA sequence from an African bat trypanosome (AJ012418) was included, although no *gGAPDH* sequence is available. Excluded characters were 1–105 991–1117 2202–2255 2287–2387 2778–2810. A trypanosome isolated from a kangaroo was used as an outgroup, since several published 18S rDNA and *gGAPDH* gene trees show it to form the deepest branch in the *T. cruzi* clade (Stevens et al., 1999a,b; Hamilton et al., 2004, 2007).

2.3.4. Alignment 4

Sequences of the D7-D8 region of 18S rDNA from the three *T. theileri* isolates (FM202489–FM202491) were aligned with those from *T. theileri*, mostly isolated from Buffalo, cattle and tabanids in South America (Rodrigues et al., 2005) and trypanosomes from the sister group, using Clustal X (Thompson et al., 1997)-(Accession ALIGN_001289). Sequences from the sister clade, which includes trypanosomes from South East Asia and Australian terrestrial leeches (Hamilton et al., 2005), were used as outgroups. All 770 characters (65 were parsimony informative) characters in the D7-D8 region were judged to be well aligned and were included in the analysis. Excluded characters were 1–676 and 1447–2248. The alignment contained 42 taxa.

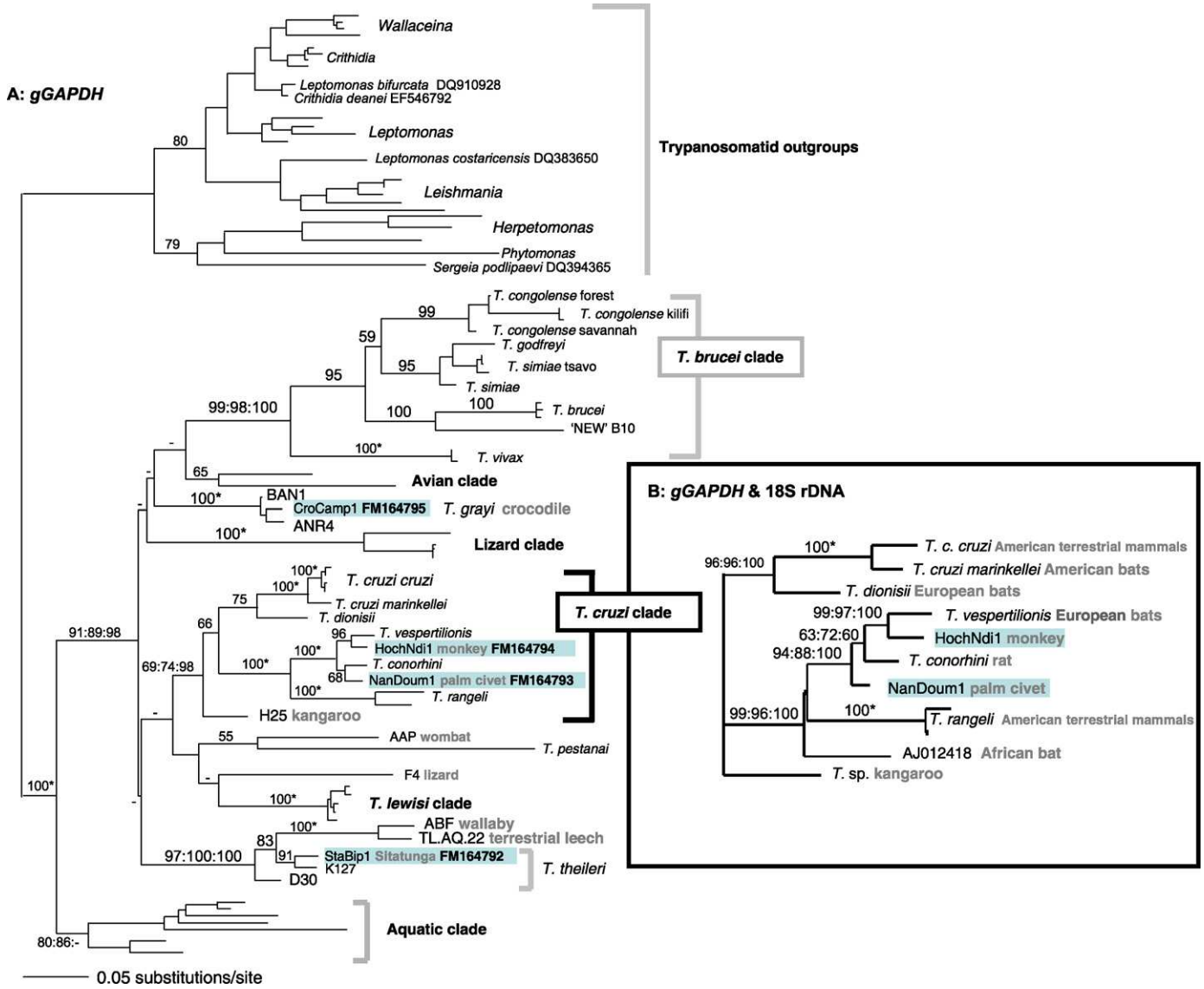

Fig. 1. (A) ML *gGAPDH* gene tree, based on an alignment of 872 characters, all of which (435 parsimony-informative) were included in the analysis. The model of nucleotide substitution was GTR + G with estimated nucleotide base frequencies. It contains 56 trypanosome taxa, representing all known clades, and was rooted using 25 other diverse trypanosomatids. −Ln = 15697.06861. Single values at nodes are ML bootstrap values. Multiple values at nodes are bootstrap values (%) in order: ML (100 replicates), MP, ML distance (1000 replicates). 100* = support values of 100 by all methods. – = support value <50. Boxed taxa were analysed in this study—for details of other isolates see (Stevens et al., 1999b; Hamilton et al., 2004, 2007). Taxa in grey are hosts. (B) Combined *gGAPDH* gene and SSU rDNA ML tree, based on an alignment of 3022 characters. −Ln = 7101.77446. The model of nucleotide substitution was TrN + G, with estimated nucleotide base frequencies. Single values at nodes are ML bootstrap values. Multiple values at nodes are bootstrap values (%) in order: ML, MP and ML distance. 100* = support values of 100 by all methods. – = support value <50.

2.4. Phylogenetic analysis

All alignments were analysed by maximum likelihood (ML), maximum parsimony (MP) and maximum likelihood distance (MLdist) analysis as implemented in the program PAUP version 4.0b10 (Swofford, 2003). ModelGenerator version 0.84 (Keane et al., 2006) was used to find optimal models for ML and ML distance analyses. Models selected were: Alignment 1—GTR + G with estimated nucleotide frequencies; Alignment 2—GTR + G with estimated nucleotide frequencies; Alignment 3—TrN + G with estimated nucleotide frequencies; and Alignment 4—K81 + G with equal base frequencies. ML bootstrap analyses were performed using 100 replicates and 100 tree bisect and reconnect (TBR) rearrangements for alignments 1 and 2 and 1000 replicates with 10,000 TBR rearrangements for alignments 3 and 4. For MP analyses, heuristic searches were performed with 10 random addition replicates and TBR branch swapping. 1000 bootstrap replicates were calculated, using a maximum of 100,000 TBR rearrangements for each replicate. The Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), as implemented in PAUP* version 4.0b10 (Swofford, 2003), was used to compare constrained trees with the optimal trees.

3. Results

3.1. Analysis of field samples by FFLB

The FFLB profiles obtained from all six isolates are shown in Table 1. These were compared with those from reference isolates from our previous study (Hamilton et al., 2008). The profile of the trypanosome from the crocodile exactly matched the reference profile of that of *T. grayi*, a tsetse-transmitted crocodile trypanosome. Since both of the reference isolates (ANR4 and BAN1) were collected from wild tsetse flies (*Glossina palpalis gambiensis*) in The Gambia (McNamara and Snow, 1991) rather than directly from crocodiles, this provides confirmation that all three isolates are indeed *T. grayi*. The profiles of the three antelope trypanosomes were most similar to those of *T. theileri*, differing from the reference isolates by a maximum of 2 bp at each locus. In contrast, the profiles of the trypanosomes from the monkey and palm civet differed from each other and those of all the reference isolates at two or more of the four loci.

3.2. Phylogenetic analysis

The 18S rRNA and *gGAPDH* genes of four of the six isolates were PCR amplified, sequenced and added to existing trypanosomatid alignments. The *gGAPDH* trees (e.g. Fig. 1A) confirmed the identification of the sitatunga trypanosome as *T. theileri*, and the crocodile trypanosome as *T. grayi*, as predicted from their FFLB profiles. Trypanosomes from the monkey and palm civet both fell in the *T. cruzi* clade, in a subclade with *T. rangeli* (a South American trypanosome transmitted by triatomine bugs and found in a wide range of mammalian hosts including humans), *T. vespertilionis* (a European bat trypanosome) and *T. conorhini* (a trypanosome of the rat *Rattus rattus* transmitted by a triatomine bug *Triatoma rubrofasciata*). The monkey trypanosome formed a monophyletic clade with *T. vespertilionis* with unequivocal support (bootstrap support (%): ML = 96: MP = 86: MLdist = 94), whereas that from the palm civet formed a monophyletic clade with *T. conorhini* with high bootstrap support (bootstrap support (%) ML = 69: MP = 73: MLDist = 78). The trees based on full length 18S rDNA sequences (Alignment 2—not shown) confirmed that the monkey and palm civet trypanosomes fall in a subclade of the *T. cruzi* clade with *T. rangeli*, *T. conorhini*, *T. vespertilionis* and a trypanosome from a bat from Gabon in West Africa (for which there is no available *gGAPDH* sequence). However there was little resolution in this subclade; no clades received bootstrap support greater than 50% in any of the trees constructed using three different methods.

Trees constructed from the combined alignment of *gGAPDH* and 18S rDNA sequences (e.g. Fig. 1B) provided unequivocal support (ML bootstrap 99%) for a clade with the monkey trypanosome and *T. vespertilionis*. However the palm civet trypanosome and *T. conorhini* did not form monophyletic clade as in the *gGAPDH* tree. Nevertheless, constrained trees, in which monophyly of *T. conorhini* and the palm civet trypanosome was forced, were not rejected, based both on the combined alignment ($p = 0.107$), or the 18S rDNA part of this alignment only ($p = 0.063$). A constrained tree in which monophyly of the trypanosomes from the palm civet and the monkey was forced was significantly suboptimal ($p = 0.017$).

To determine with greater precision the phylogenetic position of the three antelope trypanosomes identified as *T. theileri* by FFLB, sequences of the D7-D8 region of 18S rDNA were aligned with those from other *T. theileri* isolates. The three sequences differed from each other and all available *T. theileri* sequences by 0.28–1.95% (817 bp compared). Trees based on this alignment, such as the ML tree shown in Fig. 2, show two principal subclades of *T. theileri,* subdivided into a further 7 lineages A–G, that generally receive low or no bootstrap support. Lineages A–E have been described previously (Rodrigues et al., 2005), whereas F and G are new. All three antelope trypanosomes fell in subclade 2, but in different subgroups. The two isolates from duiker (*Cephalophus monticola*), CepCamp4 and 5, fell in lineages C and E respectively; lineage C also contains trypanosomes from Brazilian cattle (Rodrigues et al., 2005), while lineage E contains only a European deer trypanosome. The isolate from an amphibious antelope, the sitatunga (*Tragelaphus spekei*), separated into a new lineage, F.

4. Discussion

The use of FFLB coupled with phylogenetic analysis, has enabled us to characterize six previously unidentified trypanosome isolates from African vertebrates. Four of the six isolates were identified as either *T. theileri* or *T. grayi*, while the trypanosomes from a monkey and palm civet are of unknown species. According to Hoare (1972), there are only a handful of previous records of non-Salivarian trypanosomes described from African primates, and none from civets. As no living isolates of these monkey trypanosomes exist, it is impossible to say whether any of them are identical to the trypanosomes described here.

Both the monkey and palm civet trypanosomes fell in the *T. cruzi* clade, which contains *T. cruzi* itself, together with various other trypanosome species found in Central or South American terrestrial mammals, and also in bat species which are geographically widespread (Stevens et al., 1999a,b). The placement of a trypanosome from an Australian kangaroo on the periphery of this clade led to the idea that the clade originated on the southern super-continent comprising present day Antarctica, Australia and South America, when marsupials were the dominant mammalian fauna. In the absence of a fossil record, these observations were used to deduce two of the calibration points for dating trypanosome molecular trees. Firstly, separation of Africa and South America has been used to date the split of the *T. brucei* and *T. cruzi* clades at 100 million years ago (Stevens et al., 1999b; Stevens and Rambaut, 2001), and secondly, the split of Australia from Antarctica/South America has been used to date the spilt between the kangaroo trypanosome from the rest in the *T. cruzi* clade at approximately 80–45 million years ago (Stevens et al., 2001). Knowledge of the divergence date of *T. brucei* and *T. cruzi* would aid interpretation of information from the genomes of these species,

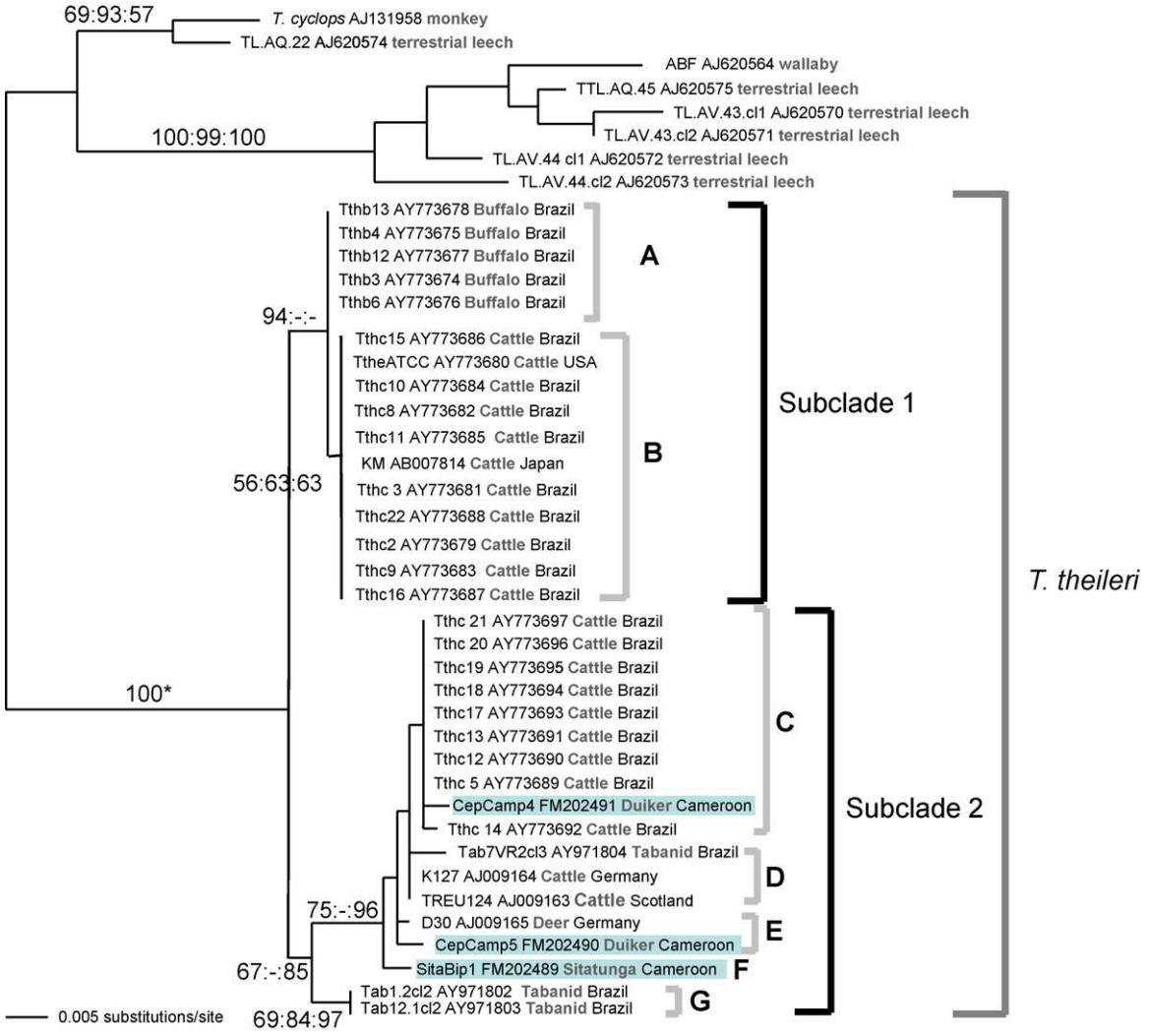

Fig. 2. ML tree, based on the D7-D8 region of SSU rDNA gene, based on an alignment of 770 (65 parsimony informative) characters. −Ln = 1845.4544. The model of nucleotide substitution was K81 + G, with equal base frequencies. It contains 42 trypanosome taxa. Single values at nodes are ML bootstrap values. Boxed taxa were analysed in this study. Taxa in grey are hosts. Multiple values at nodes are bootstrap values (%) in order: ML, MP and ML distance. 100* = support values of 100 by all methods. – = support value <50.

but our new finding – that trypanosomes of the *T. cruzi* clade occur in African terrestrial mammals – means that this hypothesis now needs to be reviewed.

It is doubtful that the trypanosomes from the monkey and palm civet represent a recently introduced lineage from South America, because, although similar to each other, the monkey trypanosome is most closely related to the bat trypanosome *T. vespertilionis*, while the civet trypanosome appears to be more closely related to *T. conorhini*; more than one introduction is therefore required. An alternate explanation is that the group has evolved independently in Africa. If so, we may expect to find more representatives of the *T. cruzi* clade in other animals indigenous to Africa.

Considering this scenario, either the *T. cruzi* clade already existed in Africa before the separation of the continents, or there have been repeated introductions. Previous reports have suggested a wider distribution of trypanosomes in the *T. cruzi* clade in terrestrial mammals; trypanosome species have been described in South East Asian primates that resemble *T. conorhini* (Weinman, 1977) and *T. cruzi* (Kuntz et al., 1970) in terms of morphology and lifecycle, although molecular taxonomic studies are required to confirm these findings. Repeated introductions are most likely to have been mediated by bats, as these highly mobile mammals are already known to harbour several different lineages of the *T. cruzi* clade. Indeed, three of these lineages are known from outside the New World, and the isolates of *T. vespertilionis* and *T. dionisii* represented in phylogenetic trees are all from Europe (Stevens et al., 1999a,b), although *T. cruzi* clade trypanosomes have not so far been recovered from European terrestrial mammals. All four bat lineages could have arisen by host jumping from New World terrestrial mammals. However, our data suggest that a bat lineage gave rise to a lineage in terrestrial mammals. Thus other lineages in terrestrial mammals, including those in the New World, may have arisen from bat lineages. Host-jumping between species could have occurred by vectorial transmission or through carnivores, such as civets, feeding on infected bats, or rats infected with *T. conorhini*. Whether already present in Africa at the time of continental separation, or introduced more recently, these results question the validity of using continental separation times to calibrate trypanosome molecular trees.

The trypanosomes from the three antelope species were identified as *T. theileri*, a trypanosome commonly found in domestic cattle and wild artiodactyls world wide (Böse et al., 1993; Rodrigues et al., 2003; Rodrigues et al., 2005) including Africa—in cattle (Gray

and Nixon, 1967; Latif et al., 2004; Goossens et al., 2006) and wild artiodactyls including duiker (Keymer, 1969; Bigalke et al., 1972). There has been considerable debate as to whether trypanosomes from wild artiodactyls, that morphologically resemble *T. theileri*, should be classified as *T. theileri* or separate species (Keymer, 1969; Böse et al., 1993; Rodrigues et al., 2005). The results of this study suggest that they should all be classified as strains of *T. theileri*. Both of the main subclades contain trypanosomes from both cattle and other artiodactyls and one of the duiker trypanosomes that fell in *T. theileri* lineage C, that previously only included isolates from cattle (Rodrigues et al., 2005). A previous study found *T. theileri* isolates from cattle are not monophyletic, although isolates from Buffalo and cattle isolated within the same region fell in different subclades (Rodrigues et al., 2005). Thus, although there is specificity of some strains to vertebrate hosts in a limited geographic region, these host associations are not stable over evolutionary time. It is also noteworthy that three of the seven lineages are found in more than one continent.

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