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Research paper

Expanding our knowledge on African trypanosomes of the subgenus *Pycnomonas*: A novel *Trypanosoma suis*-like in tsetse flies, livestock and wild ruminants sympatric with *Trypanosoma suis* in Mozambique☆

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ABSTRACT

Among the subgenera of African tsetse-transmitted trypanosomes pathogenic to livestock, the least known is the subgenus *Pycnomonas,* which contains a single species, *Trypanosoma suis* (TSU), a pathogen of domestic pigs first reported in 1905 and recently rediscovered in Tanzania and Mozambique. Analysis by Fluorescent Fragment Length Barcoding (FFLB) revealed an infection rate of 20.3% (108 out of 530 tsetse flies) in a recent study in the Gorongosa and Niassa wildlife reserves in Mozambique, and demonstrated two groups of *Pycnomonas* trypanosomes: one (14.1%, 75 flies) showing an FFLB profile identical to the reference TSU from Tanzania, and the other (6.2%, 33 flies) differing slightly from reference TSU and designated *Trypanosoma suis*-like (TSU-L). Phylogenetic analyses tightly clustered TSU and TSU-L from Mozambique with TSU from Tanzania forming the clade *Pycnomonas* positioned between the subgenera *Trypanozoon* and *Nannomonas*. Our preliminarily exploration of host ranges of *Pycnomonas* trypanosomes revealed TSU exclusively in warthogs while TSU-L was identified, for the first time for a member of the subgenus *Pycnomonas*, in ruminants (antelopes, Cape buffalo, and in domestic cattle and goats). The preferential blood meal sources of tsetse flies harbouring TSU and TSU-L were wild suids, and most of these flies concomitantly harboured the porcine trypanosomes *T. simiae*, *T. simiae* Tsavo, and *T. godfreyi*. Therefore, our findings support the link of TSU with suids while TSU-L remains to be comprehensively investigated in these hosts. Our results greatly expand our knowledge of the diversity, hosts, vectors, and epidemiology of *Pycnomonas* trypanosomes. Due to shortcomings of available molecular diagnostic methods, a relevant cohort of trypanosomes transmitted by tsetse flies to ungulates, especially suids, has been neglected or most likely misidentified. The method employed in the present study enables an accurate discrimination of trypanosome species and genotypes and, hence, a re-evaluation of the "lost" subgenus *Pycnomonas* and of porcine trypanosomes in general, the most neglected group of African trypanosomes pathogenic to ungulates.

1. Introduction

African pathogenic trypanosomes transmitted by tsetse flies are responsible for a devastating disease complex in domestic ungulates, African Animal Trypanosomiasis (AAT), which represents a major limiting factor for livestock production in Sub-Saharan Africa.

☆ Note: Nucleotides sequence data reported in this paper are available in GenBank under accession numbers listed in Supplementary Table S1.

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Comprehensive knowledge of the genetic diversity of the pathogenic trypanosomes that cause AAT, the range of wild and domestic hosts infected, and the tsetse vectors is essential for understanding the evolution, epidemiology, and pathogenicity of AAT, as well as determining control measures. Molecular diagnoses combined with phylogenetic analyses have expanded our knowledge of the genetic diversity of the extensively studied group of trypanosomes pathogenic to ungulates (Gibson et al., 2001; Hamilton et al., 2008; Rodrigues et al., 2008; Adams et al., 2010a, 2010b; Auty et al., 2012; Votýpka et al., 2015; Rodrigues et al., 2017, 2019; Garcia et al., 2018; Gaithuma et al., 2019).

The traditional method of trypanosome identification in tsetse flies relies on developmental sites of the trypanosome in dissected tsetse flies, which enables the identification of three main species: *T. brucei*, *T. congolense* and *T. vivax*. The use of generic primers that target fast-evolving regions of the ribosomal RNA locus has enabled new trypanosome genotypes to be discovered and phylogenetically characterised. Fluorescent Fragment Length Barcoding (FFLB; Hamilton et al., 2008) uncovered the new *T. brucei*-like trypanosome in *Glossina pallidipes* caught in the Msubugwe Forest Reserve, Tanzania, which was referred to as the Msubugwe trypanosome (Adams et al., 2008; Hamilton et al., 2008), was subsequently identified as *Trypanosoma* (*Pycnomonas*) *suis* by comparison with archival DNA extracted from pig blood smears of the original *T. suis* characterised by Chardome and Peel in the 1950s (Hutchinson and Gibson, 2015). Molecular information about TSU began to be reported, such as its unique SL RNA sequences and structure, and species-specific repetitive elements of satellite DNA (Hutchinson and Gibson, 2015).

Trypomastigotes of *T. suis* (TSU) in pig blood are small and monomorphic, with a short flagellum and terminal kinetoplast, overall resembling members of the subgenus *Nannomonas*. The seminal work of Peel and Chardome (1954) showed that TSU develops in the midgut and salivary glands of tsetse flies, with infective forms produced in the hypopharynx. This unique developmental cycle in the tsetse fly vector prompted Hoare (1964, 1972) to place TSU in a new subgenus of Salivarian trypanosomes: *Pycnomonas*. Experimental infections demonstrated that TSU caused severe acute disease in piglets and chronic infection in adult pigs, but was unable to infect domestic ruminants (goats, sheep, calves, and donkeys), laboratory rodents, dogs, and many small wild mammals. These results led to the prevailing view that TSU is restricted to suids, with wild suids such as warthogs, bush pigs, and forest hogs as natural hosts (Peel and Chardome, 1954; Hoare, 1972).

One decade after its rediscovery, TSU remained neglected in trypanosome surveys in both tsetse flies and ungulates, including pigs. Surveys of TSU are of great importance for gaining a better understanding of pathogenicity, transmission cycles and evolutionary histories of African trypanosomes. We previously suggested that the subgenus *Pycnomonas* might be more genetically diverse than originally thought; surveys of these trypanosomes in tsetse flies from Mozambique uncovered slightly variable FFLB profiles resembling that of TSU (Garcia et al., 2018). In addition, Votýpka et al. (2015) identified DNA sequences of a novel *Trypanosoma* sp. Makumba in tsetse flies from the Central African Republic more related to TSU than to any other trypanosome. Wide-ranging molecular surveys of trypanosomes across Sub-Saharan Africa using diagnostic assays capable of simultaneously distinguish TSU and other porcine trypanosomes, *T. simiae* (TSM), *T. simiae* tsavo (TST) and *T. godfreyi* (TGO), are still lacking.

Before the advent of molecular diagnosis, TSU could pass unreported due to its morphological similarity with *Nannomonas* trypanosomes (Hoare, 1964, 1972; Gibson et al., 2001; Adams et al., 2006). Even with the use of molecular analyses for the diagnosis of African trypanosomes, such as the widely adopted method based on the length variability of PCR-amplified ITS1 rDNA (Desquesnes et al., 2001), TSU could still pass undetected since it could not be distinguished from other porcine trypanosomes. This is because TSU, TSM, and TST share virtually identical ITS1 amplicon sizes, thus requiring further sequencing or identification based on TSU-specific satellite DNA (Adams et al., 2006; Hutchinson and Gibson, 2015). In addition, by using this method, TGO can be indistinguishable from both *T. vivax* (TVI) and *T. theileri*, which all yield ITS1 amplicons of similar lengths (Gaithuma et al., 2019). In addition to taxonomic arrangements, a comprehensive knowledge of the ranges of vertebrate hosts and tsetse vectors, especially those of the least known subgenus *Pycnomonas* still require broader surveys using sensitive and accurate molecular methods such as FFLB.

In the present study, we phylogenetically characterised TSU and the novel *Trypanosoma suis*-like (TSU-L) identified in tsetse flies from two wildlife reserves in Mozambique. We also employed FFLB to examine wild and domestic ungulates as possible hosts of *Pycnomonas* and evaluated the occurrence of mixed trypanosome infections and feeding preferences of tsetse flies harbouring trypanosomes of this subgenus. Finally, sequences of TSU and TSU-L were employed in the re-assessment of the positioning of the subgenus *Pycnomonas* in a phylogenetic analysis of all subgenera of African trypanosomes, including all recognised species of porcine trypanosomes.

2. Materials and methods

2.1. Studied areas and tsetse flies collected in Mozambique

The fieldwork for tsetse collection was performed in 2009–2012 at the Gorongosa National Park (GNP), and in 2013–2014 at Niassa Natural Reserve (NNR) (Fig. 1). The Gorongosa National Park (GNP) in the central Mozambique (MZ) province of Sofala is constituted by savannah, grassland plains, wooded forests, permanent rivers bordered by riverine forests, large lakes, and floodplains. The fauna of ungulates within the GNP includes many species of antelope (waterbuck, impala, sable, nyala, kudu, bushbuck, reedbuck, gnus, and others), buffaloes, hippopotamus, and an abundance of wild pigs. The Niassa Natural Reserve (NNR) is in the north of MZ (Fig. 1) in the provinces of Niassa and Cabo Delgado between MZ and Tanzania. This large reserve occupies a vast area covered by savannah, forests, permanent rivers, and wetlands, and is home to large numbers of elephants, buffaloes, antelopes (impala, waterbuck, hartebeest, nyala, wildebeest, duiker, eland, kudu, sable, and others), hippopotamus, zebras, and warthogs and other wild suids.

Tsetse fly collection, dissection, microscopical analysis and preservation were carried out as comprehensively detailed in our previous studies (Rodrigues et al., 2017; Garcia et al., 2018). Briefly, gut contents of 2405 tsetse from the GNP and 386 from the NNR were surveyed for trypanosome by microscopy, and positive gut samples and the heads of respective flies preserved individually in ethanol for DNA preparation. In addition, 295 tsetse flies from the NNR not examined by microscopy were preserved intact in ethanol, dissected and processed for DNA preparation exactly as described previously (Rodrigues et al., 2017; Garcia et al., 2018).

2.2. Ruminant blood samples

The blood samples examined were obtained (2006–2012) from wild suids; 6 *Phacochoerus africanus* (warthogs) and 11 antelopes: 9 *Neotragus moschatus* (suni), one *Cephalophus natalensis* (red duiker), and one *Tragelaphus angasii* (nyala) captured in a game reserve in the province of Sofala. We also tested blood samples from 97 *Syncerus caffer* (Cape buffalo) from the Marromeu Reserve (MR) in the Zambezi River Delta, Sofala (Fig. 1, Supplementary Table S1). The livestock blood samples included 43 and 50 cattle from Tete (province of Tete, north western MZ) and Matutuíne (province of Maputo, southern MZ), and 8 goats from Matutuíne. The blood samples were collected in tubes with EDTA and preserved (*v*/v) with ethanol at room temperature. Samples of livestock were collected as recommended by the Animal Ethical Committee of the University Eduardo Mondlane, and wild animals were

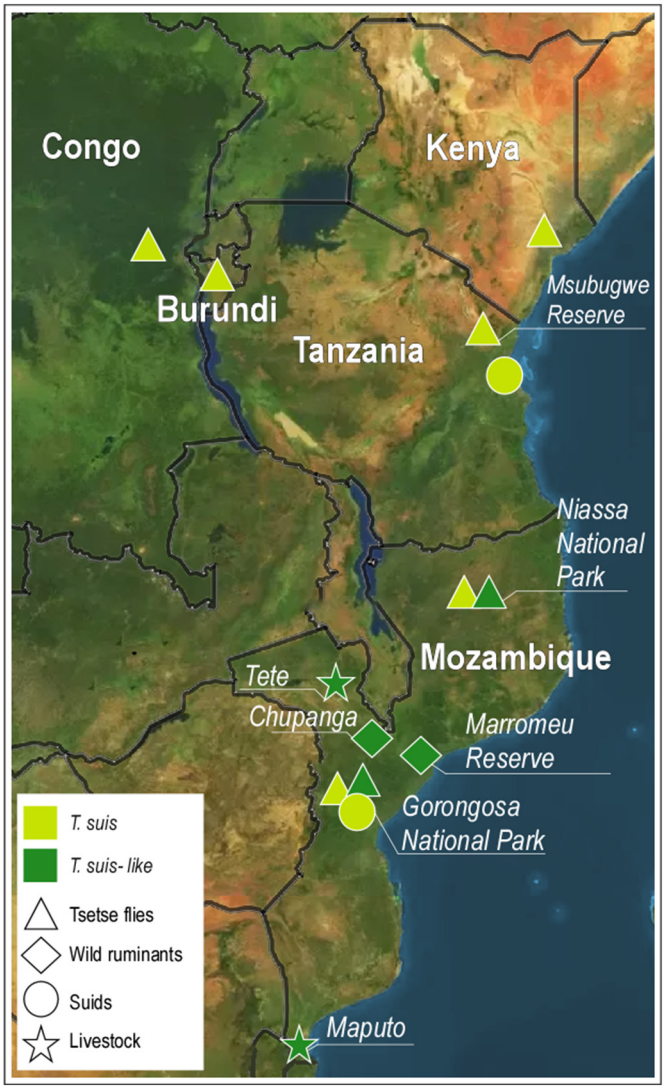

Fig. 1. Map of Central and East Africa showing all sites where *T. suis* (TSU) and *T. suis*-like (TSU-L) have been reported. Location in Mozambique of the wildlife reserves of Gorongosa National Park and Niassa National Reserve (where the tsetse flies examined in the present study were caught), the Reserve of Marromeu, and the Reserve of Msubugwe in Tanzania where the reference TSU G1–62 was obtained (Hamilton et al., 2008; Hutchison and Gibson, 2015). Remaining locations indicated on the map are the places where TSU was previously reported in Kenya (Hoare, 1972), Congo (Van Den Berghe and Zaghi, 1963) and Burundi (Peel and Chardome, 1954).

sampled under authorization of National Administration of Conservation Areas of Mozambique. Laboratory work was performed under protocols approved by the Animal Care and Use Committee at ICB, USP.

2.3. DNA preparation from tsetse and ungulate blood samples and FFLB analysis

Blood samples (~300 µl) and tsetse guts and proboscises were incubated in lysis buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl, pH 8.0, and 350 mg/mL of proteinase K) at 37 °C for 18 h, centrifuged at 14,000*g* for 5 min, and the DNA purified as described previously (Rodrigues et al., 2017, 2019; Garcia et al., 2018). All the tsetse flies were initially screened by FFLB in our previous study (Garcia et al., 2018). This approach uses a set of fluorescent primers designed to amplify four small variable regions within the 18S and 28S rRNA genes and generates a unique profile of four peaks varying in size (barcode) for each trypanosome: the FFLB profile for TSU was as follows: 18S1 (224 bp), 18S3 (237 bp), 28S1 (332 bp), and 28S2 (193–194 bp) (Hamilton et al., 2008). In our study, the FFLB profile for the TSU reference isolate G1–62 using an ABI 3500 sequencer was as follows: 18S1 (226 bp), 18S3 (239–240 bp), 28S1 (337–339 bp), and 28S2 (194 bp).

2.4. PCR amplification and sequencing of SSU rRNA and gGAPDH genes and phylogenetic analysis

DNA samples from positive tsetse flies were subject to whole genome amplification (REPLI-g Ultrafast mini kit, Qiagen) prior to PCR. The amplification of partial SSU rRNA (~700 bp of the variable V7V8 region) was performed as previously described (Noyes et al., 1999), and gGAPDH amplifications employed the primers and PCR conditions described previously by Fermino et al. (2015, 2019). The products of the PCR-amplifications were cloned, and 5–10 clones from each sample were sequenced.

To detect recombination signals or chimeras, all the sequences determined were submitted to analyses using the RDP4 programme (Martin et al., 2015). Then, the SSU rRNA and gGAPDH sequences determined in the present study were aligned using ClustalX with sequences of other African trypanosomes available in GenBank. The alignment of SSU rRNA sequences included *T. vivax* (TVI), *T. congolense* (TCO), *T. congolense* Savannah (TCS), *T. congolense* Kilifi (TCK), *T. congolense* Forest (TCF), *T. congolense* 'Dzanga-Sangha', *Trypanozoon* ssp. (TPZ), *T. simiae* (TSM), *T. simiae* Tsavo (TST), *T. simiae* 'Bai', *T. godfreyi* (TGO), *Trypanosoma* sp. 'Fly9', *Trypanosoma* sp. 'Ngbanda', *Trypanosoma* sp. 'Didon', and *Trypanosoma* sp. 'Makumba'. The alignment of gGAPDH sequences included TVI, TCO, TCS, TCK, TCF, TPZ, TSM, TST, and TGO. The hosts, geographical origin, and GenBank accession numbers of the sequences included in the analyses are listed in Supplementary Table S1.

The phylogenetic analyses were performed by parsimony using PAUP* 4.0b10 (Swofford, 2002) with 100 replicates of random additional sequence followed by branch swapping (RAS-TBR), and Maximum Likelihood using RAxMLv.2.2. (Stamatakis, 2006). The tree searches employed GTRGAMMA with 500 maximum parsimony starting trees. The model parameters were estimated in RAxML over the duration of the tree search. The nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and parsimony starting trees. To visualise the pairwise distances across sequences in the dataset, we carried out multidimensional scaling (MDS) analyses, plotted in 2D and 3D, using the Bios2mds package from the R software platform (R Development Core Team, http://www.R-project.org). The network split decomposition was inferred using the Neighbor-Net method with Kimura 2 parameters implemented, and the internode support was estimated with 100 bootstrap replicates using the parameters optimised for network inferences.

2.5. Identification of blood meal of tsetse flies

DNA from 49 *Glossina morsitans morsitans* and 21 *G. pallidipes* from both wildlife reserves were obtained from gut samples and used as templates for PCR reactions targeting the vertebrate mitochondrial cytochrome *b* (Cytb) sequences (Muturi et al., 2011). The sequences determined were compared with those available in the GenBank database using BLASTN.

2.6. Statistical analyses

To assess any association between trypanosomes of the subgenus *Pycnomonas* and other species of trypanosomes concomitantly detected in tsetse flies, we compared the number of flies infected with TSU and/or TSU-L with the numbers of flies harbouring trypanosomes known to be associated with suids (TSM, TST and TGO), ruminants (TCS and TVI), and both suids and ruminants (TPZ). For statistical analyses, we

employed the Chi-square (χ^2) test using the BioEstat v5.0 software. Significance was accepted at the 95% confidence level and the results were considered to be significant at $p < .05$.

2.7. Data accessibility

Data obtained in the present study were included in the manuscript or available as Supplementary Data. All gene sequences herein determined were submitted to GenBank, and accession numbers are shown in Supplementary Table S1.

3. Results

3.1. Identification of Trypanosoma (Pycnomonas) spp. in tsetse flies from Mozambique by FFLB

In our previous study (Garcia et al., 2018), gut contents of 2791 tsetse flies (2405 from the GNP and 386 from the NNR) were examined by microscopy and trypanosomes were detected in 235 (8.4%) flies: ~8.5% from the GNP (203 of 2405 flies) and ~8.3% from the NNR (32/386). DNA from all the 235 positive guts were submitted to FFLB and trypanosome presence confirmed in all samples. The FFLB analyses of DNA from the proboscises of 181 flies positive by microscopy (151 flies from the GNP and 30 from the NNR) uncovered the presence of trypanosomes in ~75% (136/181). In addition, FFLB analyses of DNAs obtained separately from both the guts and proboscises of 295 tsetse flies from the NNR (not examined by microscopy) revealed the presence of trypanosomes in 88.8% (262 flies) of the guts and 91.8% (271) of the proboscises. Our findings are consistent with the very low sensitivity of microscopy, uncovering trypanosomes in 8.4% (235/2791) of the flies herein examined, compared to the highly sensitive FFLB method for surveys of trypanosomes in tsetse flies (Garcia et al., 2018). Overall, flies examined by FFLB harboured different combinations of FFLB profiles previously defined for each Salivarian trypanosomes as follows: TCO (of the three main groups: 87.2% TCS, 51.5% TCK, and 12.1% TCF), 49.7% TSM, 40.4% TST, 34.4% TGO, 60.6% TPZ, 31.6% TVI, and 23.2% TSU. In the present study, a detailed analysis of all the FFLB profiles assigned to *Pycnomonas* revealed two slightly different profiles: one identical to the reference isolate G1–62 of TSU, and the other differing from the TSU profile at the 28S2 (191 and 195 bp) locus, and referred to as TSU-L. Out of the 530 flies examined by FFLB, 14.1% (75 flies) and 6.2% (33 flies) were found harbouring TSU and TSU-L, respectively. Therefore, TSU and TSU-L occurred in a ratio of approximately 2:1 in tsetse flies from MZ. TSU was identified in 9.8% (20/203 flies: 19 guts and 8 proboscises) and TSU-L in 12.3% (25/203 flies, 23 guts and 4 proboscises) of the flies from the GNP (all positive for trypanosomes by microscopy). In addition, TSU was detected in 18.6% (55/295: 39 guts and 35 proboscises) and TSU-L in 2.7% (8/295: 2 guts and 6 proboscises) of the flies from the NNR. Although results from FFLB were confirmed by PCR-sequencing of gGAPDH sequences, the presence of TSU and TSU-L in the NNR was observed only in flies that were not examined by microscopy. FFLB-positive flies were further analysed by PCR amplification, cloning, and sequencing of gGAPDH and SSU rRNA fragments (Figs. 2, 3).

3.2. Phylogenetic relationships between TSU and TSU-L inferred from gGAPDH sequences: Taxonomic insights

We determined gGAPDH sequences (~609 bp) from trypanosomes detected in 14 tsetse flies (*G. m. morsitans* and *G. pallidipes*) selected by FFLB barcoding as hosts of TSU and TSU-L (Supplementary Table S1). After discarding putatively recombinant or chimeric sequences identified by the RDP4 programme, and identical sequences of cloned amplicons from the same fly, 9 TSU and 6 TSU-L sequences were submitted to phylogenetic analysis (Fig. 2A). It is important to mention that highly similar TSU or TSU-L sequences were identified multiple times, at independent times, in different tsetse flies from distant collecting sites.

The phylogenetic analyses based on gGAPDH sequences strongly supports the subgenus *Pycnomonnas* as a monophyletic assemblage constituted by the sequences from 9 isolates of TSU (all from tsetse flies) and 20 isolates of TSU-L (6 from tsetse flies, 5 from sunis, one from red duiker, one from Cape buffalo, 5 from cattle and two from goats) within the major clade comprising all Salivarian trypanosomes (Fig. 2A). In addition, two subclades of gGAPDH sequences (separated by 7.0% of divergence) were formed: one nesting the sequences of TSU isolates from MZ and Tanzania, and other comprising exclusively TSU-L sequences. Together, the phylogenetic positioning and the degree of gGAPDH sequence divergence support TSU and TSU-L as related but distinct taxa within the subgenus *Pycnomonas*. The consistent separation between TSU and TSU-L was demonstrated in both MDS and network analyses of gGAPDH sequences. However, while TSU and TSU-L sequences clustered closely together in the MDS (Fig. 2B), as expected, in the network analysis divergences between TSU and TSU-L were more clearly evidenced (Fig. 2C). Nevertheless, in all analyses, TSU and TSU-L were more closely related one to each other than to any other trypanosome of all subgenera included in the analysis (Fig. 2C). Within TSU, small polymorphisms on gGAPDH sequences were observed among sequences of TSU isolates from MZ and those from TSU G1–62 and Msu (Msubugwe) from Tanzania, evidencing genotypes of TSU. The sequences of gGAPDH from TSU G1–62 herein determined were separated by ~1.0% sequence divergence from TSU Msu, although both isolates came from Tanzania, whereas TSU G1–62 was separated by 0.9% divergence from TSU MZ, which diverged by 0.2% from TSU Msu. The divergence of gGAPDH sequences was also small in the TSU-L clade, with only two sequences diverging by 0.6% and branching separately, thus supporting the existence of TSU-L genotypes.

The network split tree segregated into well separated branches TSU and TSU-L, as well as the trypanosomes within the subgenus *Nannomonas*, which also formed two main clusters: one comprising TCO and the other formed by the suid trypanosomes TSM, TST, and TGO (Fig. 2C). Consistent with the network topology, TSU and TSU-L greatly differed from one another when compared with the highly divergent trypanosomes in the subgenus *Nannomonas*. TSU differs more from TCO (~14.5% gGAPDH sequence divergence) and the suid trypanosomes TSM (12.7%), TST (13%), and TGO (13.3%) than TSU-L. The divergence of TSU-L sequences was considerably smaller from TSM (9.0%), TST (8.7%), and TGO (7.3%) than from TCO (12.7%).

Our phylogenetic analyses of gGAPDH sequences corroborate the positioning of TSU and TSU-L in the clade *Pycnomonas* sister to the subgenus *Trypanozoon* (Fig. 2A), despite moderate support values likely due to the similar degrees of gGAPDH sequence divergence separating *Pycnomonas* from both *Trypanozoon* (average ~13%) and *Nannomonas* (~12%). However, because sequences of gGAPDH genes are less conserved and generates more reliable alignments than the traditionally employed SSU rRNA sequences, comparison of gGAPDH sequences enabled more reliable positioning and unquestionable assessment of new species and intra-specific genotypes, as herein demonstrated for TSU and the novel TSU-L. Similar results confirming gGAPDH sequences as valuable taxonomic markers have been demonstrated for many other trypanosome species such as *T. theileri* and *T. theileri*-like (Garcia et al., 2011), and *T. vivax* and *T. vivax*-like (Rodrigues et al., 2008; Rodrigues et al., 2017, 2019).

3.3. Phylogenetic analyses of SSU rRNA sequences support a clade constituted by trypanosomes from Mozambique, Tanzania, and the Central African Republic

Aiming the comparison of *Pycnomonas* trypanosomes with many novel trypanosomes so far known just by partial SSU rRNA sequences, which have been employed as DNA barcodes, we infer their phylogenetic relationships with trypanosomes representatives of the whole polymorphism up to now uncovered in all subgenera of the major clade

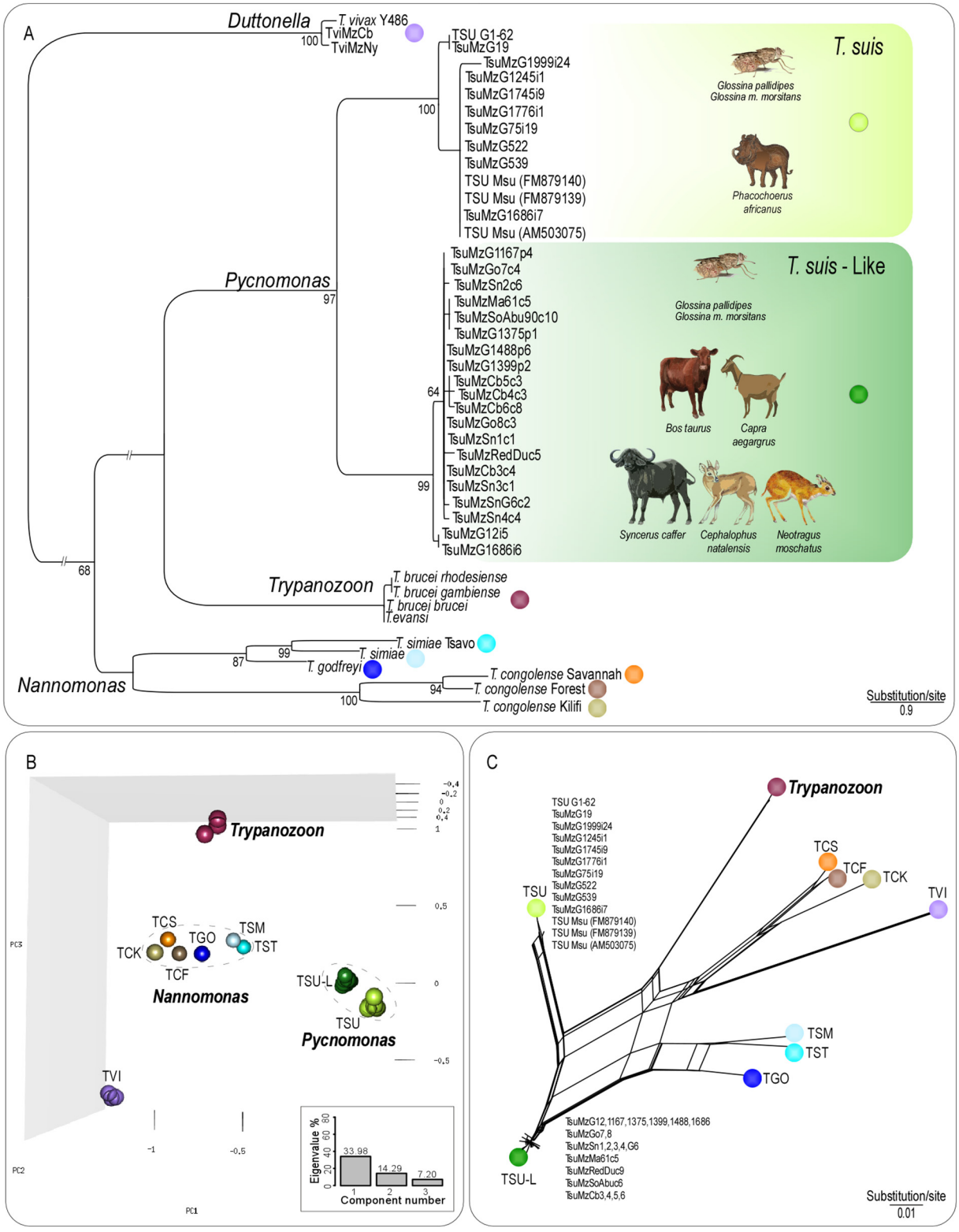

Fig. 2. Phylogenetic relationship of *Trypanosoma suis* (TSU) and *Trypanosoma suis*-like (TSU-L) compared to other African trypanosomes of ungulates based on gGAPDH sequences. (A) Phylogenetic tree (parsimony) strongly supporting TSU and TSU-L in a monophyletic assemblage, representing the subgenus *Pycnomonas*, formed by two phylogenetic lineages, TSU and TSU-L. Numbers at the nodes are support values from 500 replicates; values lower than 50% were discarded. **(B)** Multidimensional scaling analysis showing isolates of TSU clustering with those from TSU-L. **(C)** Network split decomposition evidencing the divergence between TSU and TSU-L despite their close relationships compared to the trypanosomes of other subgenera: *Trypanozoon*, *Nannomonas* and *Duttonella*. TCO, *T. congolense*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TCF, *T. congolense* Forest; TPZ, *Trypanozoon* spp.; TSM, *T. simiae*; TST, *T. simiae* Tsavo; TGO, *T. godfreyi*; TVI, *T. vivax*.

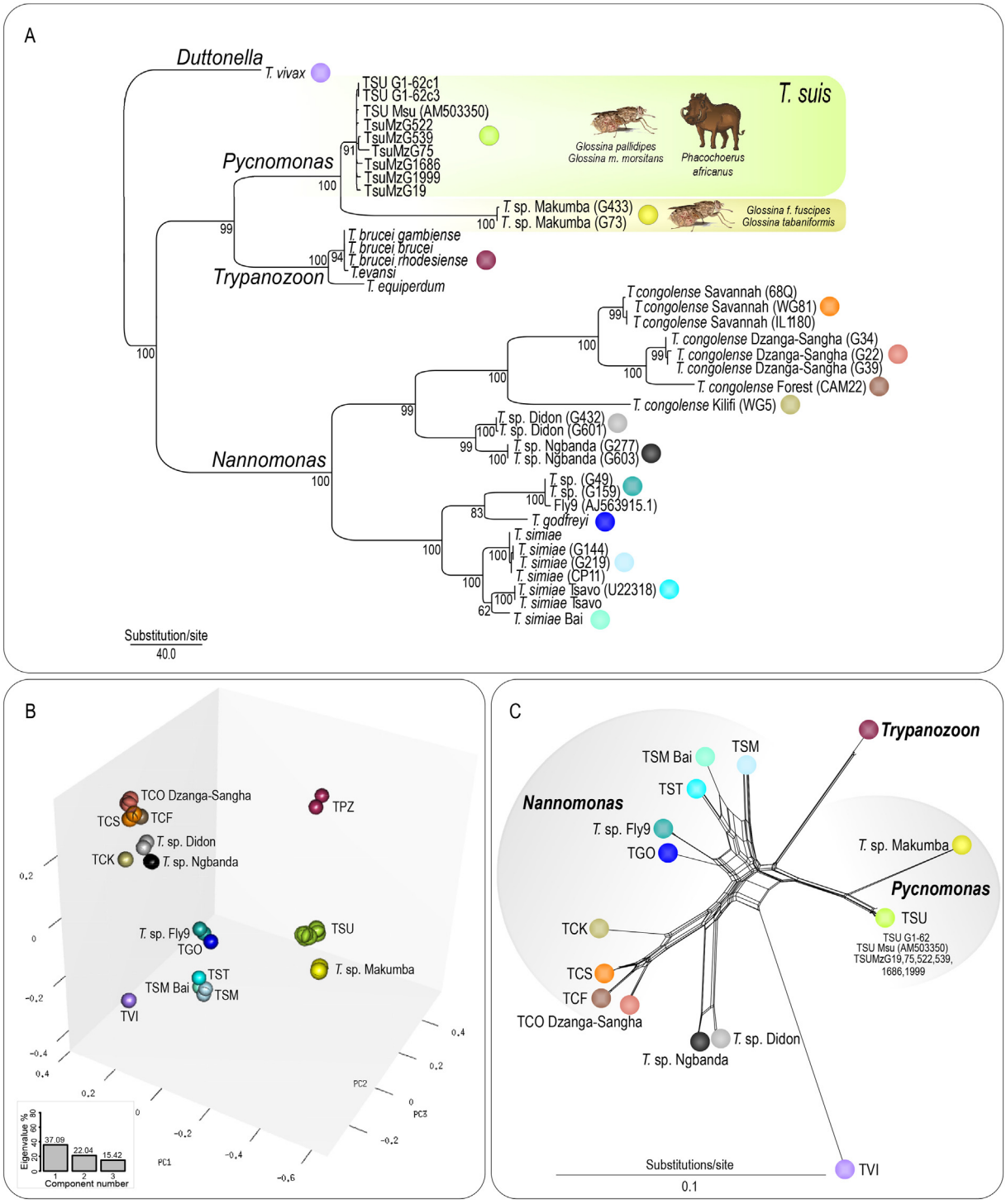

Fig. 3. Phylogenetic relationships inferred by SSU rRNA sequences of *Pycnomonas* trypanosomes. Analyses of SSU rRNA sequences of trypanosomes representative of the whole genetic diversity of African trypanosomes transmitted by tsetse flies classified in the subgenera *Pycnomonas*, *Trypanozoon*, *Nannomonas* and *Duttonella*: **(A)** parsimony phylogenetic tree, numbers at the nodes are support values from 500 replicates; values lower than 50% were discarded **(B)** multidimensional scaling analysis; and **(C)** network split tree. All analyses showed a clade clustering all *Trypanosoma suis* (TSU) sequences from Mozambique and Tanzania (TSU G1–62 and TSU Msu), which was closer to *Trypanosoma* sp. Makumba (Central African Republic) than to any other trypanosome. TCO, *T. congolense*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TCF, *T. congolense* Forest; TPZ, *Trypanozoon* spp.; TSM, *T. simiae*; TST, *T. simiae* Tsavo; TGO, *T. godfreyi*; TVI, *T. vivax*.

T. brucei. This analysis included high quality SSU rRNA sequences (~700 bp) of TSU isolates from six tsetse flies from MZ aligned with sequences of the reference TSU G1–62 (determined in this study) and TSU Msu (from GenBank), both from Tanzania (Supplementary Table S1). The SSU rRNA sequences of TSU from MZ were highly similar (0.4%) and clustered tightly with the TSU G1–62 and TSU Msu in all analyses (Fig. 3A–C), forming the clade *Pycnomonas* sister to *Trypanozoon* (Fig. 3A).

In addition, among many sequences of novel trypanosomes placed into the increasingly complex clade *T. brucei*, only those from *Trypanosoma* sp. Makumba obtained from tsetse flies from the Central African Republic (Votýpka et al., 2015) were placed closer to

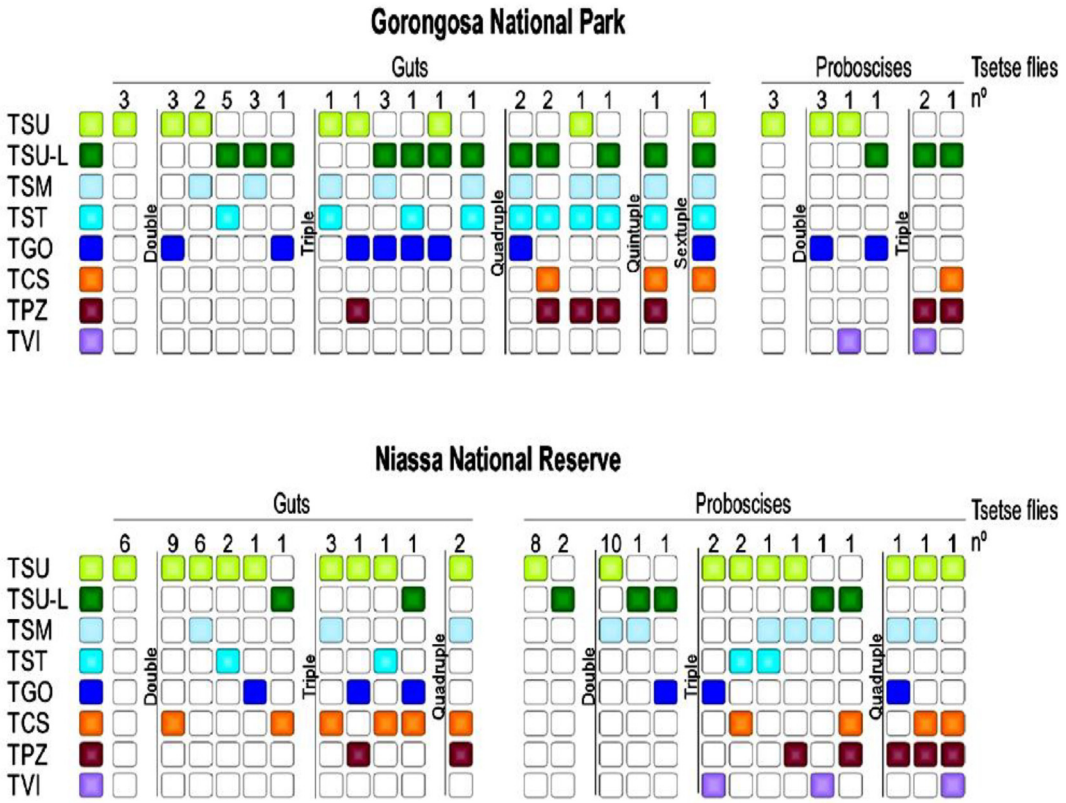

Fig. 4. *Trypanosoma suis* (TSU) and *Trypanosoma suis*-like (TSU-L) concomitant infection with other trypanosome species in tsetse flies from Mozambique. Representative illustration of TSU and TSU-L mixed infections (combinations of two to six trypanosomes) identified in tsetse flies from Mozambique. The whole dataset of trypanosomes infecting individual flies is shown in Supplementary Table S2. TCO, *T. congolense*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TCF, *T. congolense* Forest; TPZ, *Trypanozoon* spp.; TSM, *T. simiae*; TST, *T. simiae* Tsavo; TGO, *T. godfreyi*; TVI, *T. vivax*.

Pycnomonas. *Trypanosoma* sp. Makumba was strongly supported as much more related to TSU than to any other trypanosome of the clade *T. brucei*. In addition, our analyses support two main clades within *Nannomonas,* clearly clustering the porcine trypanosomes separated from TCO (Fig. 3A–C). Despite relevant SSU rRNA divergence separating TSU and *Trypanosoma* sp. Makumba (~5.7%), the average divergence between this novel trypanosome and those of other subgenera than *Pycnomonas* was much higher: 10% (TGO), 12.7% (TSM and TST), and 13% (TCO and TPZ). Together, the positioning and degrees of sequence similarity of SSU rRNA barcodes support *Trypanosoma* sp. Makumba as a different but quite phylogenetically related species of *Pycnomonas* (Fig. 3A); whether this trypanosome represents TSU-L (or an allied species) remains to be investigated, but it has been currently hampered by the lack of its gGAPDH sequence. Unfortunately, our many attempts to obtain SSU rRNA sequences from TSU-L from tsetse flies were fruitless; only sequences of other trypanosomes concomitantly infecting the flies were determined.

3.4. Domestic and wild ruminants are hosts of TSU-like whereas suids appear to be hosts of TSU

We screened blood samples from a total of 114 wild and 101 domestic ungulates by FFLB, although the success rate in obtaining all four peaks was low, probably due to low parasitaemia. The FFLB profiles compatible with TSU were detected only in warthogs (*Phacochoerus africanus*, 2 out of 6 animals tested positive). In contrast, TSU-L profiles were obtained from blood samples of Cape buffalo (*Syncerus caffer*, 12/97) and the antelopes red duiker (*Cephalophus natalensis*, 1/1) and suni (*Neotragus moschatus,* 5/9). TSU-L, but not TSU, was also found in cattle (*Bos taurus*, 5/93) and goats (*Capra aegargus*, 2/8) (Supplementary Table S1).

The sequences of cloned gGAPDH amplicons obtained from blood samples positive for TSU-L by FFLB corresponded to many species of trypanosomes, but rarely of the subgenus *Pycnomonas*. However, we determined gGAPDH sequences of TSU-L from 14 blood samples: five cattle, two goats, one Cape buffalo, and six antelopes. Although TSU was molecularly identified by FFLB in blood samples of two warthogs in the present study, we were unable to obtain a TSU sequence from this host, possibly due to the low parasitemia of TSU in wild pigs (Peel and Chardome, 1954).

3.5. Tsetse flies harbouring concomitantly TSU/TSU-L and porcine trypanosomes

In the tsetse flies examined by FFLB, detection of a single trypanosome either in guts and proboscises were scarce, 13.3% TSU (10/75 tsetse flies) and 6% TSU-L (2/33), and most flies (~88%, 96/108) harboured TSU or TSU-L together with other trypanosomes in their gut and/or proboscis. Of the 75 flies from the GNP and NNR positive for TSU, in 26 (~34%) flies this trypanosome was detected in both guts and proboscises, whereas only one fly (3%) had TSU-L in both its gut and proboscis. FFLP profiles uncovered 65 flies harbouring concomitantly up to five trypanosomes besides TSU: one additional trypanosome was detected in 33 flies (44%), two in 21 flies (28%), three in 8 flies (10.6%), four in one (1.3%), and five trypanosomes were identified just in one fly (1.3%). Similarly, 31 tsetse flies were detected harbouring TSU-L and other trypanosome species/genotypes: one additional trypanosome was detected in 12 flies (36.3%), two in 9 (27.2%), three in 6 (18.1%), four in three (9%) and five trypanosomes just in one fly (3%) (Fig. 4 illustrates different combinations of trypanosomes we have detected by FFLB, while results from the whole set of tsetse examined are shown in the Supplementary Table S2).

In most flies harbouring TSU (60%, 45/75) and TSU-L (81%, 27/33), at least one porcine trypanosome was detected: TSM, TST or TGO. Besides the commonest TSU/TSM and TSU/TGO, more complex combinations included TSU/TSM/TST/TCO/TPZ and TSU/TSU-L/TSM/TST/TGO/TCO in fly guts (Fig. 4, Supplementary Table S1). The commonest combinations including TSU-L were TSU-L/TSM/TGO, TSU-L/TSM/TST/TGO, and TSU-L/TST (Fig. 4). The association between *Pycnomonas* trypanosomes (TSU and TSU-L with the whole set of porcine trypanosomes of the subgenus *Nannomonas,* TSM, TST and TGO, was statistically significant ($p = .026$).

We also assessed the frequency of tsetse flies simultaneously harbouring TSU/TSU-L mixed with TCS or TVI, which are trypanosomes often found in ruminants. TCS, which is highly prevalent (51.5%) in the tsetse flies herein examined from MZ (Garcia et al., 2018), was detected in 34 flies (31.4%) harbouring TSU/TSU-L. Despite significant association ($p = .015$), this combination of trypanosomes was much less common in the tsetse flies examined compared to those including porcine trypanosomes and TSU/TSU-L. In contrast, TVI, which was present in 31.6% of tsetse flies examined, was seldom found together with TSU/TSU-L (Fig. 4): six flies harbouring concomitantly TSU and just three TSU-L in their proboscises, showing no significant association ($p = .850$). Finally, TPZ, which comprises *T. brucei* ssp. common in both suids and ruminants, were detected in 60.6% of the tsetse flies examined, but just 24 flies (22%) also harboured TSU/TSU-L, showing no significant association ($p = .866$).

3.6. Tsetse flies from the GNP and NNR, including those harbouring TSU and TSU-like, fed preferentially on wild suids

Sequences of Cytb obtained from DNA of 70 tsetse flies allowed for the identification of the animals providing blood meals for flies from the GNP and NNR. The DNA sequences determined from 49 *G. m. morsitans* and 21 *G. pallidipes*, randomly selected among those showing blood vestiges that were positive for trypanosomes by microscopy, revealed an overall feeding preference for the blood of wild suids both in the GNP (33% of flies examined) and NNR (36%). The flies that fed on suids revealed most Cytb sequences from *Phacochoerus africanus* (warthog), whereas in 4% of these flies we detected blood from *Potamochoerus* sp. (bush pig). This finding is consistent with high detection rates of porcine trypanosomes uncovered in the tsetse flies examined. The second most prevalent source of blood meal identified, respectively, in 5.4% and 9.6% of the flies from the GNP and NNR, was antelope blood from the following species: 10% *Tragelaphus strepsiceros* (kudu), 4% *Hippotragus niger* (sable antelope), and 3% *Kobus ellipsiprymnus* (waterbuck). In addition, Cape buffalo (*Syncerus caffer*) blood was identified in 15% of flies from the GNP. The remaining tsetse examined showed mixed blood meals in their guts, frequently including suids (species of suids cannot be determined due to low quality of mixed sequences) and rarely humans, and require further analyses of many cloned Cytb sequences. The overall predominance of suid blood mixed with other sources of blood meals in most flies regardless the trypanosomes they are harbouring (including TSU/TSU-L); alongside mixtures of different trypanosome species/genotypes hampered any clear associations of TSU and TSU-L with their ungulate hosts.

4. Discussion

Our previous survey revealed trypanosomes yielding profiles compatible with TSU in the guts and proboscises of *G. pallidipes* and *G. m. morsitans* from the GNP and NNR wildlife reserves in MZ (Garcia et al., 2018). In the present study, we further characterised trypanosomes showing FFLB profiles either identical to or slightly different from that of the reference TSU from Tanzania (Hamilton et al., 2008; Hutchinson and Gibson, 2015). The phylogenetic analyses of trypanosomes differing from the reference TSU uncovered a previously unknown species of *Pycnomonas* herein referred to as TSU-L. For the first time, the existence of TSU in MZ was confirmed by sequencing and phylogenetic analyses of two genes, SSU rRNA and gGAPDH. In all analyses, TSU from MZ clustered tightly with the reference TSU forming the clade *Pycnomonas.* This clade now also includes TSU-L and was placed between the clades *Trypanozoon* and *Nannomonas* as demonstrated previously (Hamilton et al., 2008; Adams et al., 2010b). Notably, the positioning of *Pycnomonas* was anticipated by Hoare (1964, 1972), who created the subgenus *Pycnomonas* based on the unique development of TSU in tsetse flies more akin to that of *T. brucei* (*Trypanozoon*), and the morphological resemblance of blood forms with species of *Nannomonas*. Before our studies in MZ, molecular data on TSU were only reported from Tanzanian tsetse flies (Hutchinson and Gibson, 2015). Original description of TSU came from Kenya, Tanzania, Congo, and Burundi (Peel and Chardome, 1954; Van Den Berghe and Zaghi, 1963; Hoare, 1972). Therefore, to date, TSU had been exclusively reported in East and Central Africa (Fig. 1).

TSU and TSU-L are separated by a large genetic distance (~7.0% gGAPDH sequence divergence); enough to consider them as two distinct species of the subgenus *Pycnomonas*. For comparison, within the complex clade formed by porcine trypanosomes of the subgenus *Nannomonas*, TSM and TST have diverged by ~4.0% and TSM and TGO by ~5.0%. In addition, *Trypanosoma* sp. Makumba (Votýpka et al., 2015) should be considered a more distantly related species of the subgenus *Pycnomonas*. Here, we provisionally adopted TSU-L, since the formal classification of a new species of *Pycnomonas* requires knowledge on the morphology of its blood forms and development in tsetse flies, given that this subgenus was established on the basis of the unique development of TSU in tsetse flies (Hoare, 1964, 1972). Despite the relevance of the phylogenetic positioning, genetic distance is still a controversial taxonomical parameter for the description of species within the clade *T. brucei.* In this clade, the subgenera, species, and subspecies were originally described on the basis of behaviour in tsetse and vertebrate hosts, the morphology of blood forms, and the pathogenicity and clinical manifestations in livestock (Hoare, 1972; Gibson et al., 2001; Gibson, 2007).

Tsetse flies from different genetic groups have been identified harbouring *Pycnomonas*, indicating that they are potentially able to transmit these trypanosomes: *Glossina m. morsitans*, a newly reported host of TSU in MZ, *G. pallidipes* in MZ and Tanzania, *Glossina vanhoofi* in Congo, and *G. brevipalpis* in Burundi (Peel and Chardome, 1954; Hoare, 1972; Hamilton et al., 2008; Garcia et al., 2018). Although the low prevalence rate of TSU reported in tsetse flies (Van Den Berghe and Zaghi, 1963; Hamilton et al., 2008; Adams et al., 2010b) has been confirmed by our study, the TSU infection rate was higher for *Pycnomonas* (3.5%) than for TGO (3.3%) and TCF (1.7%) (Garcia et al., 2018). The detection of TSU/TSU-L by FFLB and PCR, not only in the guts but also in the proboscises, strongly suggests the presence of mature trypanosomes in these flies and not the mere finding of these parasites present, generally in very small amounts (especially in the proboscises) in blood meals.

Possible vertebrate hosts of TSU/TSU-L were investigated by FFLB examination of blood samples from wild and domestic ungulates. TSU was detected in warthogs but not in ruminants, whereas TSU-L was identified in livestock (cattle and goat) and wild ruminants (Cape buffalo and the antelopes red duiker and suni). This is the first evidence that trypanosomes of *Pycnomonas* can infect ruminants. Although tsetse flies infected with TSU and TSU-L were captured in areas with a large warthog population, we were only able to examine six specimens of warthogs captured at the GNP, and two were positive for TSU. Unquestionably, the ability of TSU-L to infect suids remains to be further investigated using larger suid samplings. However, the absence of TSU in the large number of ruminants examined, and reports of fruitless attempts at infecting different ruminant species with TSU (Peel and Chardome, 1954; Hoare, 1972) might be due to the presumed tight association (thought to be host-restriction) between TSU and suids, similar to that reported for TST and TGO. Therefore, it might be possible

that TSU-L infects both suids and ruminants, similarly to TSM, TCO, TPZ and, very sporadically, TVI (Jamonneau et al., 2004; Simo et al., 2006, 2012; Biryomumaisho et al., 2013; Hamill et al., 2013; Karshima et al., 2016; N'Djetchi et al., 2017). Therefore, despite the limitations of our analysis, it is clear that TSU-L has a wider host range than TSU, including wild and domestic ruminants, whereas TSU appears to be restricted to suids.

Despite uncovering a large repertoire of trypanosomes pathogenic to ungulates, including the porcine trypanosomes, a recent study based on next generation sequencing of ITS1 rDNA amplicons did not detect *Pycnomonas* in tsetse flies from Zambia and Zimbabwe (Gaithuma et al., 2019). However, the absence of TSU should be confirmed by demonstrating that the employed PCR primers are able to amplify DNA from *Pycnomonas* spp. with high sensitivity. Due to its great discriminative power, FFLB clearly distinguishes between TSU and all other porcine trypanosomes and might reveal novel genotypes within these species. The fact that TSU has been detected in Tanzania and MZ in all surveys carried out using FFLB (Adams et al., 2010a, 2010b; Hamilton et al., 2008; Garcia et al., 2018) suggests that current molecular diagnostic methods have failed to detect *Pycnomonas*.

TSU is a known pathogen of domestic suids (Peel and Chardome, 1954; Hoare, 1972), and the potential pathogenicity of TSU-L to livestock remains to be investigated. In addition to TSU, domestic and wild pigs are susceptible to other trypanosomes: *T. brucei* ssp. is common, whereas TCO and TVI are rarer and not associated with acute diseases in domestic pigs (Jamonneau et al., 2004; Simo et al., 2006, 2012; Biryomumaisho et al., 2013; Hamill et al., 2013; Karshima et al., 2016; N'Djetchi et al., 2017). Overall, trypanosome-infected wild pigs lack clinical symptoms, however, domestic pigs infected with TSU, TSM and TST develop acute diseases, and TSM causes fatal disease in domestic pigs, and mild infections in ruminants (Peel and Chardome, 1954; Isoun, 1968; Joshua and Kayit, 1984; McNamara et al., 1994; Garside and Gibson, 1995; Gibson et al., 2001; Nimpaye et al., 2011; Ebhodaghe et al., 2018). Only warthogs have been identified as wild reservoirs of TSM, TST, and TGO (Hoare, 1972; Claxton et al., 1992; Gibson et al., 2001; Auty et al., 2012). The whole set of porcine trypanosomes have not been investigated using molecular diagnostic methods. In MZ, pigs are generally kept in a free-ranging husbandry system and have constant contact with warthogs (common in small villages) and tsetse flies, mainly in areas bordering wildlife reserves. In this country, infectious diseases such as African swine fever represent serious constraints to the very challenging pig production (Penrith et al., 2007; Quembo et al., 2016). The participation of warthogs, which is widespread through MZ, in the maintenance of natural transmission cycles of TSU, besides TSM, TST and TGO, point to a high infection risk to domestic pigs, and the need for surveillance of porcine trypanosomes in general aiming to improve pig production.

Information on feeding patterns is crucial to the understanding of the tripartite tsetse flies-trypanosomes-vertebrate hosts associations. To our knowledge, this is the first time that tsetse flies have been examined regarding their blood meal sources in MZ: wild suids were identified as their preferential blood meal sources. In addition, relevant rates of concomitant infection with TSU/TSU-L and porcine trypanosomes in tsetse flies that preferentially feed on suids from both wildlife reserves, pointed toward significant associations between *Pycnomonas* and suids. However, comprehensive surveys are still needed to support the association of TSU-L with suids.

Our comprehensive analyses of trypanosome diversity in tsetse flies corroborated previous reports of high prevalence rates of porcine trypanosomes concomitantly infecting tsetse flies from Tanzania, where flies were found harbouring just one out of three sets of trypanosomes: porcine trypanosomes (TSM/TST/TGO), and ruminant-associated TCO, and TVI (Lehane et al., 2000). Our analysis revealed a significant association of *Pycnomonas* and porcine trypanosomes, although association with TCO was also common, consistent with preferential blood meal sources (suids and antelopes) of the tsetse flies from the GNP and NNR, where all known trypanosomes pathogenic to ruminants and suids were previously identified (Garcia et al., 2018). Nevertheless, our findings corroborate the suggestion that tsetse flies favour concomitant infections by porcine trypanosomes, and that particular species of trypanosomes may influence the infection/maturation of other species (Lehane et al., 2000; Gibson and Peacock, 2019).

Studies addressing porcine trypanosomiasis are generally focused on trypanosomes pathogenic to livestock and humans and carried out in West African countries, where trypanosomes of *Pycnomonas* were never reported (Jamonneau et al., 2004; Simo et al., 2006, 2012; Karshima et al., 2016; Ebhodaghe et al., 2018). Thought to be the rarest African trypanosome transmitted by tsetse flies, TSU was found to be quite common and comparable to TGO in MZ, whereas the rarest trypanosome was TCF, which is common in West and Central Africa, but sporadic through East Africa (Garcia et al., 2018). Therefore, due to shortcomings of available molecular diagnostic methods, and their inability to uncover both the whole repertoire and previously unknown trypanosomes, a relevant cohort of trypanosomes transmitted by tsetse flies to ungulates, especially suids, has been neglected or most likely misclassified. Hopefully, remarkable trypanosome genetic diversity recently uncovered by comprehensive molecular surveys (Adams et al., 2008; Hamilton et al., 2008; Auty et al., 2012; Votýpka et al., 2015; Garcia et al., 2018; Rodrigues et al., 2019; Gaithuma et al., 2019) will trigger the development of more accurate diagnostic assays for trypanosomes in general both in ungulate blood samples and tsetse flies.

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Author contributions

MMGT, EPC, CMFR and HAG conceived and coordinated the study. DLP, CLP, LBV and LV were the main responsible and contribute with expertise for the fieldwork in Africa. CMFR was responsible for the phylogenetic analyses. HAG and ACC contributed with molecular characterization and data analyses. MMGT, CMFR, HAG and WG wrote the manuscript. All authors read, contributed to improve, and revised the final manuscript.

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Declaration of Competing Interest

None.

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References

Adams, E.R., Malele, I., Msangi, A.R., Gibson, W.C., 2006. Trypanosome identification in wild tsetse populations in Tanzania using generic primers to amplify the ribosomal RNA ITS-1 region. Acta Trop. 100, 103–109.

Adams, E.R., Hamilton, P.B., Malele, I.I., Gibson, W.C., 2008. The identification, diversity and prevalence of trypanosomes in field caught tsetse in Tanzania using ITS-1 primers and fluorescent fragment length barcoding. Infect. Genet. Evol. 8, 439–444.

Adams, E.R., Hamilton, P.B., Rodrigues, A.C., Malele, I.I., Delespaux, V., Teixeira, M.M.G., 2010a. New *Trypanosoma* (*Duttonella*) *vivax* genotypes from tsetse flies in

East Africa. Parasitology 137, 641–650.
Adams, E.R., Hamilton, P.B., Gibson, W.C., 2010b. African trypanosomes: celebrating diversity. Trends Parasitol. 26, 324–328.
Auty, H., Anderson, N.E., Picozzi, K., Lembo, T., Mubanga, J., Hoare, R., 2012. Trypanosome diversity in wildlife species from the serengeti and Luangwa Valley ecosystems. PLoS Negl. Trop. Dis. 6, e1828.
Biryomumaisho, S., Rwakishaya, E.K., Melville, S.E., Cailleau, A., Lubega, G.W., 2013. Livestock trypanosomosis in Uganda: parasite heterogeneity and anaemia status of naturally infected cattle, goats and pigs. Parasitol. Res. 112, 1443–1450.
Claxton, J.R., Faye, J.A., Rawlings, P., 1992. Trypanosome infections in warthogs (*Phacochoerus aethiopicus*) in the Gambia. Vet. Parasitol. 41, 179–187.
Desquesnes, M., McLaughlin, G., Zoungrana, A., Davila, A.M., 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int. J. Parasitol. 31, 610–614.
Ebhodaghe, F., Ohiolei, J.A., Isaac, C., 2018. A systematic review and meta-analysis of small ruminant and porcine trypanosomiasis prevalence in sub-Saharan Africa (1986 to 2018). Acta Trop. 188, 118–131.
Fermino, B.R., Paiva, F., Soares, P., Tavares, L.E., Viola, L.B., Ferreira, R.C., Botero-Arias, R., de-Paula, C.D., Campaner, M., Takata, C.S.A., Teixeira, M.M.G., Camargo, E.P., 2015. Field and experimental evidence of a new caiman trypanosome species closely phylogenetically related to fish trypanosomes and transmitted by leeches. Int. J. Parasitol. Parasites Wildl. 4, 368–378.
Fermino, B.R., Paiva, F., Viola, L.B., Rodrigues, C.M.F., Garcia, H.A., Campaner, M., Takata, C.S.A., Sheferaw, D., Kisakye, J.J., Kato, A., Jared, C.A.G.S., Teixeira, M.M.G., Camargo, E.P., 2019. Shared species of crocodilian trypanosomes carried by tabanid flies in Africa and South America, including the description of a new species from caimans, *Trypanosoma kaiowa* n. sp. Parasit. Vectors 12, 225–241.
Gaithuma, A.K., Yamagishi, J., Martinelli, A., Hayashida, K., Kawai, N., Marsela, M., Sugimoto, C., 2019. A single test approach for accurate and sensitive detection and taxonomic characterization of Trypanosomes by comprehensive analysis of internal transcribed spacer 1 amplicons. PLoS Negl. Trop. Dis. 13, e0006842.
Garcia, H.A., Rodrigues, A.C., Martinkovic, F., Minervino, A.H., Campaner, M., Nunes, V.L., Paiva, F., Hamilton, P.B., Teixeira, M.M.G., 2011. Multilocus phylogeographical analysis of *Trypanosoma* (*Megatrypanum*) genotypes from sympatric cattle and water buffalo populations supports evolutionary host constraint and close phylogenetic relationships with genotypes found in other ruminants. Int. J. Parasitol. 41, 1385–1396.
Garcia, H.A., Rodrigues, C.M.F., Rodrigues, A.C., Pereira, D.L., Pereira, C.L., Camargo, E.P., Hamilton, P.B., Teixeira, M.M.G., 2018. Remarkable richness of trypanosomes in tsetse flies (*Glossina morsitans morsitans* and *Glossina pallidipes*) from the Gorongosa National Park and Niassa National Reserve of Mozambique revealed by fluorescent fragment length barcoding (FFLB). Infect. Genet. Evol. 63, 370–379.
Garside, L.H., Gibson, W.C., 1995. Molecular characterization of trypanosome species and subgroups within subgenus *Nannomonas*. Parasitology 111, 301–312.
Gibson, W., 2007. Resolution of the species problem in African trypanosomes. Int. J. Parasitol. 37, 829–838.
Gibson, W., Peacock, L., 2019. Fluorescent proteins reveal what trypanosomes get up to inside the tsetse fly. Parasit. Vectors 12, 6–13.
Gibson, W.C., Stevens, J.R., Mwendia, C.M., Ngotho, J.N., Ndung'u, J.M., 2001. Unravelling the phylogenetic relationships of African trypanosomes of suids. Parasitololgy 122, 625–631.
Hamill, L.C., Kaare, M.T., Welburn, S.C., Picozzi, K., 2013. Domestic pigs as potential reservoirs of human and animal trypanosomiasis in Northern Tanzania. Parasit. Vectors 6, 322–328.
Hamilton, P.B., Adams, E.R., Malele, I.I., Gibson, W.C., 2008. A novel, high-throughput technique for species identification reveals a new species of tsetse-transmitted trypanosome related to the *Trypanosoma brucei* subgenus *Trypanozoon*. Infect. Genet. Evol. 8, 26–33.
Hoare, C.A., 1964. Morphological and taxonomic studies on mammalian trypanosomes. X. Revision of the systematics. J. Protozool. 11, 200–207.
Hoare, C., 1972. The Trypanosomes of Mammals: A Zoological Monograph. Blackwell Scientific Publications, Oxford.
Hutchinson, R., Gibson, W., 2015. Rediscovery of *Trypanosoma* (*Pycnomonas*) *suis*, a tsetse-transmitted trypanosome closely related to *T. brucei*. Infect. Genet. Evol. 36, 381–388.
Isoun, T.T., 1968. The pathology of *Trypanosoma simiae* infection in pigs. Ann.Trop. Med. Parasitol. 62, 188–192.
Jamonneau, V., Ravel, S., Koffi, M., Kaba, D., Zeze, D.G., Ndri, L., Sane, B., Coulibaly, B., Cuny, G., Solano, P., 2004. Mixed infections of trypanosomes in tsetse and pigs and their epidemiological significance in a sleeping sickness focus of Côte d'Ivoire. Parasitology 129, 693–702.
Joshua, R.A., Kayit, Y.S., 1984. The course of fatal *Trypanosoma simiae* infection in domestic sheep. Acta Trop. 41, 91–92.
Karshima, S.N., Ajogi, I., Mohammed, G., 2016. Eco-epidemiology of porcine trypanosomosis in Karim Lamido, Nigeria: prevalence, seasonal distribution, tsetse density and infection rates. Parasit Vectors 12 9 (1), 448.
Lehane, M., Msangi, A., Whitaker, C., Lehane, S., 2000. Grouping of trypanosome species in mixed infections in *Glossina pallidipes*. Parasitology 120, 583–592.
Martin, D.P., Murrell, B., Golden, M., Khoosal, A., Muhire, B., 2015. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol. 1 vev003.
McNamara, J.J., Mohammed, G., Gibson, W.C., 1994. Trypanosoma (Nannomonas) godfreyi sp. nov. from tsetse flies in the Gambia: biological and biochemical characterization. Parasitology 109, 497–509.
Muturi, C.N., Ouma, J.O., Malele, I.I., Ngure, R.M., Rutto, J.J., Mithöfer, K.M., Enyaru, J., Masiga, D.K., 2011. Tracking the feeding patterns of tsetse flies (*Glossina* genus) by analysis of bloodmeals using mitochondrial cytochromes genes. PLoS One 6, e17284.
N'Djetchi, M.K., Ilboudo, H., Koffi, M., Kaboré, J., Kaboré, J.W., Kaba, D., Courtin, F., Coulibaly, B., Fauret, P., Kouakou, L., Ravel, S., Deborggraeve, S., Solano, P., De Meeûs, T., Bucheton, B., Jamonneau, V., 2017. The study of trypanosome species circulating in domestic animals in two human African trypanosomiasis foci of Côte d'Ivoire identifies pigs and cattle as potential reservoirs of *Trypanosoma brucei gambiense*. PLoS Negl. Trop. Dis. 18 11 (10), e0005993.
Nimpaye, H., Njiokou, F., Njine, T., Njitchouang, G.R., Cuny, G., Herder, S., Asonganyi, T., Simo, G., 2011. *Trypanosoma vivax*, *T. congolense* "forest type" and *T. simiae*: prevalence in domestic animals of sleeping sickness foci of Cameroon. Parasite. 18, 171–179.
Noyes, H.A., Stevens, J.R., Teixeira, M.M.G., Phelan, J., Holz, P., 1999. A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. Int. J. Parasitol. 29, 331–339.
Peel, E., Chardome, M., 1954. Experimental study of strains of *Trypanosoma simiae*, Bruce 1912, transmitted by *Glossina brevipalpis* of Mosso, Urundi. Ann. Soc. Belg. Med. Trop. 34, 345–359.
Penrith, M.L., Lopes Pereira, C., Lopes da Silva, M.M., Quembo, C., Nhamusso, A., Banze, J., 2007. African swine fever in Mozambique: review, risk factors and considerations for control. Onderstepoort J. Vet. Res. 74, 149–160.
Quembo, C.J., Jori, F., Heath, L., Pérez-Sánchez, R., Vosloo, W., 2016. Investigation into the epidemiology of African swine fever virus at the wildlife - domestic Interface of the Gorongosa National Park, Central Mozambique. Transbound. Emerg. Dis. 63, 443–451.
Rodrigues, A.C., Neves, L., Garcia, H.A., Viola, L.B., Marcili, A., Da Silva, F.M., Sigauque, I., Batista, J.S., Paiva, F., Teixeira, M.M.G., 2008. Phylogenetic analysis of *Trypanosoma vivax* supports the separation of South American/West African from east African isolates and a new *T. vivax*-like genotype infecting a Nyala antelope from Mozambique. Parasitology 135, 1317–1328.
Rodrigues, C.M.F., Garcia, H.A., Rodrigues, A.C., Costa-Martins, A.G., Pereira, C.L., Pereira, D.L., Bengaly, Z., Neves, L., Camargo, E.P., Hamilton, P.B., Teixeira, M.M.G., 2017. New insights from Gorongosa National Park and Niassa National Reserve of Mozambique increasing the genetic diversity of *Trypanosoma vivax* and *Trypanosoma vivax*-like in tsetse flies, wild ungulates and livestock from East Africa. Parasit. Vectors 10, 337–352.
Rodrigues, C.M.F., Garcia, H.A., Sheferaw, D., Rodrigues, A.C., Pereira, C.L., Camargo, E.P., Teixeira, M.M.G., 2019. Genetic diversity of trypanosomes pathogenic to livestock in tsetse flies from the Nech Sar National Park in Ethiopia: a concern for tsetse suppressed area in southern Rift Valley? Infect. Genet. Evol. 69, 38–47.
Simo, G., Asonganyi, T., Nkinin, S.W., Njiokou, F., Herder, S., 2006. High prevalence of *Trypanosoma brucei gambiense* group 1 in pigs from the Fontem sleeping sickness focus in Cameroon. Vet.Parasitol. 139, 57–66.
Simo, G., Njitchouang, G.R., Njiokou, F., Cuny, G., Asonganyi, T., 2012. Genetic characterization of *Trypanosoma brucei* circulating in domestic animals of the Fontem sleeping sickness of Cameroon. Microbes Infect. 14, 651–658.
Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 22, 2688–2690.
Swofford, D.L., 2002. PAUP*. In: 4 V (Ed.), Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland, Massachusetts.
Van Den Berghe, L., Zaghi, A.J., 1963. Wild pigs as hosts of *Glossina vanhoofi* Henrard and *Trypanosoma suis* Ochmann in the Central African Forest. Nature. 197, 1126–1127.
Votýpka, J., Radrova, J., Skalicky, T., Jirku, M., Jirsova, D., Mihalca, A.D., 2015. A tsetse and tabanid fly survey of African great apes habitats reveals the presence of a novel trypanosome lineage but the absence of *Trypanosoma brucei*. Int. J. Parasitol. 45, 741–748.