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Research paper

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?

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ABSTRACT

In Ethiopia, home to the largest African herd of cattle, animal trypanosomiasis is a major constraint to the efforts made for food self-sufficiency. We searched for trypanosomes in tsetse flies caught in the Nech Sar National Park (NSNP), Southern Rifty Valley, Ethiopia, at the district of Arba Minch where intensive tsetse control is successfully improving cattle productivity. Despite narrow geographical and temporal scales of our survey, we found a remarkable diversity of trypanosomes using the sensitive and discriminative method of fluorescent fragment length barcoding. We also found a high density of *Glossina pallidipes* (47.8 flies/trap/day) showing relevant cytochrome oxidase I gene variability. The identification of blood meal sources through cytochrome *b* gene sequences revealed cattle and warthog as preferential ungulate hosts of tsetse flies in the study area. Our survey identified trypanosomes in 38% of the 287 flies examined (42% of proboscises and 32% of guts), and the following infection rates for each species: *Trypanosoma vivax* 23%, *T. simiae* 23%, *T. congolense* 22%, *T. theileri* 19.9%, *T.* (*Trypanozoon)* spp. 10.5%, *T. godfreyi* 9.4%, *T. simiae* Tsavo 6.3%, and mixed infections in proboscises (30%) and guts (61%). Phylogenetic analysis revealed *T. vivax* of the "West African-South American" genotype, *T. congolense* of Savannah (16.7%), Kilifi (3.5%) and Forest (2.1%) lineages, and new genotypes of *T. simiae*. To our knowledge, this is the first survey of trypanosomes in the NSNP, and the most comprehensive molecular characterisation of trypanosomes in tsetse flies of Ethiopia, including the comparison with samples from West and other East African countries. Our results support the diversification of *T. vivax* in East Africa, and the dispersion of the genotype herein identified in Ethiopia across West Africa and then in South America. Altogether, tsetse density and infection rate, repertoire of trypanosomes and feeding behavior indicate a high risk of transmission of trypanosomes pathogenic to ungulates by tsetse flies from the NSNP, a hotspot of tsetse infestation and trypanosome diversity. Our findings reinforce the need for constant surveillance, and the reliance on community efforts to prevent reinvasion of tsetse and animal trypanosomiasis in suppressed areas of Southern Rift Valley.

1. Introduction

African Animal Trypanosomiasis (AAT) is a major impediment to livestock production in Ethiopia, where the economy is largely based on integrated crop-livestock systems. The prevalence of AAT negatively impacts the overall development of the country as well as efforts to achieve food self-sufficiency (Alemu et al., 2007; Duguma et al., 2015; Shaw et al., 2017; Tesfaye et al., 2012). Ethiopia is home to the largest African herd of cattle as well as millions of goats, sheep, camels and equines (mainly donkeys). The estimated population is 435 million cattle, 24 million sheep, 23 million goats, 2.3 million camels and 6.5 million equines. In some areas, up to 25% of animals are affected by

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AAT; and more than 14 million cattle, an equivalent number of small ruminants and around 19 million camels are at risk. In addition to the 10.6 million work oxen, Ethiopia depends on its 6.5 million donkeys, mules and horses for transport and draught work (Shaw et al., 2017; Tesfaye et al., 2012; Zecharias and Zeryehun, 2012).

Except for the Ethiopian highlands where trypanosomes are rare or absent, *T. vivax*, *T. congolense*, *T. brucei brucei*, *T. evansi* and *T. equiperdum* have been reported throughout Ethiopia. The most important agents of AAT in cattle are *T. congolense* and *T. vivax* (Abebe et al., 2017; Birhanu et al., 2015; Cherenet et al., 2006; Degneh et al., 2017; Duguma et al., 2015; Fikru et al., 2012, 2016; Sheferaw et al., 2016; Terefe et al., 2015), and *T. evansi* in camels and equines (Birhanu et al., 2016; Jilo et al., 2016). In Ethiopia, *T. vivax* and *T. evansi* are prevalent both in tsetse-infested and tsetse-free regions (Birhanu et al., 2016; Dagnachew et al., 2017; Fikru et al., 2012, 2016). An important obstacle for AAT control that should be addressed in Ethiopia is the abundance of donkeys, which are known reservoirs of *T. evansi, T. vivax* and *T. congolense* and may play an important role in the dispersal of AAT throughout Ethiopia (Assefa and Abebe, 2001; Birhanu et al., 2015; Eyob et al., 2011), as demonstrated in The Gambia (Faye et al., 2001; Pinchbeck et al., 2008), Uganda (Muhanguzi et al., 2017) and the Brazilian semiarid region (Rodrigues et al., 2015).

Animals with AAT exhibit variable degrees of morbidity and mortality depending on the species and genotype of trypanosomes, and both the species/breed and management of the herds. Overall, infected cattle exhibit chronic infection which results in anaemia, lethargy, progressive loss of body condition, depressed productivity due to delayed onset of puberty, increased calving intervals, infertility, spontaneous abortion and reduced milk yield (Abebe et al., 2017; Biyazen et al., 2014; Getachew et al., 2014; Sheferaw et al., 2016; Taye et al., 2012; Terefe et al., 2015). Extensive surveys of AAT in cattle using the microhematocrit method (concentration of trypanosomes in the buffy coat) followed by morphological identification of trypanosomes have revealed high prevalence rates of AAT in tsetse-infested areas in Ethiopia: ~12–25% in north-western (Dagnachew et al., 2005, 2017; Girmay et al., 2016; Mekuria and Gadissa, 2011), ~3.0–28% in western (Biyazen et al., 2014; Degneh et al., 2017; Getachew et al., 2014), ~7.0–27% in southern (Abebe et al., 2017; Sheferaw et al., 2016; Zecharias and Zeryehun, 2012) and ~ 4.4–27% in south-western regions of Ethiopia (Lelisa et al., 2014; Teka et al., 2012; Terefe et al., 2015; Zecharias and Zeryehun, 2012). The prevalence of AAT is significantly lower (~2.0–9.0%) in areas that are not infested by tsetse flies. In these areas, *T. vivax* is prevalent, probably due to its efficient mechanical transmission by tabanids and other biting flies (Cherenet et al., 2006; Dagnachew et al., 2017; Sinshaw et al., 2006). Molecular surveys of trypanosomes in Ethiopian cattle carried out by analyses of polymorphisms on PCR-amplified 18S rRNA (PCR-RFLP) and ITS rDNA revealed *T. congolense* and *T. vivax* (Moti et al., 2015; Birhanu et al., 2016; Dagnachew et al., 2017; Fikru et al., 2012, 2016).

Five tsetse species are known to occur in Ethiopia, all of which are involved in the transmission of trypanosomes pathogenic to livestock. These species are *G. morsitans submorsitans* and *G. pallidipes* from the *morsitans* group, *G. fuscipes fuscipes* and *G. tachinoides* from the *palpalis* group and *G. longipennis* from the *fusca* group (Abebe et al., 2017; Cecchi et al., 2015; Sheferaw et al., 2016; Zecharias and Zeryehun, 2012). Despite decades of implemented control attempts in Ghibe, Didessa and the Southern Rift Valley, tsetse flies have often reinvaded cleared zones lacking effective natural barriers and non-sustained control efforts. Consequently, the distribution of AAT has been expanded in Ethiopia, with drastic effects on cattle herds (Alemu et al., 2007; Duguma et al., 2015; Shaw et al., 2017; Tesfaye et al., 2012; Vreysen et al., 2013).

The Government of Ethiopia and the Southern Nations, Nationalities and Peoples Regional State (SNNPRS), assisted by the International Atomic Energy Agency, have established the Southern Tsetse Eradication Project (STEP), which began in 1998 with entomological, veterinary, environmental and socio-economic evaluations of the project area. Field activities of the STEP aiming at drastic reductions of tsetse densities were initiated in 2002, with simultaneous spraying of insecticides, attraction of tsetse flies to insecticide-impregnated targets and insecticide spraying of cattle (Alemu et al., 2007; Shaw et al., 2017; Taye et al., 2012). The combination of STEP and community-based measures has significantly reduced both the tsetse population and the prevalence of AAT. Nevertheless, the NSNP represent a permanent concern regarding re-infestation of suppression areas by tsetse flies. Comprehensive molecular surveys aimed at analysing the genetic diversity of trypanosomes in tsetse flies from the NSNP is lacking. Assessment of tsetse density and genetic diversity, and the prevalence and repertoire of trypanosomes in tsetse flies from the NSNP is key to estimate the risk and transmission dynamics of AAT at the wildlife and livestock interface for the implementation of appropriated control strategies.

In the present study, we assessed the genetic diversity of tsetse flies caught in the NSNP and their trypanosomes using fluorescent fragment length barcoding (FFLB) to achieve sensitive identification of the trypanosomes that are pathogenic to ungulates (Garcia et al., 2018; Hamilton et al., 2008; Rodrigues et al., 2017).

2. Material and methods

2.1. Nech Sar National Park and the STEP project

The NSNP is located between 50 51′-60 05′N and 370 32′-370 48′E in the SNNPRS and includes the Gamo Gofa Zone where the district of Arba Minch is found in the Southern Rift Valley (Fig. 1). Covering an area of 514 km^2, with an altitudinal range of 1108–1650 m above sea level, mean annual temperature within the NSNP is ~21 °C, with the highest temperatures from January to March (~35 °C) and the lowest temperatures in November and December (~26 °C) (Fetene et al., 2011). With average annual rainfall of ~900 mm (the rainy seasons are from March to May and September to November), the NSNP is one of the most important biodiversity hotspots in Ethiopia, home to diverse large mammals including waterbuk, gazelle, kudu, zebra, hartebeest, bushbuck, hippopotamus, reedbuck, warthog and bush pigs. The area is characterised by a mosaic of grasslands, open and dense riverine forests and an abundance of freshwater habitats (Fetene et al., 2011). The NSNP includes the “Bridge of God”, which is an isthmus between Lake Abaya and Lake Chamo, and the Nechisar plains east of the lakes. This park is bordered to the west by the town of Arba Minch to the south and north by lakes Chamos and Abaya, respectively; and to the east by the Amaro Mountains (Fig. 1).

Tsetse flies to be examined in the present study were caught in the NSNP (Fig. 1) near to the town of Arba Minch an intensive STEP suppression area. The STEP covers an area of 25,000 km^2 of the Arba Minch district, and comprises tsetse-controlled and uncontrolled blocks separated by the town of Arba Minch. The STEP comprises fertile lowland areas, which are bordered by lakes, high escarpments in the east, north and west, and vast arid lands in the south (Fig.1). The STEP is managed by the Ministry of Science and Technology with a branch office in the SNNPR responsible for the coordination of field activities in Arba Minch (Alemu et al., 2007; Shaw et al., 2017).

2.2. Collection and identification of tsetse flies, and DNA preparation

Tsetse flies were captured over three days (in April 2016) using two Ngu traps (using old cow urine as odour bait in traps separated by ~200 m, and deployed near bush vegetation in a woodland area bordering the lake Chamo. Traps were emptied twice a day at ~ 9 AM and 5 PM, and flies preserved in ethanol (99.5%). Tsetse flies were identified by the local team based on morphological features (FAO, 1992). The average apparent density was defined as the mean number of tsetse flies caught per trap per day. Each tsetse fly was washed twice by

Fig. 1. Map of Ethiopia indicating the place in the Nech Sar National Park (NSNP) where tsetse flies were collected. Tsetse flies were collected in the NSNP (red spot), at the "Bridge of God", an isthmus between the Lakes Abaya and Chamo, close to the town of Arba Minch (arrow) and the intensively suppressed Block 1 (orange rectangle) of the Southern Tsetse Eradication Project, STEP (Alemu et al., 2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immersion in sterilised water and dried on filter paper. To prevent any contamination, dissections were carried out in the laboratory using disposable glass slides, blades and micropipette tips, also avoiding human contamination. The heads of flies were separated from the guts, and both the guts and the mouth-parts were crushed and processed for DNA extraction. Briefly, samples were digested in Digsol buffer containing proteinase K at 55 °C for 3 h, and the DNA precipitated with 4 M ammonium acetate and ethanol. Samples were then washed in 70% ethanol, dried at room temperature, re-suspended in 25 µl of TE buffer and stored at −20 °C (Garcia et al., 2018). Guts and proboscises were analysed individually for trypanosomes using FFLB (Garcia et al., 2018; Hamilton et al., 2008).

2.3. Analysis of cytochrome oxidase I sequences from tsetse flies

We ramdomly selected 125 flies caught in both traps and all collection days for barcoding using two fragments of cytochrome oxidase I (COI) sequences obtained by PCR amplification of DNA fragments (614 and 618 bp) from tsetse proboscises using primers and PCR conditions described previously (Dyer et al., 2008; Ouma et al., 2011). Amplified sequences were aligned with homologous sequences from different species of tsetse flies retrieved from GenBank. Multidimensional scaling (MDS) analyses including COI sequences of different species of tsetse flies from a range of countries or restricted to sequences of *G. pallidipes* from East African countries, were carried out as previously described

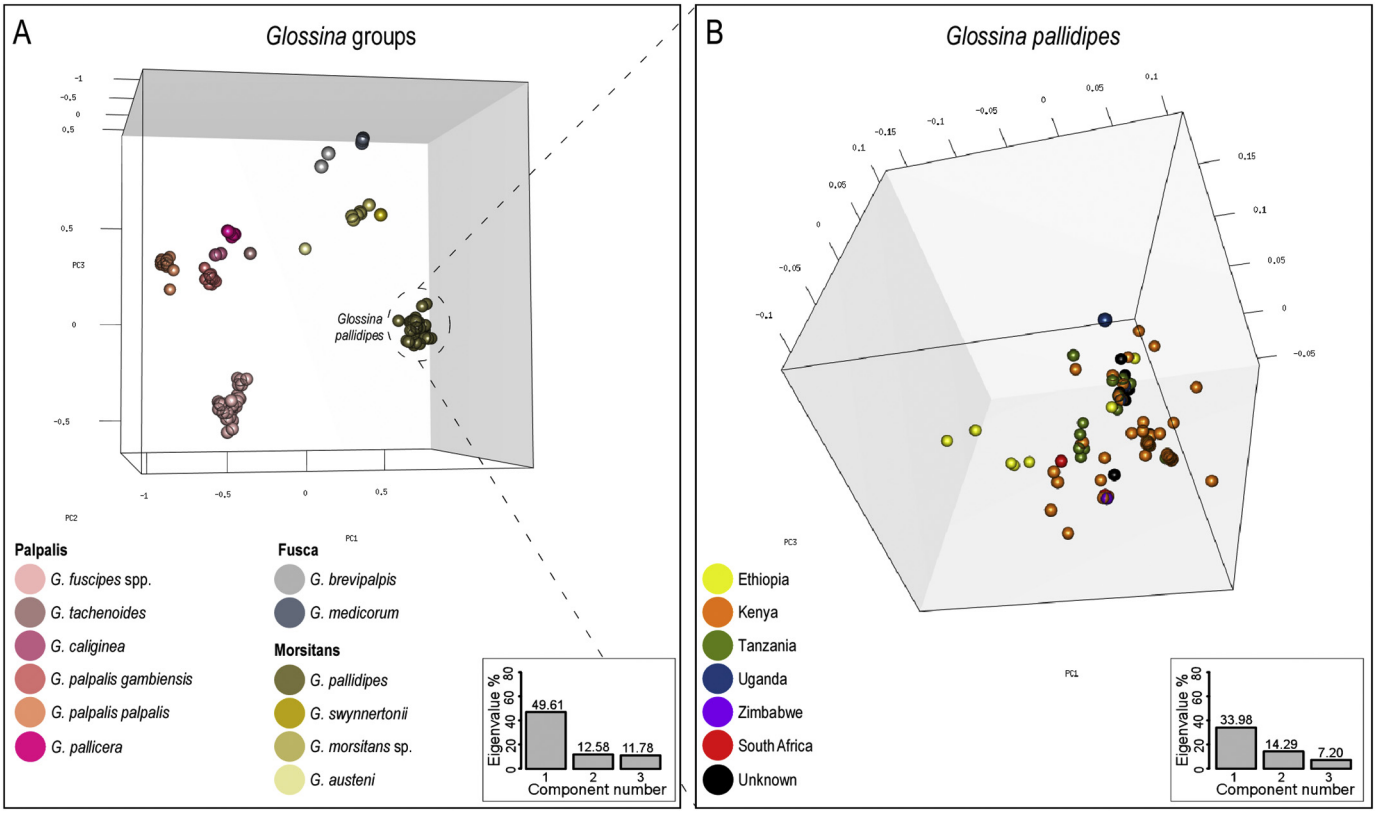

Fig. 2. Multidimensional scaling analysis of cytochrome oxidase I (COI) sequences of tsetse flies from the NSNP. (A) MDS clustering patterns of COI sequences from *Glossina* spp. of the groups *palpalis, fusca* and *morsitans.* B) MDS restricted to COI sequences of *G. pallidipes* from Ethiopia, Kenya, Tanzania, Uganda, Zimbabwe and South Africa.

(Garcia et al., 2018; Rodrigues et al., 2017).

2.4. Identification of trypanosomes using fluorescent fragment length barcoding (FFLB)

The survey of African trypanosomes in proboscises and gut contents of tsetse flies was carried out using FFLB (Garcia et al., 2018; Hamilton et al., 2008). This PCR-based method amplifies small sequences using four sets of fluorescent primers; two targeting the 18S rRNA and specific to trypanosomes, and two targeting the 28S rRNA of all trypanosomatids. The lengths of amplified fragments are used to generate profiles unique to each species, which can then be matched to profiles of reference trypanosomatid species, or to produce unknown profiles in the case of species not previously analysed by FFLB, and whose identification requires further sequencing (Adams et al., 2010; Garcia et al., 2018; Hamilton et al., 2008; Rodrigues et al., 2017).

2.5. Molecular characterisation of trypanosomes based on gGAPDH sequences

The whole data set of gGAPDH (glycosomal glyceraldehyde-3-phosphate dehydrogenase) sequences (609 bp) determined in the current study as described previously (Fermino et al., 2013; Rodrigues et al., 2017) was screened for chimera using the RDP4 package (Martin et al., 2015). For phylogenetic inferences, sequences were included in an alignment of African trypanosomes (Rodrigues et al., 2017), which included gGAPDH sequences from trypanosomes of the subgenera *Duttonella* (*T. vivax* and *T. vivax*-like), *Trypanozoon* (*T. b. brucei, T. b. rhodesiense, T. b. gambiense* and *T. evansi*), *Pycnomonas* (*T. suis*) and *Nannomonas* (*T. congolense* of the Savannah, Forest and Kilifi genotypes, *T. simiae, T. simiae* Tsavo and *T. godfreyi*). The final alignment was employed for phylogenetic analysis by parsimony (P) using the PAUP*4.0b10 package, and maximum likelihood (ML) using RAxML with GTRGAMMA (500 maximum parsimony starting trees) as described previously (Fermino et al., 2013; Rodrigues et al., 2017). The model parameter was estimated in RAxML over the duration of the tree search, and nodal support was estimated with 500 bootstrap replicates. Visual representation of the level of similarity among gGAPDH sequences was carried out by multidimensional scaling (MDS) analysis using the Bios2mds package (Pelé et al., 2012).

2.6. Identification of vertebrate source of blood in guts of tsetse flies

Because the number of recently fed tsetse flies was very low, we additionally selected flies lacking evidence of recent blood meals for blood source identification; according to their thinness they were starving flies. DNA samples obtained from the gut contents of tsetse flies (previously examined for trypanosome surveys) served as templates in PCR reactions carried out for the amplification of vertebrate mitochondrial cytochrome *b* (Cytb) sequences using previously standardized protocol (Muturi et al., 2011). Cytb sequences obtained from tsetse guts were compared with those available in GenBank database using BLASTN.

3. Results and discussion

3.1. Glossina pallidipes comprising a set of haplotypes was the only tsetse species detected in the NSNP

Tsetse flies caught in the NSNP during three days, using two traps mounted ~200 m apart and emptied twice a day, were identified by morphological parameters as *G. pallidipes*. We selected flies from both traps and the three collecting days for identification by COI barcoding (Dyer et al., 2008; Ouma et al., 2011). Comparative analysis of the COI

sequences (Dyer et al., 2008) of 125 selected tsetse flies (GenBank accession numbers MK120125-38) with those from a range of *Glossina* spp. (GenBank) corroborated previous identification of tsetse flies caught in the NSNP as *G. pallidipes* (Fig. 2A). Additionally, COI sequences (Ouma et al., 2011) determined in the present study (GenBank: MK120139-45) were compared with those obtained from analysis of *G. pallidipes* throughout East African countries (Fig. 2B). COI sequences of *G. pallidipes* from Ethiopian tsetse flies, including those we obtained from the NSNP plus sequences from tsetse that were caught in Arba Minch (GenBank) and sequences from Kenya, Uganda, Tanzania, Zimbabwe and South Africa that are available in GenBank (Ouma et al., 2011) were clustered in a single clade (Fig. 2B). This comparison confirmed important genetic diversity across East African *G. pallidipes* populations and uncovered a set of haplotypes of *G. pallidipes* in the NSNP regardless small temporal and spatial scales of our sampling. Noteworthy, the haplotypes found within the NSNP were reported from other East African countries such as Kenya and Tanzania (Ouma et al., 2011; Okeyo et al., 2017) (Fig. 2B).

Our findings, which are far from representative of the entire NSNP, suggest that a non-isolated *G. pallidipes* population exists in the NSNP despite extensive control activities in nearby areas. The present study was not designed to investigate tsetse population dynamics and structure, nor the relationships of tsetse flies from the NSNP with populations outside of the park. This has been carried out for Kenyan population through microsatellite analyses, which despite evidencing genetic bottlenecks in some places, did not reveal a significant drop in the genetic diversity levels of *G. pallidipes* following extensive and long-lasting tsetse control measures (Okeyo et al., 2017). Recently, microsatellite analysis of *G. pallidipes* population dynamics that underline limited control success revealed two clusters separated by the Great Rift Valley, with evidence of admixture and migration between the two populations (Okeyo et al., 2018).

The choice of Arba Minch by STEP was based on the potential social-impacts for the agro-pastoral district, its geographical location (flanked by tsetse-free mountains and arid zones) and the occurrence of only one species of tsetse fly, *G. pallidipes*. Our barcoding demonstrated that *G. pallidipes* is the only (or the highly predominant) species of tsetse in the NSNP, in concordance with previous studies based on morphological identification of tsetse flies caught in either the NSNP or in cattle farms of Arba Minch (Alemu et al., 2007; Girma et al., 2014; Sheferaw et al., 2016; Teka et al., 2012; Zecharias and Zeryehun, 2012). It is notable that, even considering the uneven distribution of tsetse flies and our limited capture efforts, 287 flies were captured, with an average of 47.8 flies/trap/day. Our finding is in agreement with the high apparent density of *G. pallidipes* that was previously estimated by entomological surveys in the NSNP during the wet seasons (up to ~70 flies/trap/day); whereas in the Arba Minch district, tsetse density varied from 0.3 to 29 flies/trap/day depending on the magnitude of community control efforts (Gechere et al., 2012; Girma et al., 2014; Terefe et al., 2015). Large surveys of tsetse flies at different seasons, using a larger number of traps (different types) widely distributed in the NSNP, including ecologicaly different sites, are required to investigate tsetse density and genetic diversity across the NSNP.

In STEP areas of intensive suppression, low numbers or even the absence of tsetse flies have been reported (Alemu et al., 2007; Gechere et al., 2012; Girma et al., 2014; Taye et al., 2012; Zecharias and Zeryehun, 2012). However, tsetse flies are highly resilient, and their populations are quickly restored when control procedures are interrupted. Suppression activities require constant, expensive and controversial measures such as systematic insecticide spraying and **treatment of tsetse targets**, which may lead to tsetse resistance as well as environmental contamination. In addition, treatment of infected animals and the use of preventive drugs may induce trypanosome resistance (Dagnachew et al., 2017; Girmay et al., 2016; Moti et al., 2012, 2015; Vreysen et al., 2013; Zecharias and Zeryehun, 2012).

3.2. Blood meal profiling in tsetse flies from the NSNP reveals feeding in cattle, human and warthog

Sequences of Cytb gene obtained from DNA of tsetse guts were employed for blood meal profiling in 96 selected tsetse flies caught during three days in the NSNP. Most tsetse flies collected did not exhibit evidence of recent blood meals, and Cytb sequences obtained from some flies were not of enough quality maybe due to degradation of the ingested blood. Consequently, only 27.1% ($n = 26$ flies) of 96 flies examined turned out positive for PCR-amplification that allowed for sequencing of high-quality vertebrate Cytb sequences. BLAST analysis revealed that the tsetse flies examined fed in cattle (30.8%, 8 flies), human (23%, 6 flies), warthog (*Phacocerus africanus*) (19.3%, 5 flies) and birds (11.6%, 3 flies); 15.3% (4 flies) showed mixed blood meals. To our best knowledge, feeding patterns have not previously been reported for tsetse flies in any place in Ethiopia, and our preliminary findings agreed with host availability in the studied area. Host choice of tsetse flies depends on available animal fauna and tsetse fly preferences. Blood of both domestic and wild mammals have been identified in *G. pallidipes* from Uganda, Tanzania and Kenya. In *the* Serengeti National Park (Tanzania), buffalo, elephant and giraffe are the preferential blood sources of *G. pallidipes* (Auty et al., 2016).

Information on the source of blood meals of tsetse flies is crucial in understanding the relationship between flies and hosts. Our findings demonstrated that tsetse flies caught in the NSNP often feed on cattle. As most of the land is intensively cultivated in the district of Arba Minch, cattle are often moved to the NSNP where grassland is available through the year, thus facilitating both transmission and dispersion of AAT. In addition, the studied area in the NSNP has been invaded by human dewllings and domestic animals.

3.3. Prevalence and diversity of trypanosomes in tsetse flies from the NSNP assessed by FFLB

In contrast with many parasitological surveys reporting *T. vivax*, *T. congolense* and *T. brucei brucei* in cattle (Birhanu et al., 2016; Dagnachew et al., 2017; Duguma et al., 2015; Fikru et al., 2012, 2016; Moti et al., 2015), both prevalence and diversity of trypanosomes in tsetse flies remains to be widely investigated in Ethiopia. In the present study, the highly discriminative method of FFLB was applied to assess trypanosomes in DNA samples of *G. pallidipes* proboscises and guts. This method revealed ungulate trypanosomes in 38% (109 flies) of the 287 flies examined with 9 trypanosome species/genotypes identified in 120 (42%) proboscises and 93 (32%) guts; 53 flies showed both guts and proboscises positive for trypanosomes (Fig. 3). The trypanosomes identified in these flies were as follows: *T. vivax* (TVI), *T. congolense* (TCO) Savannah (TCS), *T. congolense* Kilifi (TCK), *T. congolense* Forest (TCF), *T. brucei* ssp. (TPZ), *T. simiae* (TSM), *T. simiae* Tsavo (TST), *T. godfreyi* (TGO) and *T. theileri* (Tth). Altogether, the analyses of guts and proboscises of *G. pallidipes* yielded the following prevalence rates (decreasing %): TSM, 23.3; TVI, 23; TCO, 22.3 (taking into account the genotypes TCS, TCK and TCF), TPZ, 10.5; TGO, 9.4 and TST, 6.3 (Fig. 3A). The estimated detection rate of each trypanosome species in proboscises by FFLB were (%): TVI, 22; TGO, 7.7; TPZ, 4.5; TCS, 4.5; TST, 4.5; TSM, 3.8; TCF, 1.4; TCK, 0.7. A similar overall species composition but showing different prevalence rates was detected for each trypanosome species in the tsetse guts (decreasing %): TSM, 20.2; TCS, 13.6; TPZ, 7.0; TCK, 3.8; TVI, 2.4; TGO, 2.1; TCF, 0.3. The non-pathogenic trypanosome Tth, which is common in domestic and wild ruminants throughout the world (Garcia et al., 2011), was detected in 19.9% of the tsetse flies: 10.1% of proboscises and 9.8% of guts (Fig. 3B).

The FFLB method also identified mixed trypanosome infections of two or three different trypanosomes (including the three TCO genotypes) infective to ungulates. Examination of the proboscises revealed 30% of mixed infections. Twenty-five flies were identified with eight

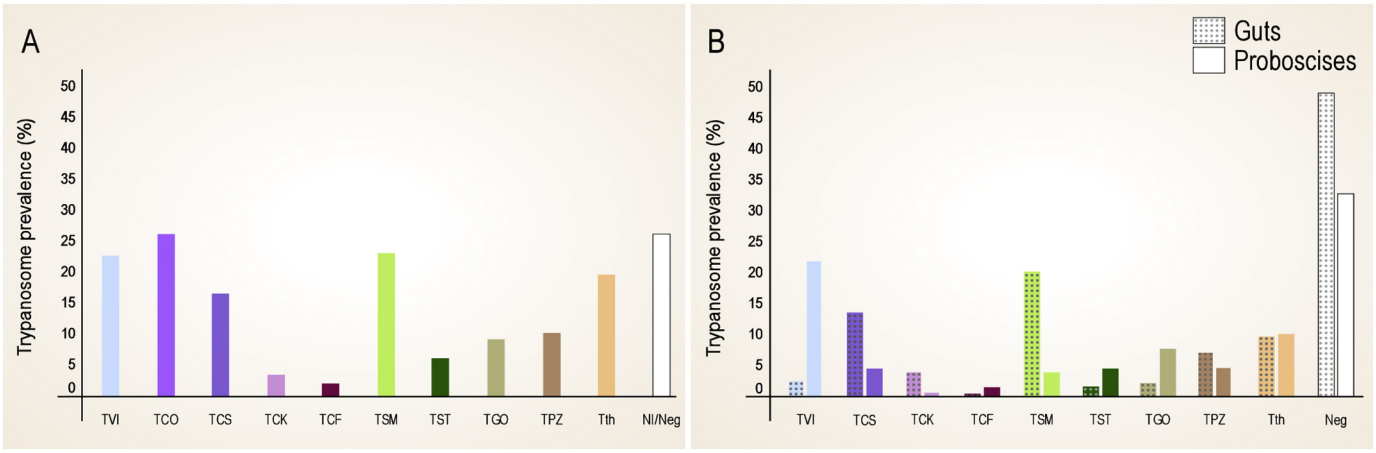

Fig. 3. Prevalence rates of trypanosomes in tsetse flies from the Nech Sar National Park. (A) Prevalence rates of trypanosome species in *Glossina pallidipes* (n = 287) from the Nech Sar National Park in Southern Ethiopia assessed by fluorescent fragment length barcoding. (B) Prevalence rates in individual guts and proboscises. TSM, *T. simiae*; TVI, *T. vivax*; TCO, *T. congolense*; TCS, *T. congolense* Savannah; TCK, *T. congolense* Kilifi; TCF, *T. congolense* Forest; TPZ, *Trypanozoon* spp.; TGO, *T. godfreyi*; TST, *T. simiae* Tsavo; Tth, *T. theileri*; NI/Neg, non-identified/negative.

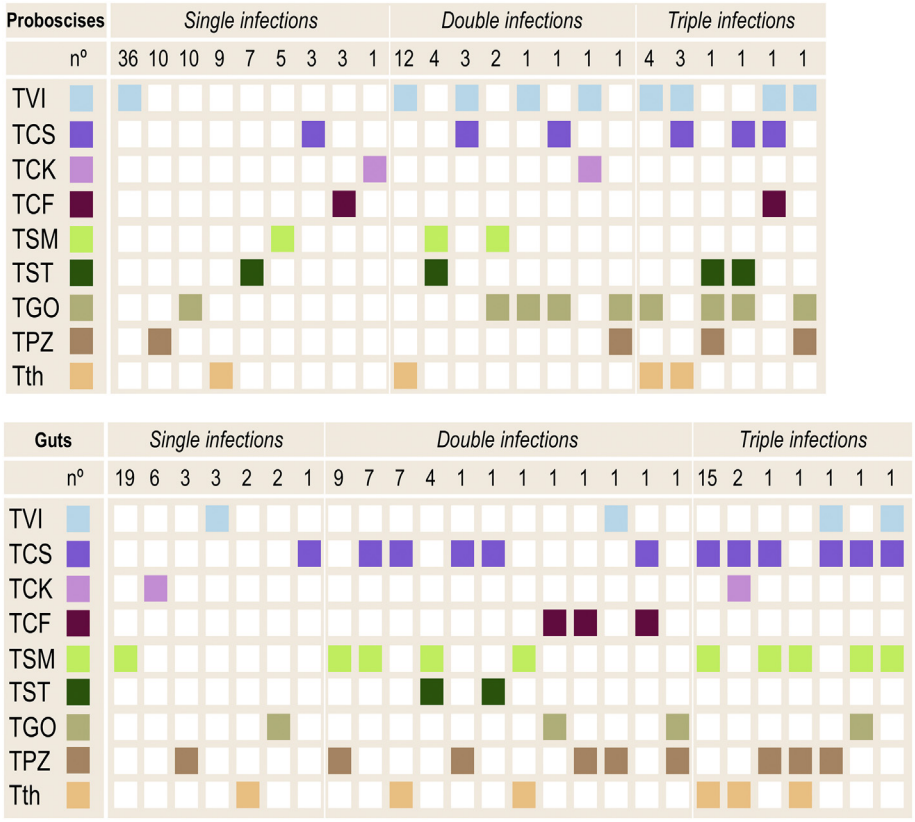

Fig. 4. Schematic illustration of mixed trypanosome infections in tsetse flies from the Nech Sar National Park. Diversity of trypanosomes in mixed infected tsetse guts (61%) and proboscises (30%) of *Glossina pallidipes* identified by FFLB (fluorescent fragment length barcoding). n, numbers of tsetse flies that exhibited each parasite combination. TSM, *T. simiae*; TVI, *T. vivax*; TCS, *T. congolense* Savannah; TPZ, *Trypanozoon* spp.; TGO, *T. godfreyi*; TST, *T. simiae* Tsavo; TCK, *T. congolense* Kilifi; TCF, *T. congolense* Forest; Tth, *T. theileri*.

different combinations of two trypanosomes, and 11 flies showed six combinations of three trypanosome species/genotypes (Fig. 4). The most common combinations of two trypanosomes in proboscises were TVI/Tth (48%), TSM/TST (16%) and TVI/TCS (12%); whereas the most common combinations of three species were TVI/TGO/Tth (36.3%) and TVI/TCS/Tth (27.2%) (Fig. 4). The majority (61.2%) of trypanosome-positive guts carried mixed infections: 35 flies (61.4%) were identified with 12 different combinations of two trypanosomes, and 22 flies (38.5%) were found to have 7 different combinations of three trypanosomes. The predominant combinations of double infections in the guts examined were TSM/TPZ (25.7%), TSM/TCS (20%) and TCS/Tth (20%); and the most common combination of three trypanosomes was TSM/TCS/Tth (68.1%) (Fig. 4).

Altogether, FFLB profiles (including partial profiles composed of 2–3 peaks) indicating the presence of trypanosome DNA were obtained from guts and proboscises of 211 flies. However, FFLB profiles from just 109 flies (51.6%) could be associated to known trypanosome species/genotypes. We searched for profiles of *T. grayi* of crocodilians, which is commonly found in its vector tsetse flies (Fermino et al., 2013; Ngomtcho et al., 2017), but did not identify this species among the FFLB profiles of the flies examined.

A possible link between the species of trypanosomes identified in *G. pallidipes* from the NSNP and those previously identified in livestock of bordering areas is supported by a cross-sectional study (using the MH technique) which was carried out in Arba Minch and eight nearby districts. This study revealed that 7.2% of cattle were AAT-positive

using the MH methods, with a higher prevalence observed in Arba Minch, where 66.9% and 33.1% of cattle were found to be infected with TCO and TVI, respectively (Sheferaw et al., 2016). Notably, Arba Minch is the closest district to the NSNP, where abundant tsetse flies may mediate the transfer of trypanosomes between wild and domestic animals that often use the park for foraging. Therefore, the NSNP continuously provides diverse trypanosomes to livestock. In Ethiopia, AAT are usually diagnosed in the fresh blood of livestock by the microhematocrit method, and species identification is based on morphology and movement of blood trypomastigotes, and/or by morphology in stained blood-smears (Abebe et al., 2017; Degneh et al., 2017; Duguma et al., 2015; Gechere et al., 2012; Teka et al., 2012). Accurate use of these parameters requires skilled technicians to distinguish trypomastigote forms at subgenus and species levels. However, these parameters are unsuitable for reliable identification of trypanosome in tsetse flies, which often harbour multiple trypanosome species.

To increase our knowledge about TCO and TVI, the main agents of AAT in Ethiopia (Abebe et al., 2017; Biyazen et al., 2014; Dagnachew et al., 2017; Degneh et al., 2017; Lelisa et al., 2014; Moti et al., 2012; Sheferaw et al., 2016; Taye et al., 2012; Zecharias and Zeryehun, 2012) likewise all Sub-Saharan Africa (Morrison et al., 2016), we selected tsetse samples harboring TVI and TCO for further molecular analyses. Aiming at the characterisation of the three equally prevalent trypanosomes found in tsetse flies caught in the NSNP, we additionally analysed TSM. To this end, samples that generated FFLB profiles that were compatible with these three species were characterised using gGAPDH sequences.

3.4. Genotyping of Trypanosoma vivax from the NSNP revealed exclusively the "West African-South American" genotype

Previous studies have revealed that a greater diversity of TVI exists in East Africa compared to the much more homogeneous and closely related populations so far indentified in West Africa and South America (Adams et al., 2010; Cortez et al., 2006; Garcia et al., 2014, 2018; Rodrigues et al., 2008; Rodrigues et al., 2017). Phylogeographical analysis based on gGAPDH and ITS rDNA of TVI isolates from East African countries including Mozambique, Tanzania, Kenya and Ethiopia have supported two main lineages separated by relevant genetic distance, and referred as to *T. vivax vivax* (TVV) and *T. vivax*-like (TVL) (Cortez et al., 2006; Garcia et al., 2018; Rodrigues et al., 2017). The analysis of proline racemase (PRAC) gene sequences revealed genetic differences between the TVI isolates obtained from cattle living in tsetse-infested and tsetse-free areas of Ethiopia (Fikru et al., 2014). However, these differences, which have been suggested to be linked to different transmission routes, were not corroborated by the analysis of additional isolates using ITS rDNA sequences (Fikru et al., 2016).

The FFLB profiles herein obtained from 66 tsetse flies from the NSNP did not reveal any TVL genotypes, and all samples were assigned to the TVV lineage. Selected DNA samples from tsetse flies that generated FFLB profiles typical of TVI were characterised by PCR-sequencing of gGAPDH genes (GenBank accession numbers MK120146–70). Confirming the FFLB profiling, the MDS analysis of 62 cloned sequences revealed exclusively TVV sequences, all tightly clustering with TVI sequences from West African and South America (Fig. 5A). Previously, ITS rDNA sequences of TVI from Ethiopian cattle (Fikru et al., 2016) were found to be very similar to the sequences of all the West African and South American TVI isolates that have been characterised to date (Rodrigues et al., 2017). Additionally, a few sequences of the TVL lineage were identified among sequences obtained from Ethiopia cattle by Fikru et al. (2016) in our previous analysis (Rodrigues et al., 2017). More comprehensive analyses of highly polymorphic markers such as ITS rDNA and microsatellites, and SNPs (single nucleotide polymorphisms) of whole genomes are required to better understand population structure of cyclically and mechanically transmitted TVI in Ethiopia. These wide-ranging studies would enable to track the origin and dispersion of TVI populations in East and West Africa and their introduction into the Americas (Cortez et al., 2006; Garcia et al., 2014, 2018; Rodrigues et al., 2017). The importance of TVI mechanical transmission in Ethiopia has been highlighted by high prevalence rates in areas of low tsetse density and even completely tsetse free, where transmission relies on tabanids and other biting flies (Cherenet et al., 2006; Dagnachew et al., 2017; Duguma et al., 2015; Fikru et al., 2012; Sinshaw et al., 2006). Tabanid flies are in general abundant in wildlife reserves, and they were common in tsetse traps mounted in the NSNP, suggesting that mechanical transmission of TVI could also be relevant in the studied area.

3.5. Tsetse flies from the NSNP harboured all species of the subgenus Nannomonas, including Trypanosoma congolense Savanah, Kilifi and Forest genotypes

The FFLB-based survey of trypanosomes in *G. pallidipes* from the NSNP revealed that all trypanosome species of the subgenus *Nannomonas* were present, with relevant prevalence rates for TSM (23.3%), TCO (22.3%), TGO (9.4%) and TST (6.3%) (Fig. 3A). We also detected the presence of three genotypes of TCO: TCS (16.7%), TCK (3.5%) and TCF (2.1%) (Fig. 3, 4). In agreement with our data, previous molecular surveys based on the PCR-RFLP analysis of 18S rRNA of cattle from the Ghibe valley, in the south-western region of Ethiopia, revealed a high prevalence of TCS (17.8–59.9%) but low rates of TCK (4.7%) (Moti et al., 2012, 2015). Molecular surveys conducted in Ethiopian cattle have shown TCS to be the most prevalent genotype of TCO, thus corroborating the high prevalence and wide-range of TCS across Sub Saharan countries. Small populations of TCK and TCF have been also reported, mainly in East and West/Central Africa, respectively (Garcia et al., 2018; Malele et al., 2011; Mamabolo et al., 2009; Moti et al., 2015; Simo et al., 2012).

In our MDS analysis of gGAPDH sequences from TCO isolates of different genotypes (determined in this study or retrieved from GenBank and genome databases) all sequences of TCS from East Africa (Ethiopia, Mozambique, Kenya, Uganda and Tanzania) and West Africa (The Gambia, Nigeria and Burkina Faso) clustered together (Fig. 5B). Interestingly, comparison of genome-wide SNPs revealed a cluster of TCS isolates from Ethiopia, Uganda and Burkina Faso, which differs from the cluster containing isolates from The Gambia, Mali, Togo and Cameroon (Tihon et al., 2017). In contrast with TCS (Rodrigues et al., 2014; Tihon et al., 2017), there are very few available DNA sequences from TCK. In the present study, we determined gGAPDH sequences of TCK found in tsetse flies that showed FFLB profiles compatible with TCK, previously defined using the reference isolates WG5 and WG84 from Kilifi on the Kenyan coast (Knowles et al., 1988). The gGAPDH sequences that we obtained from Ethiopian TCK (GenBank: MK120176-90) were clustered with those of TCK from Kenya and Mozambique (GenBank: MK120191-92) (Fig. 5B). Our analysis of TCO sequences from Ethiopia, Kenya and Mozambique provides important insight toward a remarkable genetic diversity, likely suggesting the existence of additional genotypes of TCO (Fig. 5B). The influence of genetic exchange on the increasingly genetic diversity of TCO populations requires wide-ranging analyses of microsatellite and SNP polymorphisms (Morrison et al., 2009; Rodrigues et al., 2014; Tihon et al., 2017).

Epidemiological data has suggested that TCS and TCK share cattle and tsetse flies, as we have confirmed for *G. pallidipes* in Ethiopia and *G. morsitans* in Mozambique (Garcia et al., 2018). Our findings suggest that Ethiopia may represents the extreme northern distribution of TCK. Since the first report from Kenya (Knowles et al., 1988), TCK has been identified using PCR-ITS in cattle across East African countries, from Ethiopia to South Africa, although at much lower prevalence rates compared with TCS (Garcia et al., 2018; Malele et al., 2011; Mamabolo et al., 2009; Moti et al., 2015; Rodrigues et al., 2014). It is known that TCK is less virulent than TCS in experimentally infected mice, but very little is known about livestock infected with TCK, and TCK wild

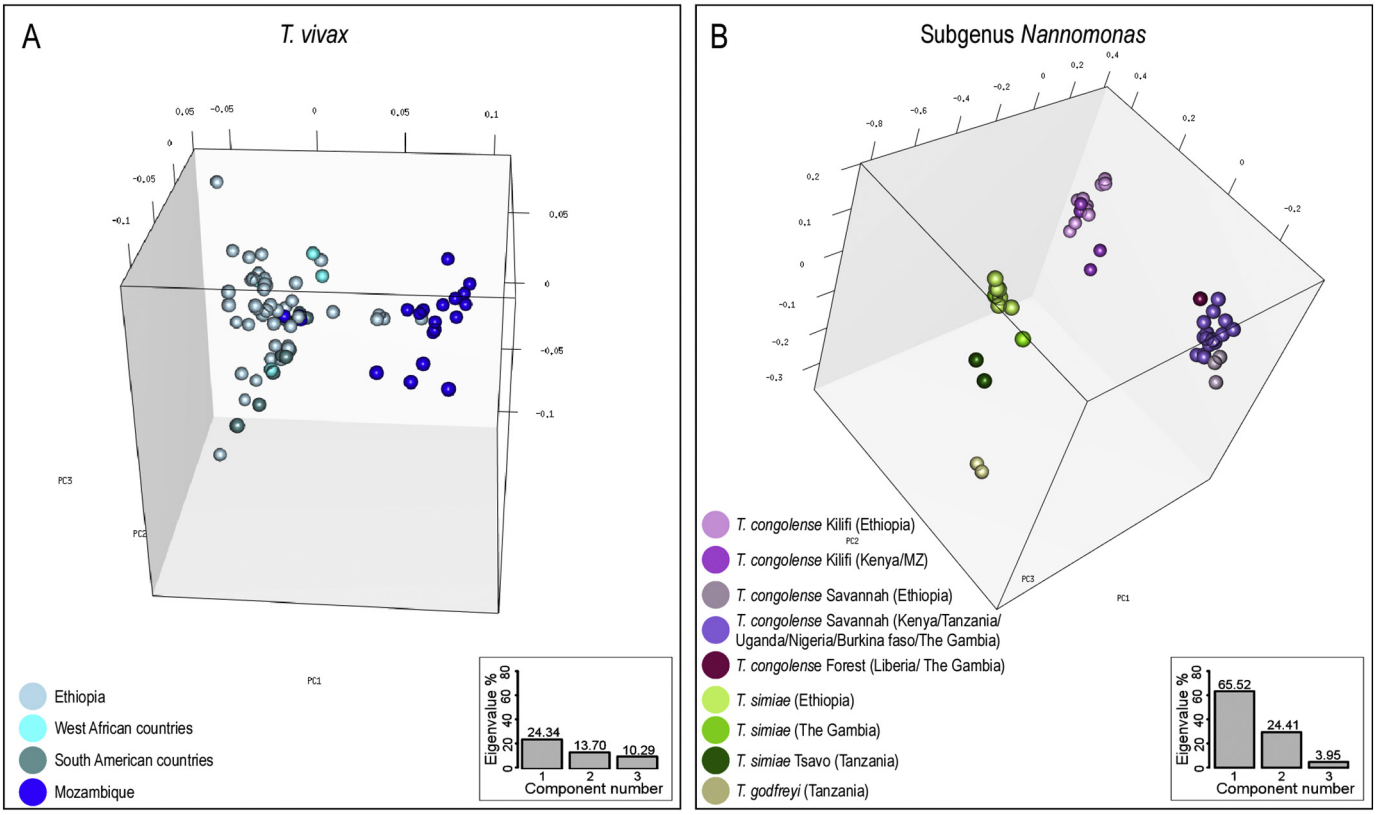

Fig. 5. Multidimensional scaling analysis of gGAPDH sequences of trypanosomes from tsetse flies caught in the Nech Sar National Park. (A) All sequences of *T. vivax* from Ethiopian tsetse flies obtained in the present study clustered into a highly cohesive group comprising all sequences from West Africa and South America, and part of previously identified isolates from Mozambique (Rodrigues et al., 2017). (B) Sequences of *T. congolense* from Ethiopia clustered with those from West, Central and other East African *T. congolense* Savannah (Rodrigues et al., 2014; Tihon et al., 2017) or with the distantly-related *T. congolense* Kilifi from Kenya and Mozambique. Heterogeneous sequences related to *T. simiae* from Ethiopia clustered with sequences from *T. simiae* from The Gambia and Mozambique near to *T. simiae* Tsavo.

reservoirs (Bengaly et al., 2002; Motloang et al., 2014).

Our analysis of *G. pallidipes* from the NSNP revealed high prevalence rates of the suid-associated TSM, TST and TGO. To our knowledge, this is the first report using molecular techniques to identify trypanosomes that are pathogenic to domestic pigs in tsetse flies from Ethiopia. Tsetse samples which were positive by FFLB for the high prevalent TSM were selected for trypanosome characterisation using gGAPDH sequences. Results of our comparative analysis revealed heterogeneous gGAPDH sequences (sequence divergence ranged from 0.2 to 1.1%, with an average of ~0.6%). The comparison of sequences from Ethiopian isolates of TSM determined in the present study (GenBank: MK120171–73), with those available of TSM from The Gambia as well as those herein obtained by us from Mozambique (GenBank: MK120174–75) confirmed the high genetic diversity of TSM (Fig. 5B). Previously, relevant genetic diversity of TSM were reported in tsetse flies from other wildlife natural reserves in Tanzania (Hamilton et al., 2008), and Central African Republic (Votypka et al., 2015). Therefore, comprehensive phylogenetic analyses of TSM sequences are required to better investigate this underestimated genetic diversity.

3.6. Trypanozoon spp. in tsetse flies from the NSNP

Among the tsetse flies caught in the NSNP, ~10.5% were found to have FFLB profiles compatible with trypanosomes of the subgenus *Trypanozoon* (Fig. 3A). *Trypanosoma brucei* spp. and *T. evansi* are indistinguishable by this method or by comparisons of gGAPDH, SSU rRNA and ITS rDNA sequences, as all these sequences are highly conserved among all *Trypanozoon* spp. Human trypanosomiasis is not reported in Ethiopia. Most likely, flies of the NSNP are infected with *T. b. brucei,* a species commonly detected in cattle either in the studied area or other regions in Ethiopia (Dagnachew et al., 2005; Degneh et al., 2017). However, we cannot discard the possibility that the highly sensitive FFLB detects *T. evansi* (unable to develop in tsetse flies) arising from recent blood meals. This mechanically transmitted species is the leading cause of camel trypanosomiasis in Ethiopia (Birhanu et al., 2015, 2016; Fikru et al., 2016; Jilo et al., 2016).

3.7. Trypanosoma (Megatrypanum) theileri is common in tsetse flies from the NSNP

The cosmopolitan Tth is highly prevalent in African cattle (Fikru et al., 2012, 2016; Moti et al., 2015; Ngomtcho et al., 2017). In Ethiopia, Tth has been identified in cattle by the morphology of blood forms (Birhanu et al., 2015; Girmay et al., 2016) and PCR amplification of ITS rDNA (Fikru et al., 2012, 2016). Recently, Tth has been detected by PCR in tsetse flies, as reported in surveys conducted in the Central African Republic and Mozambique (Garcia et al., 2018; Votypka et al., 2015). Here, we identified FFLB profile typical of Tth from DNA obtained from – 20% tsetse flies caught in the NSNP (Fig. 3). However, whether Tth-like species from wild ruminants (Garcia et al., 2011) may have been present in tsetse flies needs further phylogenetic analysis because Tth (cattle) and Tth-like (domestic and wild ruminants) are indistinguishable by FFLB profiling. Tth is known to be cyclically transmitted by tabanid flies, and whether it can be transmitted cyclically or mechanically by tsetse flies remains to be investigated. Another feature that requires further investigation is the potential pathogenicity of Tth. Negative effects of Tth infection have been reported in South American and African cattle concomitantly infected with pathogenic

trypanosomes (TVI and/or TCO) and other haemoparasites (Jaimes-Dueñez et al., 2017; Ngomtcho et al., 2017).

4. Conclusions

In some places of the District of Arba Minch, Ethiopia, the tsetse population has been reduced by more than 90% and AAT prevalence has decreased by 58% due to intensive suppression activities supported by communities highly committed to control efforts such as those living in the first block of STEP (Fig. 1). Significant reductions of both tsetse flies and AAT in this suppression area have led to improved survival and growth of cattle. The present study conducted in an area of the NSNP bordering the town of Arba Minch confirmed *G. pallidipes* to be the sole species present in this area. However, our findings also demonstrated relevant genetic diversity, suggesting a potential wide connectivity between *G. pallidipes* populations inside and outside of the NSNP. Trypanosome agents of AAT were identified in 38% of *G. pallidipes* from the NSNP, with comparable infection rates for TVI (23%), TSM (23%) and TCO (22%), in addition to relevant infection rates of TPZ (11%), TGO (9.5%), TST (6.3%) and Tth (19.9%). Our phylogenetic analysis uncovered a relevant genetic diversity of both TCO and TSM, and corroborated a conserved TVI genotype, positioned in the lineage TVV by this and previous studies (Fikru et al., 2014, 2016; Rodrigues et al., 2017) widespread in West Africa and South America. In East Africa, in addition to Ethiopia, TVI genotypes closely related to those from West Africa/South America have been found in Mozambique, Zambia and Uganda (Rodrigues et al., 2017; Garcia et al. in preparation). The diversity of trypanosomes uncovered in tsetse flies by FFLB reflects the notable repertoire of parasites circulating in the blood of vertebrates in the studied area. Even though the transmission of the trypanosomes that herein were identified only by DNA detection depends on their intricate establishment and differentiation in the tsetse flies, our main findings agreed with those from parasitological surveys of trypanosomes in cattle, thus providing reliable insight into the epidemiology of AAT in the studied area. The high density of tsetse flies alongside their feeding pattern and repertoire of trypanosome species/genotypes indicate the need for more comprehensive and systematic evaluation of tsetse flies in the NSNP and nearby areas. **Suppression of tsetse flies is very difficult to sustain in areas near to natural wildlife reserves**, where the invasion of abundant wild tsetse flies into the controlled area must be prevented uninterruptedly. Insecticide-based control of tsetse flies in the NSNP is highly questionable for reasons of adverse ecological consequences and cost-effectiveness. The surveillance of tsetse flies and livestock foraging both in the NSNP and tsetse controlled areas is fundamental to reduce the risk of both tsetse and AAT re-emergence in suppressed areas. The information provided by this study reinforces the need of continuous vigilance and tsetse control, and appropriated livestock management practices, especially at the interfaces between the NSNP and farm areas, in order to sustain tsetse/AAT suppression areas in the Southern Rift Valley in Ethiopia.

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