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Research paper

Molecular characterization of *Trypanosoma vivax* in tsetse flies confirms the presence of the virulent *Tvv4* genotype in Kenya: Potential implications for the control of trypanosomiasis in Shimba Hills

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ABSTRACT

Trypanosoma vivax is a vector-borne protozoan parasite of livestock endemic to Africa and South America. To date, fifteen genotypes of the parasite have been described in vertebrate and insect hosts in East Africa. However, information regarding *T. vivax* diversity remains limited in many endemic countries in the sub-region, including Kenya. Such information could deepen insight into the local epidemiology of animal trypanosomiasis in Shimba Hills, a wildlife area in southeast Kenya where *T. vivax* is endemic and infects livestock. We employed two-gene conventional-PCR-sequencing and phylogenetic analysis to characterize *T. vivax* genotypes in tsetse flies collected between November 2018 and September 2019 in the wildlife-livestock interface of the Shimba Hills National Reserve. Phylogenetic analysis of Internal Transcribed Spacer-1 (ITS-1) sequences of *T. vivax* isolates confirmed the presence of two *T. vivax* genotypes in Shimba Hills of which >80% of *T. vivax* isolates from tsetse flies clustered within the virulent *Tvv4*-genotype clade. Tsetse infections with the *Tvv4* genotype were also confirmed based on 18S *r*RNA gene sequencing. Expanded gene characterization identified three closely related haplotypes within the *Tvv4*-clade. The *Tvv4*-isolates were detected in male and female *Glossina pallidipes* tsetse flies, most of which were collected from grasslands and within two kilometres of the Shimba Hills National Reserve boundary. Considering that *T. vivax* is the most common trypanosome in the Shimba Hills area and causes severe clinical conditions in livestock, the *Tvv4* genotype reported here for the first time in Kenya contributes to our understanding of these pathologies. The effectiveness of trypanocidal drugs in the management of *Tvv4* is presently not clearly understood. Therefore, the parasite management in Shimba Hills should focus on vector control to reduce the density of *G. pallidipes,* especially in grasslands near the wildlife protectorate.

1. Introduction

Trypanosoma vivax is a pathogenic tsetse-transmitted trypanosome parasite of livestock in sub-Saharan Africa. The parasite is also transmitted by other biting flies such as *Stomoxys* sp. and tabanids. This has enabled its sustained transmission outside the tsetse-belt of sub-Saharan Africa (Jones and Dávila, 2001). In sub-Saharan Africa, *T. vivax, T. congolense* and *T. brucei sl* account for the major trypanosome burden on livestock health and production in agro-pastoralist communities, many of which are located along the interface of wildlife reserves (Auty et al., 2016; Anderson et al., 2011; Squarre et al., 2020; Lord et al., 2018; Lord et al., 2020; Auty et al., 2012; Votýpka et al., 2015). Other trypanosomes, including *T. simiae, T. simiae* Tsavo, and *T. godfreyi* have also been described to cause pathology in livestock though mainly in suids (Auty et al., 2012; Votýpka et al., 2015; Adams et al., 2006; Garcia et al., 2018; Njiru et al., 2005; Hamilton et al., 2008; Gaithuma et al., 2019; Ng'ayo et al., 2005; Njiru et al., 2004; Von Wissmann et al., 2011).

Trypansoma vivax is genetically diverse and different genotypes show subtle differences in transmission patterns and variable responses to chemotherapy with trypanocides. These genotypes also exhibit non-

Abbreviations: SHNR, Shimba Hills National Reserve; KWS, Kenya Wildlife Service; KENTTEC, Kenya Tsetse and Trypanosomiasis Eradication Council; DNA, Deoxyribo-Nucleic Acid; PCR, Polymerase Chain Reaction; *icipe*, International Centre of Insect Physiology and Ecology.

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uniform virulence in infected animal hosts (Rodrigues et al., 2008; Rodrigues et al., 2017; Fasogbon et al., 1990; Giordani et al., 2016). It is believed that *T. vivax* isolates from East Africa are less virulent than isolates from West Africa (Losos and Ikede, 1972). However, some acute outbreaks and severe hemorrhagic cases have been reported in cattle in Kenya and Uganda following infections with *T. vivax* (Gardiner et al., 1989; Magona et al., 2008). In Mozambique, a *T. vivax* isolate from nyala was reportedly highly virulent with severe conditions observed in an experimentally infected goat less than three weeks after inoculation (Rodrigues et al., 2008). Using molecular techniques for trypanosome examination, Rodrigues et al. (Rodrigues et al., 2008) generated DNA sequences of the nyala-derived trypanosome. A subsequent taxonomic revision, reporting novel and highly diverse *T. vivax* genotypes from Mozambique (Rodrigues et al., 2017), placed the isolate within the *Tvv4*-genotype of *T. vivax* (Rodrigues et al., 2017). To date, reports are scanty regarding the distribution of this highly virulent genotype.

In Kenya, epidemiological studies showed the genotype diversity of *T. brucei* and *T. evansi* (Echodu et al., 2015; Kamidi et al., 2017). However, relatively little is known about the molecular diversity of *T. vivax*. A prior study using isoenzyme polymorphisms confirmed that at least two genotypes occur in Kenya (Fasogbon et al., 1990). A recent review of *T. vivax* genotype distribution in Africa reported one genotype in Kenya based on the ITS rDNA gene (Rodrigues et al., 2017). However, in Ethiopia and Tanzania, four and three genotypes were reported, respectively. These results contrast with findings in Mozambique, where most studies have been conducted, and ten *T. vivax* genotypes are described (Rodrigues et al., 2017).

We used the conventional-Polymerase Chain Reaction (PCR) technique, molecular sequencing and phylogenetic analysis to describe genotypes of *T. vivax* in tsetse flies from the wildlife-livestock interface of the Shimba Hills National Reserve on the coast of Kenya. *Trypanosoma vivax* is known to be the main trypanosome circulating in populations of tsetse flies in Shimba Hills, where high parasitological rates of trypanosome infections in cattle are associated with anaemic conditions (Ebhodaghe F, unpublished). Currently, there is no clear understanding of *T. vivax* genetic diversity in Shimba Hills. A study that addresses this knowledge gap can help explain trypanosome pathologies in cattle and guide interventions against animal trypanosomiasis.

2. Materials and methods

2.1. Ethical clearance

The study was done according to guidelines stipulated by the International Centre of Insect Physiology and Ecology *icipe* Nairobi, Kenya. Tsetse fly samples were collected in collaboration with the Kenya Wildlife Service (KWS) and the Kenya Tsetse and Trypanosomiasis Eradication Council (KENTTEC).

2.2. Study setting and tsetse flies sampling

The Shimba Hills National Reserve (SHNR) in Kwale County on the coast of Kenya is one of East Africa's biodiversity hotspots (Malonza et al., 2018). Extending over 200 km^2, the SHNR accommodates a large community of wildlife species, including the critically endangered sable antelope (*Hippotragus niger*), warthog (*Phacochoerus africanus*), buffalo (*Syncerus scaffer*), and bushbuck (*Tragelaphus scriptus)* and is remarkable for its high elephant (*Loxodonta africana*) density. Historically, conflicts

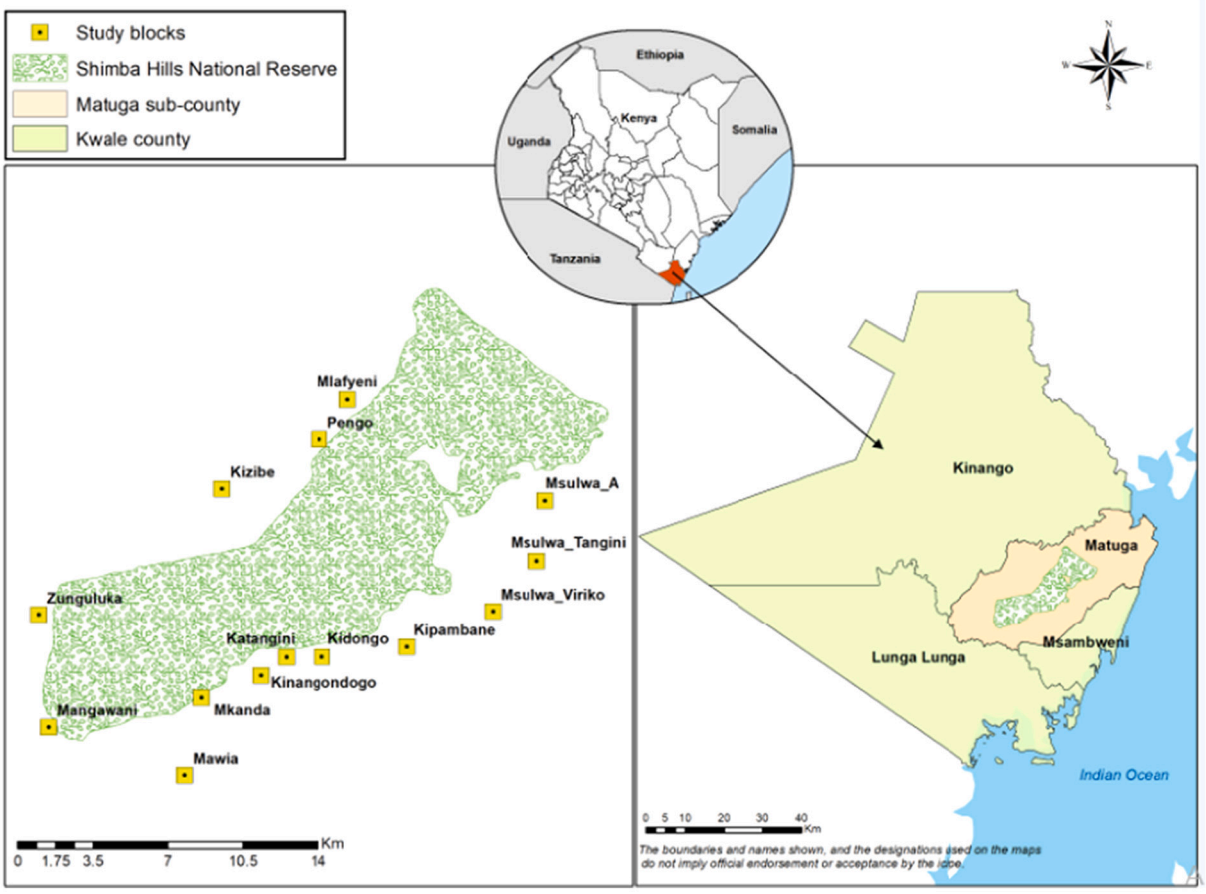

Fig. 1. Map of Kenya showing Kwale county. Right: Map of Kwale County showing Matuga district and location of Shimba Hills National Reserve. Left: Map of Shimba Hills National Reserve showing block locations where sampling was done.

between wildlife and humans/livestock were common around the SHNR. Such conflicts still arise, albeit not physically but due to disease spillover. Pathogens from wildlife in Shimba Hills that impact the health of livestock include trypanosomes transmitted by tsetse flies. In the present study, tsetse flies were collected in bimonthly entomological surveys between November 2018 and September 2019 in the SHNR (Fig. 1). Briefly, tsetse fly samples were collected using cow urine and acetone-baited biconical traps deployed at a density of one per km grid-cell over a ~ 230 km^2 area stratified into 14 blocks along the wildlife-livestock interface of the SHNR. Trapped flies were collected after 48 h of trap deployment and identified using established taxonomic keys (Pollock, 1982). All tsetse fly samples were sorted according to sex and species and then preserved in 95% ethanol until further analysis. Tsetse flies identified as male and female *Glossina pallidipes* Austen, 1903, *G. brevipalpis* Newstead, 1910, and *G. austeni* Newstead, 1912 were selected from traps deployed across the 14 study blocks and screened for *T. vivax* infection. Overall, 696 tsetse flies from 113 traps were randomly selected for screening. The number of fly samples from each trap ranged from 1 to 45 (average: 6 flies per trap).

2.3. DNA extraction and trypanosome detection and identification

DNA was extracted from dry, crushed homogenates using the Bioline Genomic DNA extraction kits (London, UK) following the manufacturer's instructions for animal tissues. Briefly, individual tsetse flies were removed from alcohol and allowed to air-dry on paper towels and afterwards crushed in a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK, USA). DNA amplification was conducted in a 10 µl reaction volume comprising of 0.5 µl (concentration: 10 µM) each of Forward and Reverse ITS-1 primers (CF: CCGGAAGTTCACCGATATTG, BR: TTGCTGCGTTCTTCAACGAA) (Njiru et al., 2005), 3 µl nuclease-free water, 5 µl DreamTaq, and 1 µl DNA template with the following cycling conditions: initial denaturation for 1 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 20 s, and extension at 72 °C, followed by a final extension at 72 °C for 7 min. PCR products were run against a Gene-Ruler 100 bp DNA ladder (Thermo Scientific, Lithuania) on a 1.5% agarose gel stained with 5 µg/ml ethidium bromide. Tsetse fly extracts that produced ~250 bp band were scored as positive for *T. vivax* infection (Njiru et al., 2005). Positive samples were subjected to 18S rRNA amplification using published primers and thermal cycling conditions (Maslov et al., 1996).

2.4. Nucleotide sequencing

We conducted unidirectional sequencing of ITS-1 amplicons with the ITS1 CF (Njiru et al., 2005) primer to confirm the identity of trypanosomes detected. Similarly, unidirectional sequencing of the ~200 bp 18S rRNA amplicons was carried out using 18S *rRNA* primer GACCRTTG-TAGTCCACACTG (Maslov et al., 1996) to confirm *T. vivax* genotype identity. Amplicons were cleaned using EXO-SAP to remove unincorporated dNTPs and primers. Alternatively, DNA bands were excised from gels using clean scalpel blades under blue light illumination and purified using Qiagen Gel Extraction Kits following the manufacturer's instructions. Sanger sequencing was outsourced to Macrogen (Macrogen Europe B.V., The Netherlands).

2.5. Phylogenetic analysis

DNA sequences were inspected for quality based on chromatograph profiles and edited using the BioEdit software *v7.2.5* (Hall, 1999). BLAST searches were conducted against the GenBank-*nr* database to identify sequences with the highest levels of sequence identity using the NCBI BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were undertaken using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The dataset was complemented with reference sequences from Kenya, Nigeria and Burkina Faso, and closely related sequences before removing end-unaligned sequences and regions corresponding to primer-binding sites. Maximum-Likelihood trees were inferred in the Molecular Evolution and Genetic Analysis *MEGA-X* software (Kumar et al., 2018) using the Hasegawa-Kishino-Yano HKY model of sequence evolution (Hasegawa et al., 1985), selected as the best-fit model using the Smart Model Selection in PhyMl (Lefort et al., 2017). The Nearest Neighbour Interchange was used to estimate tree topologies and nodal support was estimated from 1000 bootstrap replications. The kinetoplastid *Bodo caudatus* in the Bodonidae family (GenBank accession number: AY028450) was selected as outgroup to root trees.

2.6. DNA sequence and haplotype analysis of T. vivax isolates

We estimated *T. vivax* population diversity indices [haplotype diversity (*Hd*), polymorphic (or segregating) sites (*S*), parsimony-informative sites, and nucleotide diversity (π)] in DnaSP *v6* (Rozas et al., 2017). Median-joining networks (Bandelt et al., 1999) were constructed in the PopART software (Leigh and Bryant, 2015) to visually explore relationships among haplotypes from Shimba Hills and available closely related sequences in the GenBank database.

3. Results

Overall, 2.44% (17/696) of tsetse flies were positive for *Trypanosoma vivax* based on ITS-PCR. Infections were detected in one female *Glossina brevipalpis* (0.77%, 1/130) and the other cases in males and females of *G. pallidipes* (3.04%, 16/526). All *T. vivax*-positive tsetse flies were collected in traps within 3 km of the Shimba Hills National Reserve border.

3.1. Genotypes of T. vivax in Shimba Hills and Kenya

3.1.1. Based on ITS-1 DNA

A total of ten *T. vivax* isolates were sequenced based on the ITS1 gene and confirmed by BLAST analysis to be *T. vivax*. However, we excluded three of these isolates from the study due to the poor quality of sequences revealed by their chromatographs, thus leaving us with 7 ITS1 sequences (Table 1).

Trypanosoma vivax ITS1 phylogeny revealed three major genotype clades for the parasite in Kenya, with the first and second clades present in the Shimba Hills National Reserve area (Fig. 2, S1). The first clade consists of Kenyan isolates (GenBank Accession Nos.: DQ316042, DQ316043, DQ316040, DQ316039, DQ316038, DQ316037, DQ316044, and DQ316041) from cattle and an isolate GP105 from our study (Fig. 2) (GenBank Accession Number: MW689621).

The second clade comprises of *T. vivax* Tvv4 genotype first identified from a wild-caught nyala antelope in the Sofala Province in Mozambique (GenBank Accession No.: EU482078) and a *Morsitans* tsetse fly from the Gorongosa National Park in the Central region of Mozambique (GenBank Accession No.: KX584844), as well as samples from this study (GP49, GP306, GP464, GP586, GP870 and GP525) (GenBank Accession Number: MW689622- MW689627).

The third clade is made up of isolates from Nigeria and Burkina Faso in West Africa and from Kenya in East Africa. Isolates from Kenya were obtained from camel flies (GenBank Accession Nos.: MK880189, MH247152, MH247150, and MT586222) and dromedary camels (GenBank Accession Nos.: MK880188, MH247145, MH247149, MH247147, MH247140, and MH247142) in the tsetse-free region of northern Kenya. The isolates from Nigeria (GenBank Accession No.: U22316) and Burkina Faso (GenBank Accession No.: JX910379, JX910377) were obtained from cattle.

3.1.2. 18S rRNA gene phylogeny

We selected the isolates GP49, GP306, GP464, GP586, and GP525 (shown on the ITS1 phylogeny as Tvv4) and 2 other isolates GP599 and

Table 1
Identification of nucleic acid sequences of *T. vivax* detected in tsetse flies from Shimba Hills (2018 to 2019).

Sample ID	Sequence length (bp)	Closest match on GenBank (host, country)	Sequence identity (%)
ITS-1 DNA			
GP464	221	KX584844 (Tsetse fly, Mozambique)	100
GP49	209	KX584844 (Tsetse fly, Mozambique)	99.42
GP586	208	KX584844 (Tsetse fly, Mozambique)	100
GP105	215	KM391825 (Cattle, Ethiopia)	98.58
GP306	210	KX584844 (Tsetse fly, Mozambique)	100
GP870	210	KX584844 (Tsetse fly, Mozambique)	98.82
GP525	203	KX584844 (Tsetse fly, Mozambique)	95.86
18S rRNA			
GP49	163	EU477537 (Nyala, Mozambique)	99.38
GP306	176	EU477537 (Nyala, Mozambique)	97.74
GP464	186	EU477537 (Nyala, Mozambique)	92.47
GP525	170	EU477537 (Nyala, Mozambique)	97.63
GP586	169	EU477537 (Nyala, Mozambique)	98.21
GP599	213	EU477537 (Nyala, Mozambique)	98.27
GP788	173	EU477537 (Nyala, Mozambique)	98.28

GP788 for 18S *rRNA* gene amplification and sequencing. Nucleotide BLAST searches performed with each of the resulting sequencing revealed that each of the 7 18S *rRNA T. vivax* isolates (GenBank Accession No.: MW686915-MW686917, MW812256-MW812259) are closest in identity to a sequence for a nyala antelope-derived *T. vivax* isolate in Mozambique (GenBank Accession No.: EU477537) (Table 1).

Two major genotype clades emerged on the 18S *rRNA* phylogeny (Fig. 3, S2). Isolates GP49, GP306, and GP586 (GenBank Accession Number: MW686915-MW686917) selected to represent the Tvv4 genotype (as shown on the ITS1 phylogeny) clustered within the first clade. This first clade also features an isolate from a nyala antelope in Mozambique (GenBank Accession No.: EU477537).

The second genotype group consists of isolates from Africa and the Americas. Isolates from Nigeria obtained from a rat (GenBank Accession No.: KM391828) and cattle (GenBank Accession No.: U22316) clustered with those from cattle in Ethiopia (GenBank Accession No.: KM391826 and KM391827) in East Africa and Brazil (GenBank Accession Nos.: MH184518, MN966706, MN966728, MN966709, MN966707, MN966705, MN966704) in South America.

3.2. Variation among DNA sequences of T. vivax isolates from Shimba Hills

Haplotype and nucleotide diversity were respectively 0.714 and 0.123 for *ITS-1* sequences (174 base-pair long) for seven *T. vivax* isolates from Shimba Hills. We noted a total of 68 polymorphic sites in the set of aligned sequences with 5.88% (4/68) of these sites being parsimony informative (S3).

Haplotype diversity for the seven 18S *rRNA* isolate sequences (165 base-pair) was 0.714, and nucleotide diversity 0.058. The entire set of aligned sequences had 28 polymorphic sites with 21.43% (6/28) appearing as parsimony informative sites (Table 1, S3).

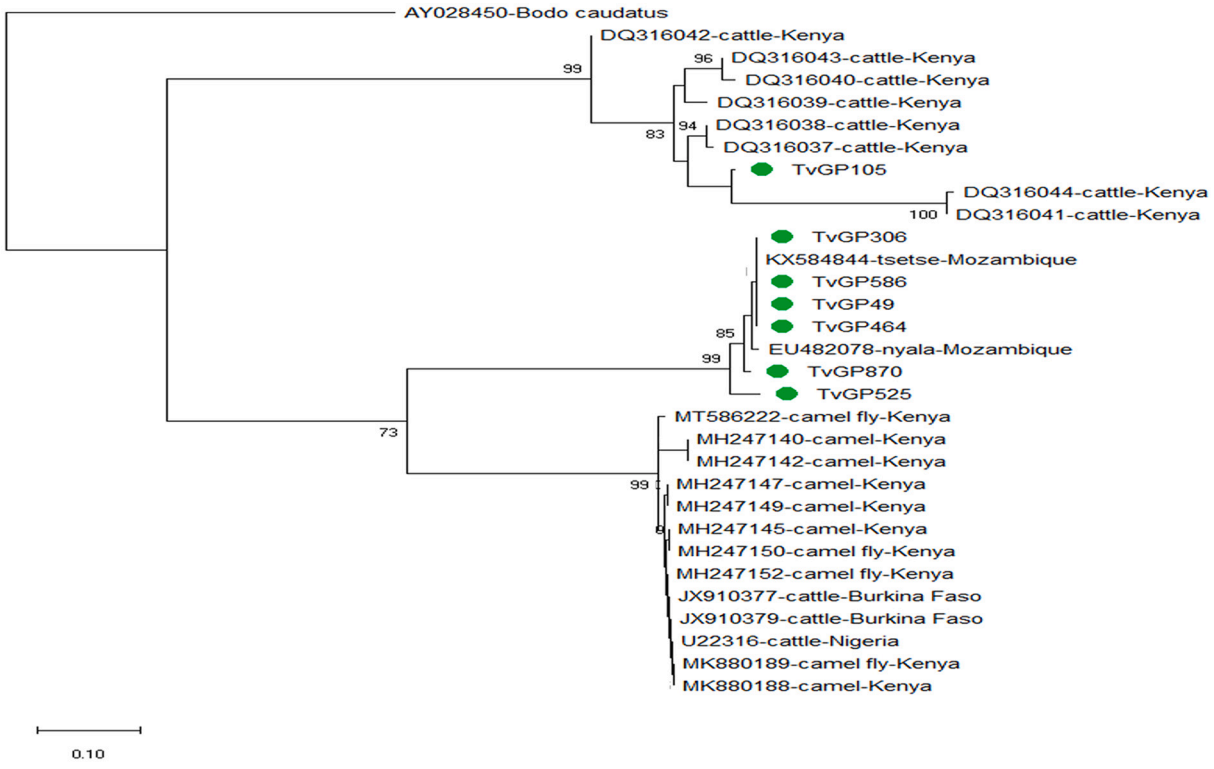

Fig. 2. Maximum-Likelihood phylogenetic tree inferred using partial ITS-1 *T. vivax* sequences (220 base-pairs) from tsetse flies collected in Shimba Hills, Kenya. Sequences from this study are bulleted in green and 'Tv' captioned before the sample ID to indicate that they are *T. vivax* species. Other sequences were obtained from GenBank. 'Host' and 'country' of isolation are added to each of the GenBank sequences. *Bodo caudatus* is designated as out-group. Nodal support values >70% based on 1000 bootstrap replicates are indicated next to the relevant nodes. The branch length represents substitution per site.

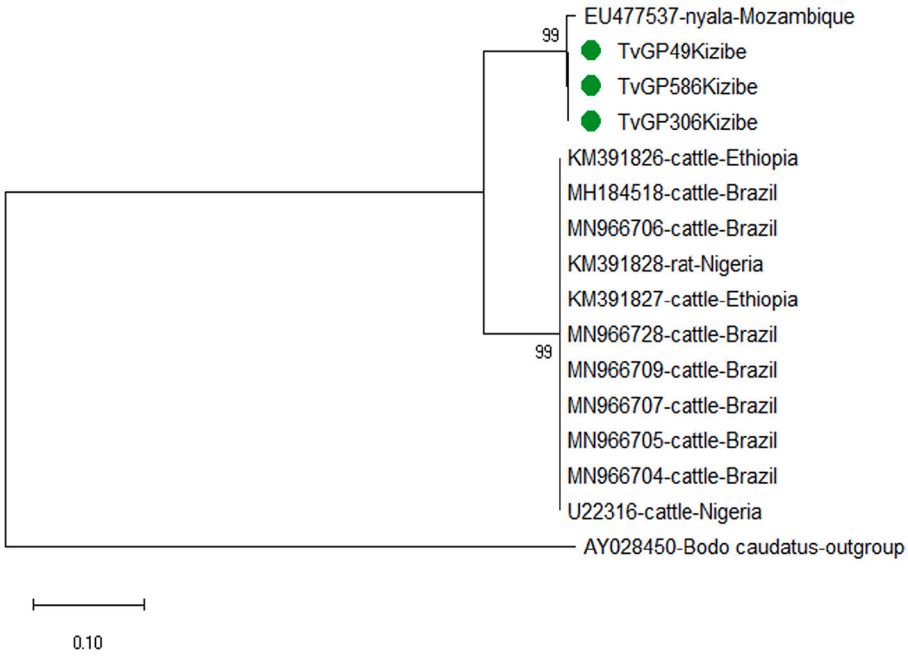

Fig. 3. Maximum-Likelihood phylogenetic tree inferred using partial 18S *rRNA T. vivax* sequences (159 base-pair) from tsetse flies collected in Shimba Hills, Kenya. Sequences from this study are bulleted in green and 'Tv' captioned before the sample ID to indicate that they are *T. vivax* species. Other sequences without the green bullets are from the GenBank database. 'Host' and 'country' of isolation are added to each of the GenBank sequences. *Bodo caudatus* is designated as an out-group. Nodal support >70% based on 1000 bootstrap replicates is indicated next to the relevant nodes. The branch length represents substitution per site.

3.3. Median-joining haplotype network analysis of *T. vivax* isolates from Shimba Hills and other locations in sub-Saharan Africa and Brazil in South America

Three major groups emerged from our median-joining network analysis based on ITS-1 DNA sequences (Fig. 4). The first group was the Tvv4 comprising of GP49, GP306, GP464, GP525, GP586, and GP870 from Shimba Hills and the nyala trypanosome isolate from Mozambique (EU482078). The Tvv4 group had four distinct haplotypes, the main haplotype comprising of GP49, GP306, GP464, and GP525 differing from the nyala isolate from Mozambique by a single mutation. GP870 and GP 525 differed from the main haplotype by two and seven mutations, respectively. The second group (TvL1-G) contained the sample from Shimba Hills (GP105) and the *IL3905* strain (DQ316041) from Kenya, which differed by five nucleotide substitutions and a single nucleotide indel from each other. Finally, the third group was made up of isolates from Kenya (MH247149), Nigeria (U22316), and Burkina Faso (JX910379), all sharing a common haplotype.

When analyzed based on the 18S *rRNA* sequences, *T. vivax* isolates GP49, GP306 and GP586 from Shimba Hills shared same haplotype, differing from the nyala isolate from Mozambique (EU477537) by a single mutation (Fig. 5). Isolates from Brazil (MN966728), Ethiopia (KM391827), and Nigeria (U22316) belonged to the same Tvv1 group having a common haplotype.

3.4. *T. vivax* diversity in relation to tsetse fly and location in Shimba Hills

The *T. vivaxTvv4* genotype was detected in both male and female tsetse flies (Table 2), while the single *TvL1-G* genotype was found in a female tsetse fly. Both genotypes were observed in *Glossina pallidipes*. The tsetse fly positive for the *TvL1-G* genotype was collected from a fruit orchard three kilometres from the boundary of the Shimba Hills National Reserve in Msulwa A (Fig. 1 and Table 2. S4). Tsetse flies positive for the *Tvv4* were collectedly mostly in grasslands. However, some were also found in fruit orchards, shrubland and cultivated fields within two kilometres from the fence of the Shimba Hills National Reserve in Pengo, Kizibe, and Mlafyeni (Fig.1).

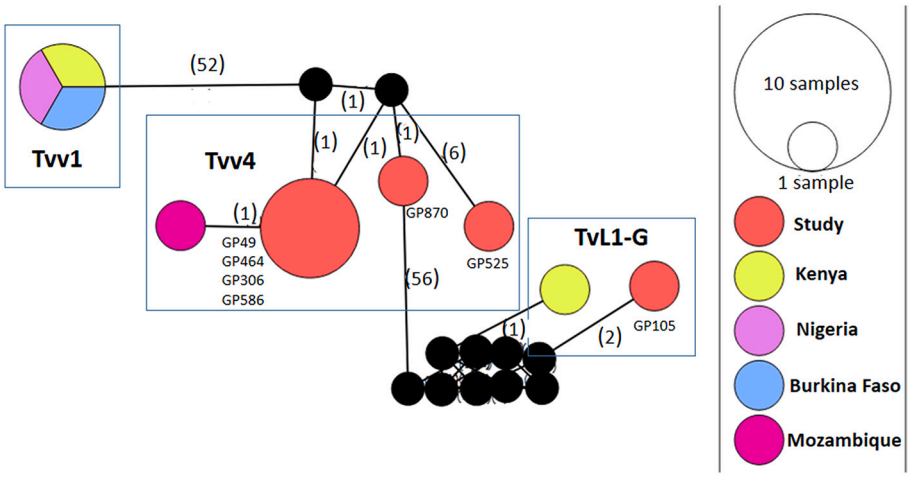

Fig. 4. Median-joining haplotype network of *T. vivax* isolates from Shimba Hills and different African countries, including Kenya, based on the ITS-1 gene sequences. Unique colours denote the haplotypes according to the location of origin. The black circles are median vectors (i.e. hypothetical haplotypes). Circle sizes are proportional to the haplotype frequencies. The number in parentheses on branches indicates the number of mutation changes segregating haplotypes. **TvL1-G**: Study (Shimba Hills) (GP105) and Kenya (GenBank Accession No.: DQ316041); **Tvv1**: Nigeria (GenBank Accession No.: U22316), Burkina Faso (GenBank Accession No.: JX910379) and Kenya (GenBank Accession No.: MH247149); **Tvv4**: Mozambique (GenBank Accession No.: EU482078) and Study (Shimba Hills) (GP49, GP306, GP464, GP525, GP586, and GP870).

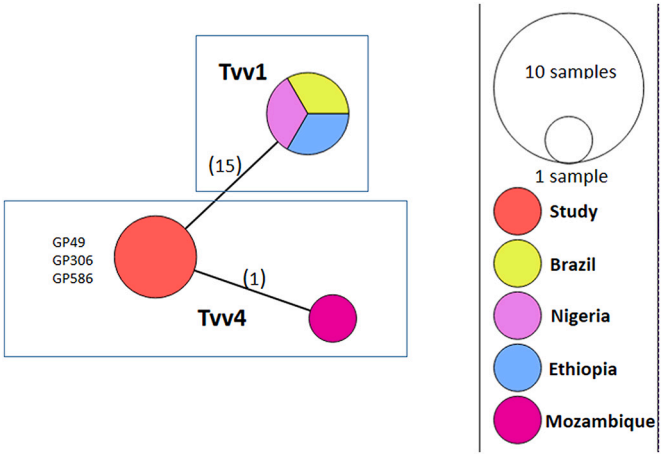

Fig. 5. Median-joining haplotype network of *T. vivax* isolates from Shimba Hills and different African countries including Kenya, and from Brazil in South America based on the 18S *rRNA* gene sequences. The haplotypes are denoted by unique colours according to the location of origin. The black circles are median vectors (i.e. hypothetical haplotypes). Circle sizes are proportional to the haplotype frequencies. The number in parentheses on branches indicates the number of mutation changes segregating haplotypes. **Tvv1**: Nigeria (GenBank Accession No.: U22316), Brazil (GenBank Accession No.: MN966728), and Ethiopia (GenBank Accession No.: KM391827); **Tvv4**: Study (GP49, GP306 and GP586) and Mozambique (GenBank Accession No.: EU477537).

4. Discussion

Our study in Shimba Hills, Kenya, characterized *T. vivax* infections in tsetse flies, a major vector for trypanosomiasis in the area. Importantly, the study provided valuable insights into the parasite diversity in Shimba Hills and reported the presence of two *T. vivax* genotypes, one of which is here reported for the first time in Kenya. Using molecular markers common to previous studies, it was possible to evaluate this diversity in a regional and global context.

Trypanosoma vivax is ubiquitous in sub-Saharan Africa: The pathogen occurs in wildernesses and livestock-producing areas where tsetse flies and biting flies such as *Stomoxys* sp. and tabanids are present and act as vectors. Our detection of *T. vivax* in tsetse flies from Shimba Hills shows that the area provides suitable conditions to sustain the parasite. That we detected infections in tsetse flies at a rate higher than reported in some similar ecologies such as the Msubugwe and Tarangire conservation areas in Tanzania adds evidence to this (Adams et al., 2010). The higher rate of *T. vivax* in Shimba Hills than in Msubugwe and Tarangire could also have been because investigators in Msubugwe and Tarangire used the relatively less sensitive microscopy methods. In contrast, we used sensitive molecular tools to screen tsetse flies for trypanosomes. Nonetheless, our results re-affirm *T. vivax* endemicity in Shimba Hills (Channumsin et al., 2018), with potential for transmission to cattle in smallholder agropastoral communities along the wildlife-livestock interface where we collected the tsetse flies.

The two genotypes *Tvv4* and *TvL1-G* described in Shimba Hills comprised of four haplotypes, three haplotypes for *Tvv4* and a single haplotype for *TvL1-G*. Haplotypes for the *Tvv4* genotype had eight within-genotype polymorphic sites but 65 polymorphic sites when considered together with the single haplotype for the *TvL1-G* genotype. This reveals a wide genetic variation between both genotypes in Shimba Hills. It also shows the close genetic relationship existing among all the three haplotypes for the *Tvv4* genotype and support the haplotypes' assignment into a single phylogenetic clade named by Rodrigues et al. (2017) as *Tvv4*.

Trypanosomes belonging to the *Tvv4* genotype clade were originally isolated from a wild-caught nyala antelope in the Sofala Province of Mozambique (Rodrigues et al., 2008). Subsequent studies in wildernesses in Mozambique detected the *Tvv4* trypanosome infections in cattle and tsetse flies (Rodrigues et al., 2017). Nakamura et al. (2021) also recently detected the *Tvv4* (reported as TviCatL7 based on the Cathepsin L-like cysteine protease gene sequence) in cattle in Kafue, Zambia's largest and oldest National Park. These results and those from our study suggest that the *Tvv4* genotype is widely distributed over East to Southern Africa.

The study by Nakamura et al. (2021)) reported anaemia in cattle positive for different *T. vivax* genotypes, including the TviCatL7 (*Tvv4*). The *Tvv4,* following its first detection in Nyala, was inoculated into a goat (Rodrigues et al., 2008). The goat developed a range of pathological conditions –severe anaemia, high parasitaemia, fever, and emaciation. The findings by Rodrigues et al. (2008) showed that the *Tvv4* is highly virulent in livestock hence may be a major cause for pathology in cattle in Shimba Hills especially given the high rates of *T. vivax* infections in tsetse flies and livestock in Shimba Hills (*unpublished*) and the *Tvv4* dominance among isolates in our study. Additionally, a high proportion of *Glossina pallidipes* feeds are from bovids (including cattle) (Weitz, 1963) and the *Tvv4*-infected fly individuals were mostly collected from grasslands where cattle grazing activities are ongoing.

All the *G. pallidipes* found positive for the *Tvv4* trypanosome were collected within the two-kilometre interface area outside of the Shimba Hills National Reserve. This closeness to wildlife suggests that wildlife could potentially be the reservoir and source of infection especially since the *Tvv4* pathogens has to date only been detected in wilderness areas (Rodrigues et al., 2008; Rodrigues et al., 2017; Adams et al., 2010). *Tvv4* detection in wildlife, cattle, and tsetse flies in Mozambique (Rodrigues et al., 2017) adds evidence that tsetse flies, after acquiring the parasite from wildlife, are likely to transmit infections to livestock. In a separate unpublished study that analyzed the blood meal sources of tsetse flies, we found evidence of ongoing trypanosome transmission between wildlife and livestock in Shimba Hills and showed that individual tsetse

Table 2
Details about tsetse flies from which *T. vivax* isolates were recovered.

Sample ID	Genotype	Block	latitude	longitude	Fly species	Sex	Fly collection date	Vegetation landscape	Elevation (m)	Distance from the SHNR (m)
GP464	*Tvv4*	Pengo	−4.24723	39.36326	*G. pallidipes*	M	February 2019	Grassland	178.00	775.00
GP49	*Tvv4*	Kizibe	−4.27402	39.30951	*G. pallidipes*	F	December 2018	Grassland	181.50	557.08
GP586	*Tvv4*	Kizibe	−4.2715603	39.33925	*G. pallidipes*	M	April 2019	Grassland	178.10	228.81
GP105	TvL1-G	Msulwa A	−4.26385	39.46818	*G. pallidipes*	F	June 2019	Fruit orchard	126.00	2292.57
GP306	*Tvv4*	Kizibe	−4.28925	39.27263	*G. pallidipes*	M	September 2019	Fruit orchard	170.30	0.00
GP870	*Tvv4*	Mlafyeni	−4.1903429	39.3789	*G. pallidipes*	F	June 2019	Grassland	121.60	243.00
GP525	*Tvv4*	Mlafyeni	−4.21615	39.39431	*G. pallidipes*	F	February 2019	Cultivated field	171.00	149.48
GP599	*Tvv4*	Pengo	−4.2427876	39.37366	*G. pallidipes*	F	April 2019	Shrub land	206.00	117.70
GP788	*Tvv4*	Mlafyeni	−4.1903355	39.37814	*G. pallidipes*	F	December 2018	Cultivated field	122.80	1679.37

M: male. F: female.
*Elevation: metre above sea level.
SHNR: Shimba Hills National Reserve.

flies with *T. vivax* isolates GP49 and GP586 had respectively fed on warthog and cattle. This finding further strengthens the argument of wildlife as reservoirs for the genotype and with the possibility of transmission to livestock.

The *TvL1-G* genotype comprised of the *T. vivax IL3905* strain from cattle in Kenya. The genotype infectivity to cattle (Nakamura et al., 2021) demonstrates its epidemiological importance and its detection in our study, portraying it as a potential threat to livestock production along the wildlife interface in Shimba Hills. However, the characterization of the *TvL1-G* genotype infection in Shimba Hills was based on a single tsetse fly, suggesting this genotype may be less common than the *Tvv4* genotype. The TS06009 isolate that also clustered within the *TvL1-G* clade was from buffalo in the Serengeti National Park in Tanzania (Auty et al., 2012). This lends support to the likely interplay between wildlife and livestock in the circulation of *T. vivax*. Wildlife may be playing a pivotal role as a source of infections to livestock in the Shimba Hills area (Channumsin et al., 2019), similar to what has been documented in the Luangwa Valley of Zambia (Anderson et al., 2011) among other wildernesses in sub-Saharan Africa (Fetene et al., 2021).

Sequences of *T. vivax* isolates from the tsetse-free camel-keeping areas in the northern region of Kenya segregated into an entirely different *Tvv1* genotype clade, thus bringing the total number of *T. vivax* genotypes identified based on ITS-1 sequences studied in Kenya to three (*Tvv4, TvL1-G,* and *Tvv1*). These isolates were supported in the same clade with those from cattle in Nigeria and Burkina Faso. A similar observation has been made in Ethiopia, where *T. vivax* isolates from a tsetse-free region clustered with West African isolates (Fikru et al., 2016). Thus, our study re-affirms the genetic relatedness of *T. vivax* isolates from East and West Africa (Fikru et al., 2016).

A major limitation in our study was that tsetse flies from within the Shimba Hills National Reserve were not included in our sampling. Findings from wilderness areas in Mozambique (Rodrigues et al., 2008; Rodrigues et al., 2017), Kenya (Majiwa et al., 1993), Tanzania (Malele et al., 2003), Central Africa Republic (Votýpka et al., 2015), and recently Zambia (Nakamura et al., 2021) uncovered novel genotypes of trypanosomes, unveiling the potential of sylvatic ecologies to host a wide range of genotypes. Our finding in the Shimba Hills wildlife-livestock interface of the *Tvv4* genotype in Kenya is further evidence of this. Thus, we may expect that analysis of tsetse flies from within the Shimba Hills Wildlife Reserve will in subsequent studies, uncover additional genotypes of *T. vivax*. Such studies should in addition to the ITS-1 DNA and 18S *rRNA* in the current work consider targeting other gene markers for example the glyceraldehyde 3-phosphate dehydrogenase and Cathepsin L-like cysteine protease genes. Consideration of isoenzymes will also be important as it will allow for comparisons with the findings by Fasogbon et al (1990) in coastal Kenya of a *T. vivax* stock (MID 627) with unique enzyme banding patterns distinct from those of West and other East African stocks.

5. Conclusion

This is the first report of the *Tvv4* genotype of *T. vivax* in Kenya to the best of our knowledge. The *Tvv4* genotype is dominant in Shimba Hills, a wilderness area where livestock experience severe pathological conditions due to animal trypanosomiasis. The genotypes of *T. vivax* in Shimba Hills may vary in key phenotypes, such as response to available trypanocidal drugs. Since there are currently no clear guidelines for treating *Tvv4* genotype infections with trypanocides, we recommend vector control particularly targeting *G. pallidipes* within 2-3kms of the SHNR boundary to prevent *T. vivax* infections in the Shimba Hills area. More studies will be required to further describe the diversity and pathogenicity of *T. vivax* in Shimba Hills, and the role of wildlife species in the transmission thereof.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104953.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article. The dataset used and/or analyzed during the current study are available from the corresponding author FIE on reasonable request. Trypanosome sequences generated during the current study are available in the GenBank.

Ethics approval

The study was conducted in collaboration with the Kenya Wildlife Service and the Kenya Tsetse and Trypanosomiasis Eradication Council. Local communities gave oral consent for the deployment of biconical traps for tsetse fly collection.

Consent for publication

Not applicable.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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