Molecular characterization of pathogenic African trypanosomes in biting flies and camels in surra-endemic areas outside the tsetse fly belt in Kenya

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Abstract

African animal trypanosomosis (nagana) is becoming prevalent beyond its traditionally defined geographical boundaries in African tsetse belts. However, knowledge of clinically important trypanosomes and infection rate in non-tsetse hematophagous flies and domestic animals are limited. This study characterized the potential mechanical vectors, their host feeding patterns, and trypanosome infection in them and domestic animals outside the tsetse belt in northern Kenya. Field-trapped flies and blood from camels, cattle, donkeys, goats, and sheep were screened for trypanosome infection by microscopy and polymerase chain reaction (PCR) of the internal transcribed spacer 1 region. Blood-fed specimens were analysed using PCR-HRM and/or sequencing of 16S rRNA gene to identify vertebrate blood-meal host sources. *Hippobosca camelina*, *Stomoxys calcitrans, Tabanus* spp., and *Pangonia rueppellii* were identified as potential vectors of trypanosomes outside the tsetse belt in Marsabit County. The trypanosome species, *Trypanosoma vivax*, *T. evansi*, *T. brucei*, and *T. congolense* were recovered in biting flies as well as in camels (*Camelus dromedarius*). The diversity of parasites in the biting flies was similar to that detected in the tsetse fly *Glossina pallidipes* collected from the tsetse-infested Shimba Hills, in coastal Kenya, suggesting a wide geographic distribution of the trypanosomes in Kenya. The biting flies fed on camels, cattle, goats, and sheep. Furthermore, we identified diverse clinical outcomes based on PCV (anemia), heamorrhagia) associated with infection with disparate *Trypanosoma* species. Thus, infection of flies and camels by diverse *Trypanosoma* species could contribute to the complex epidemiology of observed trypanosomosis in camels.

Keywords: Surra; Camel; Non-tsetse transmitted trypanosomes; Biting flies; Tsetse belt; Kenya

Abbreviations

HRM: High-resolution melting

icipe : International Centre of Insect Physiology and Ecology

ITS1: Intergenic transcribed spacer subunit 1

LNA: Lymph node aspirate

PCV: Packed Cell Volume

PCR: Polymerase chain reaction.

Introduction

Trypanosoma evansi is one of the most important *Trypanosoma* spp. infecting livestock globally. Its wide geographic distribution (Hoare 1972; Auty et al. 2015; Aregawi et al. 2019), mode of transmission (Desquesnes et al. 2013; Kamidi et al. 2017), zoonotic potential (Joshi et al. 2005; Shah et al. 2011), pathogenicity to several domestic animals (Misra et al. 1976), high genetic diversity, and variation in virulence (Kamidi et al. 2017, 2018; Kimenyi et al. 2021) make it an important parasite. Animal trypanosomosis caused by *T. evansi* is called surra in camels and is the most lethal disease of camels worldwide (Njiru et al. 2004a, b; Kassa et al. 2011; Desquesnes et al. 2013). In addition to causing camel mortality, *T. evansi* infections reduce production of milk, an important staple food and the primary source of protein for pastoralists. Furthermore, *T. evansi* is an important pathogen in cattle and buffalo that results in morbidity and mortality and induces high rates of abortion in domestic animals as reported in Asia (Jittapalapong et al. 2009; Kassa et al. 2011).

Basic knowledge of the epidemiology and diversity of clinically important trypanosomes (such as *Trypanosoma vivax*, *T. congolense*, and *T. evansi*) in non-tsetse hematophagous flies and domestic animals from tsetse-free areas is significantly outweighed by that of tsetse and trypanosomes studied in tsetse-infested areas. However, several laboratory and semi-field experiments have demonstrated that different *Trypanosoma* spp., including *T. congolense* (Desquesnes and Dia 2003), *T. vivax* (Gardiner 1989), and *T. evansi* (Oyieke and Reid 2003), are potentially transmitted to domestic animals by various biting flies, such as *Stomoxys* spp. and *Tabanus* spp.

To determine the presence of various *Trypanosoma* spp. and the potential role of non-tsetse biting flies in their transmission within a tsetse-free area of northern Kenya, we investigated: (i) the diversity of hematophagous biting flies that could be involved in the mechanical transmission of trypanosomes; (ii) the identity, and diversity of economically and clinically important pathogenic trypanosomes in randomly collected flies and domestic animals. Furthermore, we identified (iii) the blood-meal sources of the trapped biting flies to identify potential reservoir hosts, and (iv) assessed clinical symptoms associated with camels infected with different trypanosomes.

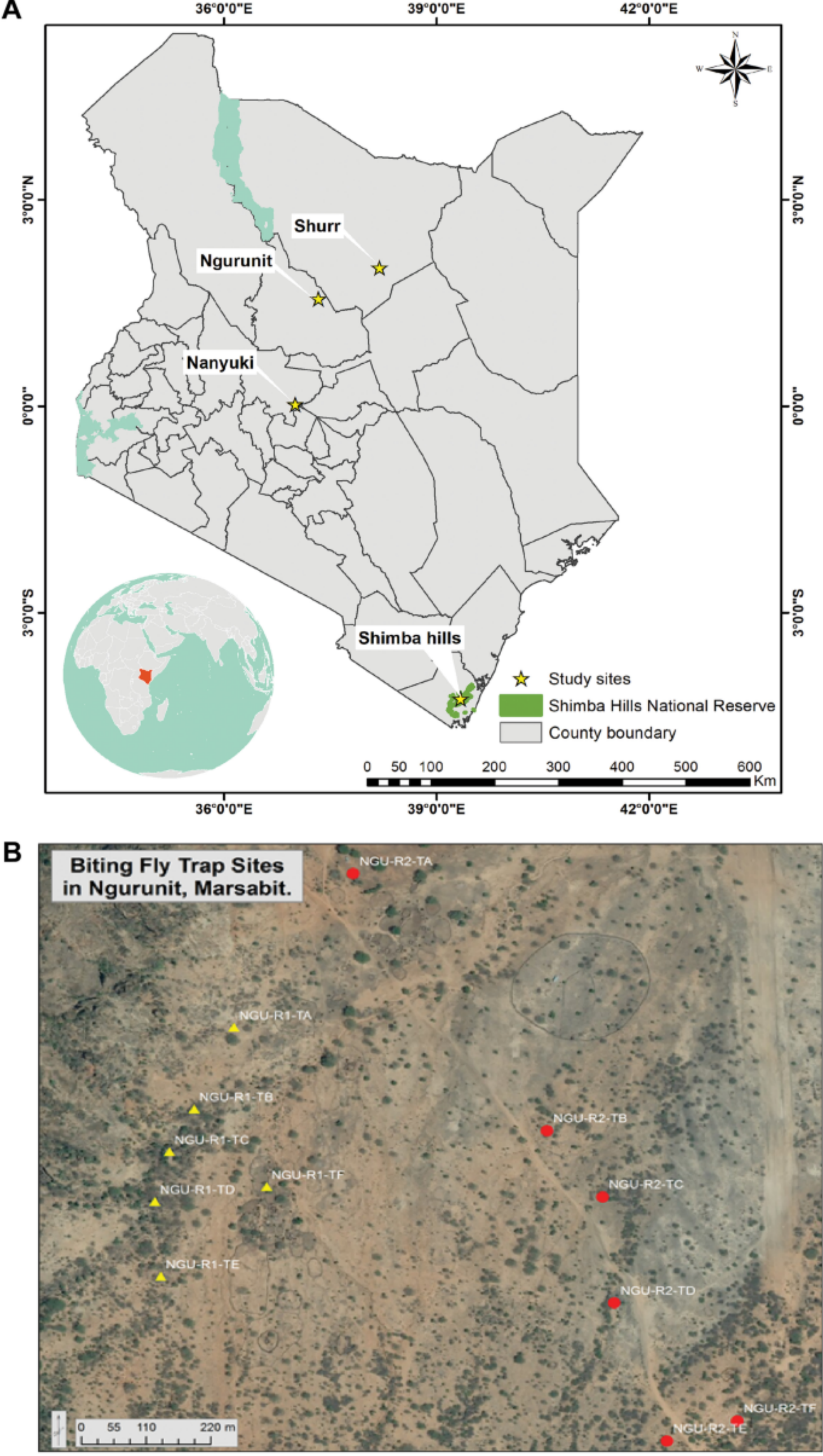

Fig. 1. (**A**) Map of Kenya showing the study sites (**B**) biting flies trap sites in Ngurunit village in Marsabit County. Yellow triangles represent fly trapping in a forested area, and red circles, trapping in a sparsely vegetated area in Ngurunit

Materials and methods

Study sites

The study was conducted in three sites. These included two sites in Marsabit County, northern Kenya, namely Ngurunit (N01°0.74’, E 037.29’) at the edge of the tsetse distribution map and Shurr, a tsetse free area (N02°0.08’, E038°0.27’). Nanyuki in central Kenya represented the third site which is also a tsetse free area (N00°0.41’, E036°0.90’) (Fig. 1A). All the three study sites are characterized by semi-arid to arid climatic conditions. The main means of communities’ livelihood is animal husbandry. The areas have suitable biomass, especially for browsers such as goats and camels. Ngurunit falls within the tsetse distribution map, although a previous study found no tsetse flies in the area (Oyieke and Reid 2003). Marsabit County has high density of camels, 1.3 – 1.9 camels/km^2 (Corman et al. 2014). Tsetse flies (*G. pallidipes*) were collected from Shimba Hills in Kwale County, coastal Kenya for *Trypanosoma* spp comparisons.

Trapping of biting flies

Flies were trapped using monoconical traps (Laveissière and Grèbaut 1990), placed ~ 150 m apart. Camel urine odour dispensed from plastic bottles (release rate not quantified) was used as an attractant. Biting flies feeding on camels were collected using sweep nets and preserved in absolute ethanol and identified using standard published keys (Kirk-Spriggs and Sinclair 2017). Camels from which biting flies were collected were randomly selected regardless of their sex, age, or health status. The flies were trapped during both dry, November 2017 and rainy seasons, May 2018 using 25 monoconical traps daily per site for five consecutive days at each season, and emptied every 24 h. The 25 traps were randomly distributed in thick bushland, *Acacia* spp. woodlands, watering points, animal enclosures, and open grassland areas (Fig. 1B). The traps had fixed positions, as we were interested in presence of biting flies.

Fly density per camel

Hippobosca camelina and *Stomoxys calcitrans* counts were made by approaching ten randomly selected camels slowly from the side. The number of *S. calcitrans* and *H. camelina* was made by counting the total number of flies on the legs and belly of each camel. Counts were made from 0.5–1 m by observation with naked eyes independently by two experienced technicians with expertise in distinguishing between the target flies. Stable flies could be differentiated morphologically from house flies by their distinctive feeding posture on camel’s body (Mullens et al. 2006).

Blood sampling and microscopy

Approximately 5–10 mL of blood was drawn from the jugular vein of camels, goats, sheep, donkeys, and cattle into vacutainer tubes containing disodium salt of ethylene diamine tetra-acetate (EDTA) (Plymouth PLG, UK). An aliquot from each vacutainer tube was transferred into heparinized capillary tubes (75 × 1.5 mm) and spun in a micro-haematocrit centrifuge at 12,000 rpm for 5 min to separate the red and white blood cells and plasma, hence concentrating the trypanosomes (Murray et al. 1977). Packed cell volume (PCV), an indicator of the animal’s anaemic status, was measured using Haematocrit Reader (Hawksley & Sons Limited, England) and expressed as a percentage of PCV to total blood volume. The buffy coat plasma interface

was placed onto a microscope glass slide and examined under Zeiss compound microscope for the presence of moving trypanosomes. The trypanosome species were provisionally identified based on cell motility and morphology using wet blood film examination (Murray et al. 1977; Bargul et al. 2016). Furthermore, thin blood smears were prepared from the samples, fixed with methanol, and stained with 10% Giemsa (Murray et al. 1977). The stain was flushed with running tap water and allowed to dry for 35 min. Slides were then examined under the 100 × oil immersion objective for trypanosomes and positive cases recorded. The rest of the well-mixed blood contents were appropriately labelled and stored in liquid nitrogen for transportation to Nairobi-based *icipe* laboratories for further screening. Domestic animals (including camels) were randomly sampled from various herds, for up to a maximum of 10% of the herd population to accommodate sampling of more herds. The mean number of camels per household varied from four to 35 on average in Ngurunit and Shurr sites, respectively. However, in Nanyuki, with relatively smaller population of camels, only one herd was sampled. All the identified infected camels were treated with triquin (Vetoquinol®) at a dose of 5 mg/kg body weight (Njiru et al. 2004a, b).

To obtain lymphnode aspirate, a sterile needle of gauge 18 was used to pierce the precapular swollen lymph node and lymph fluid was aspired. The lymph node fluid was used to check for the presence of trypanosomes using microscopy and PCR.

DNA extraction from blood and biting flies

Genomic DNA was extracted from blood samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), as per the manufacturer's recommendations. The freshly eluted DNA samples were stored at -20 °C until PCR analysis. Similarly, the DNA from the crushed guts of biting flies were extracted and purified after brief surface sterilisation with 70% ethanol and rinsed twice with distilled water. A negative extraction control, comprising extraction buffer was also performed for contamination assessment.

Identification of trypanosomes in the blood and fly samples

A random subset of the biting flies by species were screened for trypanosomes by PCR. To check trypanosomes in blood we combined light microscopy with more sensitive DNA-based approaches (Masiga et al. 1992) that enabled differentiation of trypanosome species and their subgroups. We employed PCRs targeting a portion of the internal transcribed spacer (ITS-1) which is conserved across all African trypanosomes (Desquesnes et al. 2001). PCR assays were carried out in 10-µL reaction volume containing 5 µL 2 × DreamTaq mix, 3 µL PCR water, 0.5 µL ITS-1 primers (F: 5'-CCGGAAGTTCACCGATATTG-3'; R: 5'-TTGCTGCGTTCTTCAACGAA-3') (Njiru et al. 2005) and 1 µL DNA template. PCR amplification conditions were programmed as follows: 95 °C denaturation step for 1 min, 35 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min and final extension of 72 °C for 10 min. Additionally, primers designed to amplify kinetoplast 9S ribosomal RNA subunit (kDNA 12 (modified): 5′-TTAATGCTATTAGATGGGTGTGG-3′; kDNA 13: 5′-CTCTCTGGTTCTCTGGGAAATCAA-3′) (Masiga et al. 2006) and Cytochrome c oxidase 1 (*CO1)* (Tb_kDNA_COI_Max1: 5'-CCCTACAACAGCACCAAGT-3'; Tb_kDNA_COI_Max2: 5'-TTCACATGGGTTGATTATGG-3') (Balmer et al. 2011) genes were used to differentiate *T. brucei* from *T. evansi* as previously described. To identify the *T. evansi* subtypes A and B, we used type A-specific primers targeting the VDG RoTat 1.2 gene (F: 5′-GCGGGGTGTTTAAAGCAATA-3′; R: 5′-ATTAGTGCTGCGTGTGTTCG-3′) and type B-specific primers targeting the minicircle gene (EVAB-1: 5′-

ACAGTCCGAGAGATAGAG-3′; EVAB-2: 5′-CTGTACTCTACATCTACCTC-3′) (Birhanu et al. 2016; Ngaira et al. 2005). For each PCR, a negative PCR control (non-template control) was set up alongside the samples (SF1). PCR products were resolved through 1% ethidium bromide-stained agarose gel at 80 V for 1.5 h and visualized using a gel documentation system.

The PCR amplicons were purified using Quickclean II gel extraction kit (GeneScript USA Inc., Piscataway, USA) according to the manufacturer's instructions. Confirmation of the purified DNA was performed by gel electrophoresis followed by both forward and reverse sequencing of ITS1 marker at Macrogen Inc. (Amsterdam, Netherlands).

All the obtained nucleotide sequences were edited and aligned using the MAFFT plugin in Geneious software version 11.1.4 (Kearse et al. 2012). Sequence identities were revealed by querying in the GenBank database using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST/). The aligned ITS-1 sequences were used to construct a maximum likelihood phylogenetic tree using PHYML v. 3.0 (Guindon et al. 2010). The phylogeny employed the Akaike information criterion (Lefort et al. 2017) for automatic model selection and tree topologies were estimated using nearest neighbor interchange (NNI) improvements over 1,000 bootstrap replicates. The phylogenetic tree was visualized using FigTree v1.4.2 (Drummond and Rambaut 2007).

Bloodmeal source identification in biting flies by PCR – High-resolution melting (HRM) analysis

Genomic DNA from individually blood-fed *H. camelina* and *S. calcitrans* and known vertebrate whole blood samples (positive controls) were isolated using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. High-resolution melt profiles from PCR amplicons of different vertebrate 16S rRNA DNA in 100 *H. camelina* were analysed in Applied Biosystems QuantStudio 3 real-time PCR system (Thermo Scientific, USA) and used to identify the various vertebrate bloodmeal sources as previously described (Omondi et al. 2015; Peña et al. 2012). Blood-meal profiles of vertebrates were specifically matched to selected domestic and wild animal positive controls. Vertebrate host samples including human (*Homo sapiens*), cow (*Bos taurus*), sheep (*Ovis aries*), warthog (*Phacochoerus africanus*), African buffalo (*Syncerus caffer*), goat (*Capra aegagrus hircus*), elephant (*Loxodonta africana*), Sprague Dawley rat (*Rattus norvegicus*), and camel (*Camelus dromedarius*) served as controls. The 10-µL PCRs reaction consisted of 1 µL DNA template, 6 µL of PCR water, and 2 µL of 5 × HOT FIREpol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia) and 0.5 µM concentrations of each primer (Omondi et al. 2015; Peña et al. 2012). The PCR thermal cycling conditions for cytochrome b were set as follows: initial denaturation 95 °C for 15 min, 35 cycles of denaturation at 95 °C for 30 s, annealing 58 °C for 20 s, extension 72 °C for 30 s and final extension of 72 °C for 7 min with final PCR products kept at 4 °C. The annealing temperature of the 16S rRNA gene was 56 °C. Following PCR amplification, HRM analysis was performed within normalised temperature regions of between 65 °C−78 °C and 88 °C−95 °C. The different melt curve profiles of the samples were compared to the reference standards, and representative samples under each peak were selected for gene sequencing.

Data analysis

Biting fly densities on camels were compared using the Mann–Whitney test as the data was not normally distributed following normality test according to Levene's test of homogeneity of

variance. We used the following formula developed by Cameron and Baldock (1998): $n = \ln(\alpha)/\ln(1-p)$ to determine the minimum number of camels and biting flies to be sampled for trypanosomes detection. We used sensitive molecular tools for pathogen detection, and we assumed that 3% of field collected flies and camels were infected at 95% confidence limit, thus, $\alpha = 0.05$, $p = 0.03$ (probability of detecting infected biting flies, camel).

Chi-squared tests were used to compare differences in the number of trypanosome infection among biting fly species, and the different domestic animals. The independent t-test was used to compare PCV values between infected and non-infected camels. The proportion of blood-meal from various animals they fed on was compared using the chi-squared test. All analyses were performed using GraphPad software (GraphPad Software, Inc, USA). The Shannon diversity index (H) was used to characterise the diversity of *Trypanosome* spp. in biting flies using percent prevalence data and the diversity index of biting flies between sites was analyzed using number of individuals per trap calculated using PAST 3.11 (www.folk.uio.no/ohammer/past/) (Hammer et al. 2001). The relative feeding index of *H. camelina* and *S. calcitrans* was calculated according to Kay et al. (1979), and Russel et al. (2016) as follows; $W_i = O_i/P_i$, where W_i = feeding ratio for livestock i, O_i = percentage of livestock, I, in the blood meals, P_i = proportion or percentage of livestock i available in the environment. We calculated the relative feeding preference of *H. camelina* to camel against sheep, the most abundant livestock per household in Shurr.

Results

Diverse biting flies identified as potential trypanosome vectors in the tsetse-free area

We collected a variety of biting flies of the order Diptera from the study areas using monoconical traps (Table 1). Similar hematophagous flies were observed feeding on camels, including *H. camelina* (Leach), *S. calcitrans* (L), *Pangonia rueppellii* (Jaenn), *Haematopota pluvialis*, and *Tabanus* spp. (Fig. 2A–F). *Hippobosca camelina* and *S. calcitrans* were present all year-round and observed feeding together on the same camel. The abundance of biting flies on a given camel varied between biting fly species. For instance, more *H. camelina* flies per camel (n = 10) were recorded as compared to *S. calcitrans* ($P < 0.005$, Mann–Whitney Test) (Fig. 2G). However, the number of other biting flies on camels (*P. rueppellii, Tabanus* spp., and *H. pluvialis*) were too low to allow for meaningful comparisons.

Table 1 Diversity of biting flies at three sites

Site	*H. camelina*	*S. calcitrans*	*Tabanus* spp.	*P. rueppellii*	*Hae. pluvialis*	Shannon index (H)
Ngurunit	+	+	+	+	+	1.3
Shurr	+	+	+	-	+	0.6
Nanyuki	-	+	-	-	-	0

(+) indicates the specific biting fly was detected; (-) indicates not detected

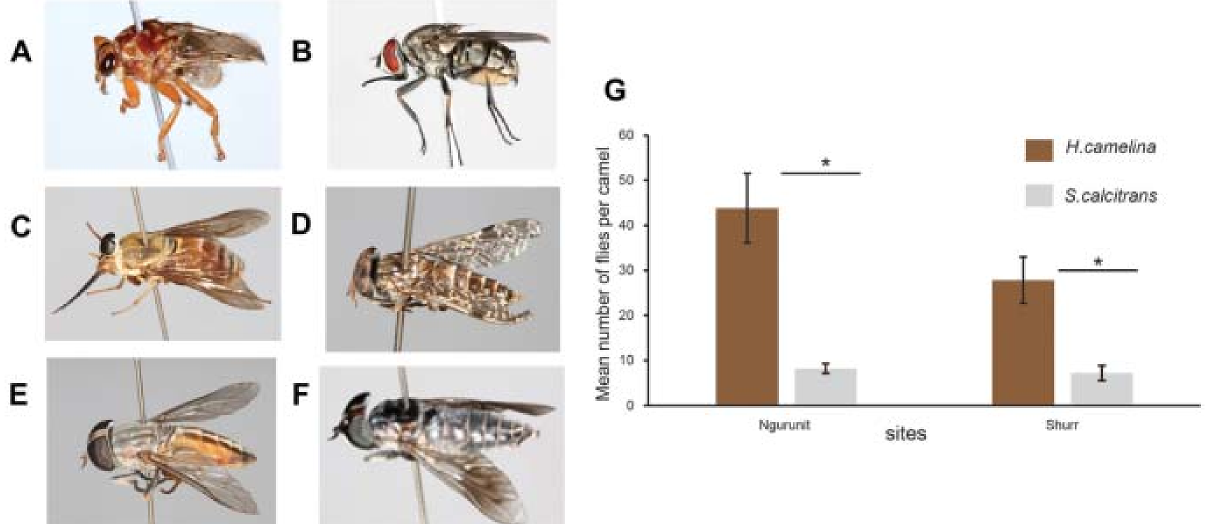

Fig. 2. Diversity and abundance of biting flies (**A**) *H. camelina*, (**B**) *S. calcitrans*, (**C**) *P. rueppellii*, (**D**) *H. pluvialis*, (**E**, **F**) *Tabanus* spp., (**G**) Mean number of *H. camelina* and *S. calcitrans* per camel at Ngurunit and Shurr sites, bars represent standard error of the mean, *depicts significant differences in fly density, $P < 0.05$, sample size (N) = 10

The diversity of biting flies varied between study sites; Ngurunit had diverse species of biting flies (Shannon index, $H = 1.3$), including *H. camelina, P. rueppellii*, *Hae. pluvialis, S. calcitrans* and *Tabanus*. Shurr (Shannon index, $H = 0.6$) had all the species of biting flies, except *P. rueppellii*, while Nanyuki (Shannon index, $H = 0$) had only *S. calcitrans.*

Trypanosoma spp. identified in biting flies from the tsetse-free area

Among all biting flies analysed (except *Tabanus* spp.), *T. vivax* was the most abundant trypanosome species, followed by *Trypanozoon* (Fig. 3B). No trypanosomes were identified in 50 *H. pluvialis* analysed (Fig. 2D).

Trypanosomes diversity in *H. camelina*

Out of the 150 *H. camelina* flies analysed from Shurr and Ngurunit (Fig. 2A), 3% were infected with *T. congolense* savannah, 39% had *T. vivax,* 9% had *Trypanozoon*, 6% had mixed infection with *T. vivax* and *Trypanozoon* DNA, 3% had mixed infection with *T. congolense* and *Trypanozoon* DNA, and 1% had DNA of all three species (*T. congolense*, *Trypanozoon*, and *T. vivax*) (Fig. 3A and B). *Trypanosoma vivax* was more common compared to *Trypanozoon* ($\chi^2 = 36.88$, df = 1, $P < 0.001$) and prevalence of *Trypanozoon* was significantly higher than *T. congolense* ($\chi^2 = 4.77$, $P = 0.03$). Occurrence of mixed parasite DNA was low (Fig. 3A–B).

The prevalence of trypanosomes in *H. camelina* collected from Ngurunit and Shurr in northern Kenya was variable. Out of the 150 flies analysed (75 flies from each site), 33% of flies from Ngurunit carried *T. vivax* (25/75); *Trypanozoon* was detected in 2.7% of flies (2/75), whereas about 5% of flies harboured *T. congolense* savannah (4/75). Forty-five percent (45%) of *H. camelina* from Shurr were positive for *T. vivax* (34/75) while 14.7% of the flies tested positive for *Trypanozoon* (11/75). Overall, a significantly higher proportion of *H. camelina* infected with trypanosome parasites was observed in Shurr than Ngurunit ($\chi^2 = 6.23$, $P = 0.013$).

However, *T. congolense* was not detected in flies sampled from Shurr flies. No *H. camelina* flies were found in Nanyuki (Table 1).

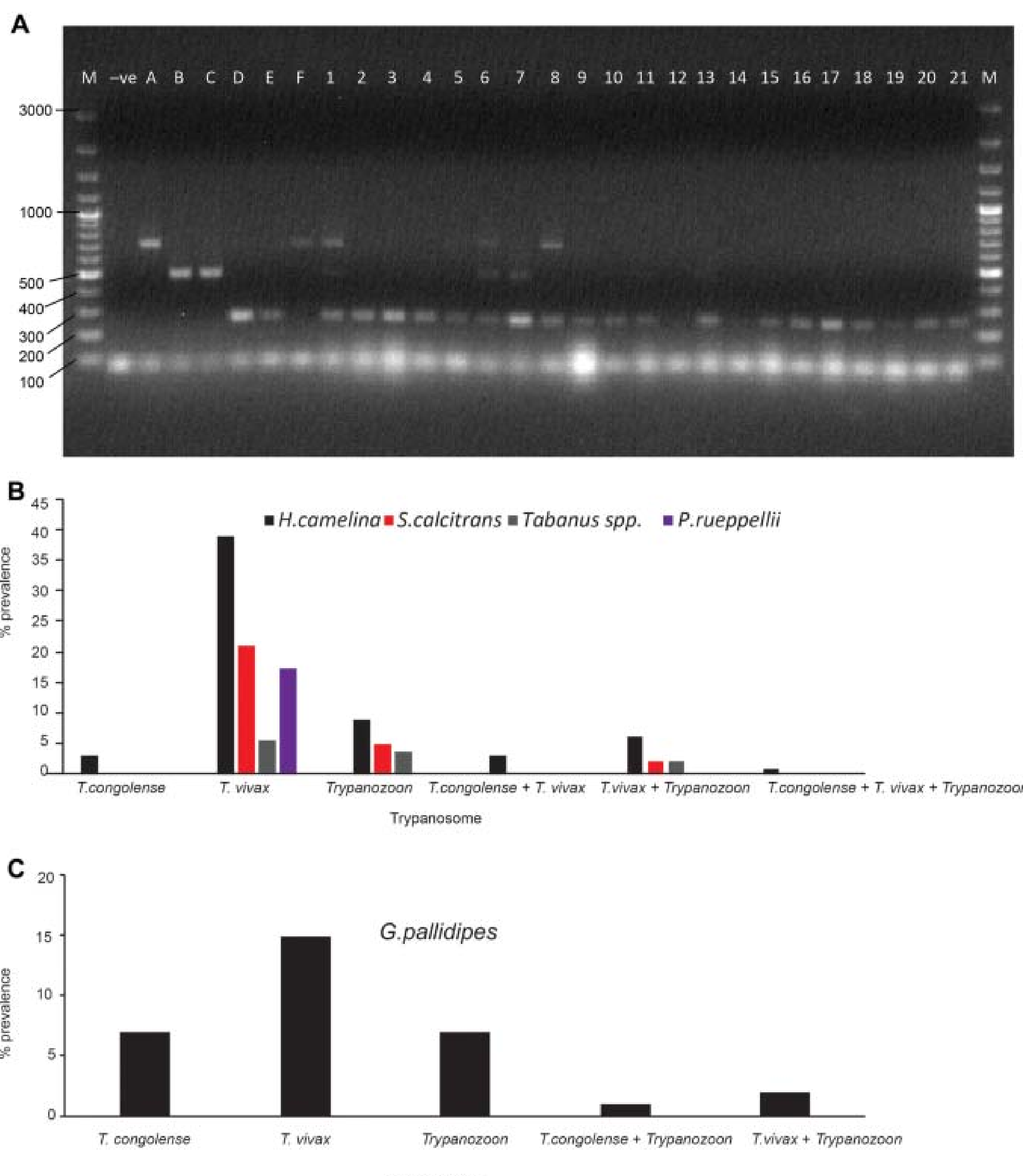

Fig. 3. Prevalence and diversity of trypanosomes in biting flies (**A**) A gel image of resolved trypanosome PCR products. Lane M 100-bp marker (Thermo Scientific, USA); Lane '-ve': negative control; Lane A – D contained positive controls, Lane A: *T. congolense* savannah (IL3000); Lane B: *T. brucei* ILTat 1.4; Lane C: *T. evansi* KETRI 2479; Lane D: *T. vivax* IL 2136; Lanes E and F: Trypanosome-infected camel blood from the same site as that of the flies; Lanes 1 – 21: selected infected *H. camelina* samples. (**B**) The prevalence of trypanosome species in biting flies. (**C**) The prevalence and diversity of trypanosomes detected from field collected *G. pallidipes* (n = 101)

Trypanosomes detected in *S. calcitrans*

Field-trapped *S. calcitrans* (n = 100), 50 from each of the two sampling sites in Marsabit (Fig. 2B), were analysed for the presence of trypanosomes. One fly from Ngurunit and four flies from Shurr were positive for *Trypanozoon* in total (5%) and in 21 flies, *T. vivax* DNA was detected, eight from Ngurunit and 13 from Shurr. Similarly, more *S. calcitrans* were positive with *T. vivax* as compared to *Trypanozoon* ($\chi^2 = 9.85$, $P = 0.002$). Approximately 2% of the *S. calcitrans* from Shurr were positive for mixed *Trypanozoon* and *T. vivax* DNA (Figs. 3B and 4).

Biting flies	*Trypanozoon*	*T. vivax*	*T. congolense*	Mixed DNA	Shannon diversity index	sample size
H. camelina	9%	39%	3%	10%	1.1	150
S. calcitrans	5%	21%	-	2%	0.7	100
P. rueppellii	-	17.4%	-	-	0.0	50
Tabanus sp.	3.6%	5.4%	-	2%	0.7	50
H. pluvialis	-	-	-	-	0.0	50
G. pallidipes	7%	15%	7%	2%	1.4	101

Fig. 4. Diversity and percent of trypanosome species in biting flies. (-) indicates negative for that specific trypanosome species

Trypanosomes detected in *Tabanus* spp.

Field trapped *Tabanus* spp. (Fig. 2E and F) harboured *T. vivax* DNA (5.4%; n = 50 flies) and *Trypanozoon* (3.6%; n = 50 flies), trypanosomes DNA detected were relatively lower in *Tabanus spp*. than in *H. camelina* and *S. calcitrans* (Figs. 3B and 4), and no significant difference was observed between the proportion of *T. vivax* and *Trypanozoon* DNA detected in the *Tabanus spp.* flies (χ^2, $P > 0.05$).

Trypanosomes detected in *P. rueppellii*

We also identified another potential trypanosome vector, *P. rueppellii* (Fig. 2C). Of the flies (n = 50) analysed, 17.4% harboured *T. vivax* DNA and no other species of trypanosome was detected (Figs. 3B and 4).

Trypanosomes detected in *G. pallidipes*

The trypanosomes diversity and prevalence were analysed in field-trapped trypanosome biological vector, *G. pallidipes*, from Shimba Hills in coastal Kenya (Fig. 1A). In 32% of 101 field-trapped *G. pallidipes*, DNA of various trypanosomes were detected, 7.14% of which were *T. congolense* savannah, 14.8% were *T. vivax*, and 6.9% *Trypanozoon*. However, no significant differences in prevalence were recorded for the three trypanosome species, namely *T. congolense*, *T. vivax*, and *Trypanozoon* (either *T. brucei* or *T. evansi*) ($P = 0.08$). Moreover, 1% *T. congolense* and *Trypaanozoon*, and 2% *T. vivax* and *Trypanozoon* mixed DNA were detected in the analysed *G. pallidipes* (Fig. 3C). The diverse non-tsetse hematophagous biting flies from the tsetse-free area harboured similar trypanosomes as *G. pallidipes* (Fig. 3C). The diversity of trypanosomes indicated by Shannon index H varied between the different biting flies; *H. camelina* and *G. pallidipes* harboured more diverse trypanosome DNA (Fig. 4).

Trypanosome prevalence and diversity in camels

Thin blood smears from the jugular vein using microscopic techniques showed an active infection of *Typanozoon* in 16 camels (7.2 ± 3.4%, mean ± standard error) of the sampled camels (n = 222). Trypanosomes were morphologically identified as belonging to *Trypanozoon* subgenus based on their morphology and motility. Trypanosome infections in camels varied in the parasite load, ranging from 10^6 to 5×10^8 trypanosomes/mL blood (Figs. 5A–B).

To confirm the morphological identification, molecular analysis of microscopically positive and negative samples was performed. Figure 5C shows selected samples that were microscopically positive (+) and negative. Morphologically, *Trypanozoon*-positive samples 25 (GenBank accession MH247163), 31 (GenBank accession MH247168), 42 (GenBank accession MH247174), and 47 (GenBank accession MH247157) were also *Trypanozoon* positive by PCR and sequencing (Fig. 5C). As depicted in Fig. 5C, six of the microscopically negative samples (26, 32, 37, 38, 41, and 45) remained negative by PCR. However, thirteen samples (27–30; 33, 34–36; 39, 40, 43, 44, 46) that were negative microscopically, were found to be *Trypanozoon* positive by PCR. A clear difference was observed in those microscopically positive and negative samples, with relatively sharp and intense PCR bands in those samples that were microscopically positive (+) as compared to those microscopically negative (Fig. 5C)). For further confirmation, six microscopically *Trypanozoon* positive (GenBank accessions MH247157, MH247162, MH247163, MH247168, MH247170, and MH247174) and 10 microscopically negative, but *Trypanozoon* positive by PCR (GenBank accessions MH247155, MH247158, MH247159, MH247160, MH247161, MH247166, MH247167, MH247169, MH247173 and MH247177) were sequenced.

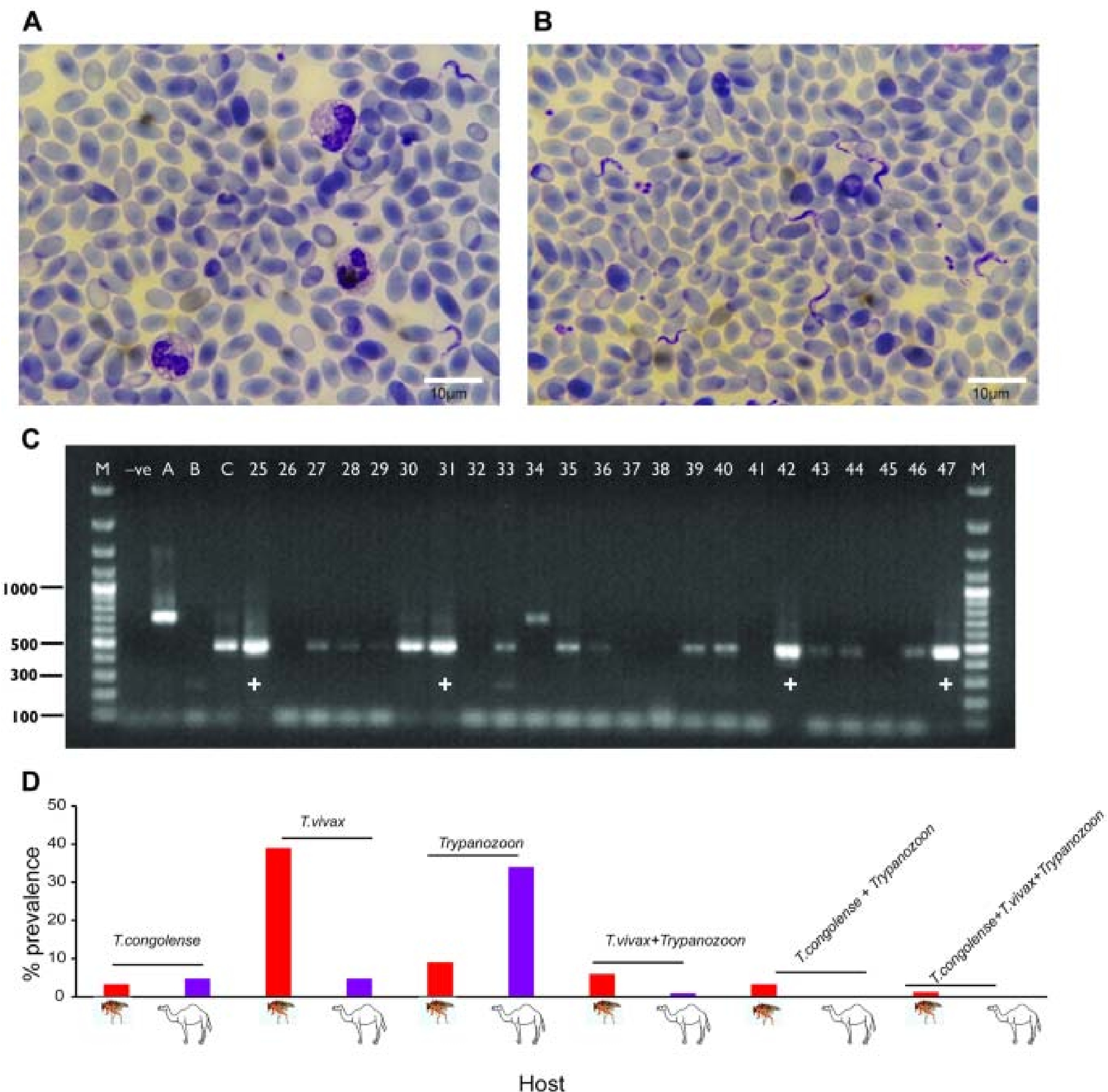

Fig. 5. Detection of trypanosomes in camels and *H. camelina*. (**A**, **B**) Representative light micrographs of Giemsa-stained camel blood sample smears from two different camels showing varying parasitemia (**A**) low and (**B**) high parasitemia, *T. evansi* (GenBank accessions MH247174) with a small sub-terminal kinetoplast at the pointed posterior end, a long free flagellum and a well-developed undulating membrane. (**C**) Agarose gel electrophoresis (1.2%) performed on ITS1 PCR amplicons. Lane M: 100-bp marker; Lane '-ve': negative control (non-template control); Lane A: *T. congolense* savannah IL3000; Lanes B: *T. vivax* IL2136; Lane C: *T. evansi* KETRI 2479; Lanes 25–47: selected camel blood samples, + shows camel that was trypanosome-positive by microscopy (D). Prevalence of trypanosome species in *H. camelina* and camels

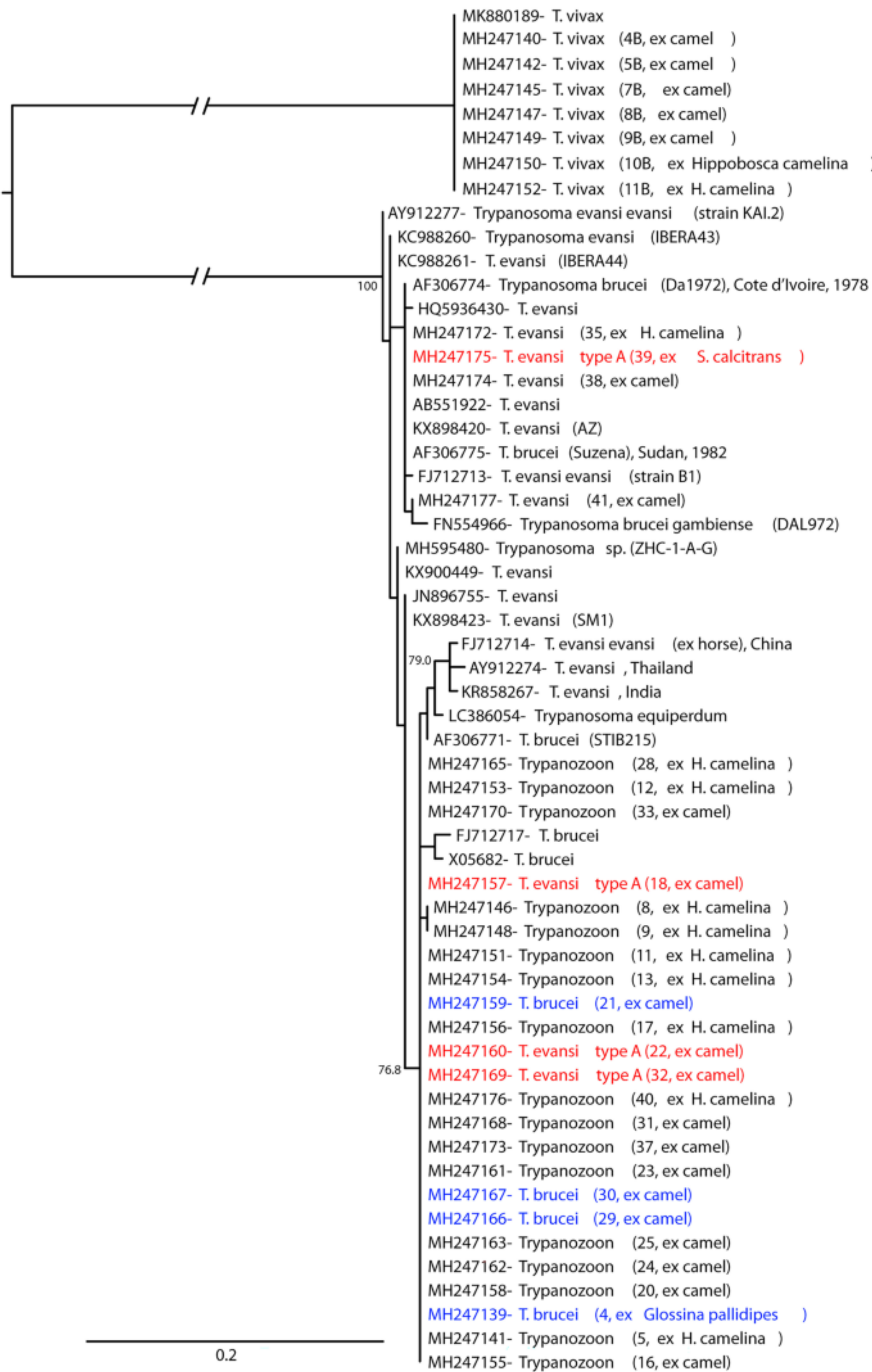

Fig. 6. Maximum likelihood phylogeny of trypanosome ITS-1 nucleotide sequences. GenBank accession numbers and isolation sources are indicated. Sequences from this study are indicated in bold; trypanosomes were isolated from *G. pallidipes*, *S. calcitrans*, *H. camelina*, and camels. Sequences associated with samples confirmed to harbour *T. evansi* type A by amplification of the VDG RoTat 1.2 gene are highlighted in red. Sequences associated with samples confirmed to harbour *T. brucei* by maxicircle kDNA amplification are highlighted in blue. Bootstrap values at the major nodes represent agreement among 1000 replicates. The branch length scale represents substitutions per site. Branch gaps in the mid-point root branches represent 2.7 substitutions per site

PCR-based trypanosome characterization in camels

Trypanosomal ITS-1 PCR showed 34% (75/222) of the camels were infected with *Trypanozoon* at a significantly higher prevalence than *T. vivax* (4.8%; $\chi^2 = 14.4$, $P < 0.001$), and *T. congolense* savannah infections (4.8%). Mixed infections of *T. vivax* and *Trypanozoon* (*T. evansi* and /or *T. brucei*) accounted for 1.4% of the 222 camel samples analysed from the three sites. For further confirmation, 32 samples randomly selected from PCR amplicons of each trypanosome species, with good quality DNA were sequenced including those trypanosomes with known clinical symptoms. The sequences of the 32 samples clustered with known *T. vivax* or *T. evansi* and *T. brucei* isolates (Fig. 6). Sequences of *T. vivax* detected in five camels (GenBank accessions number MH247140, MH247142, MH247145, MH247147, MH247149) and two *H. camelina* (GenBank accession number MH247150, MH247152) were 100% identical to nucleotide sequences of *T. vivax* in the GenBank accession number MK880189. Two camels, one *H. camelina*, and one *S. calcitrans* were infected with trypanosomes (GenBank accessions MH247172, MH247174, MH247175, MH247177) sharing > 99% ITS-1 nucleotide sequence identity with *T. evansi* sequences. Twenty-four sequences (GenBank accessions number MH247139, MH247141, MH247146, MH247148, MH247151, MH247153-MH247163, MH247165-MH247170, MH247173, MH247176) clustered with *T. brucei*, sharing 99.3% identity with *T. brucei* (GenBank accession X05682), but also > 98% identity with *T. evansi* reference sequences. Further attempts to amplify and sequence *T. brucei*-specific kinetoplast maxicircle sequences, which are absent in *T. evansi*, confirmed four of the samples, one from *G. pallidipes* and three camel samples, as harbouring *T. brucei* (GenBank accessions MH247139, MH247159, MH247166, MH247167). We were unable to determine conclusively whether the other 17 samples with trypanosome ITS-1 sequences clustering among *T. brucei*, were *T. brucei* or *T. evansi* (Fig. 6).

Further characterization of the samples using the *T. evansi* type A-specific primers differentiated the *T. evansi* in the *S. calcitrans* (GenBank accession number MH247175) as *T. evansi* type A. Three of the camel samples with ITS-1 sequences (GenBank accessions number MH247157; MH247160 and MH247169) that clustered among *T. brucei* also amplified using the *T. evansi* type A-specific primers, indicating either that *T. brucei* and *T. evansi* can share identical ITS-1 sequences or could be indicative of mixed infections with *T. brucei* and *T. evansi*. We failed to amplify *T. evansi* type B from *Trypanozoon* positive samples.

Anaemia associated with *Trypanozoon* infection

To determine the association between *Trypanozoon,* active infection and PCV, a measure of anaemia, which is one of the consequences of trypanosome infection (Mossaad et al. 2017; Murray et al. 1977) and a symptom of surra, we compared the mean PCVs between camels with active *Trypanozoon* infection and those that were negative by microscopy. *Trypanozoon* infection affected the PCV values significantly; camels that had active *Trypanozoon* infection were anaemic with the mean PCV value of 24.64 ± 6; however, microscopically negative camels had a higher PCV value of 30.14 ± 6, independent t-test, $t = 3.303$, $P = 0.002$, $n = 222$). Surra-infected camels were considerably anemic, an indication of trypanosomosis severity (Getahun et al. 2022). Camels that were haemorrhagic, one infected with *T. vivax* (PCV 9%) and another with *T. evansi* type A (PCV 11%), were both severely anemic. However, other camels with high *T. evansi* parasitemia, but feeding well and in good body conditions (PCV 21%).

Diversity of trypanosomes in other domestic animals co-herded with camels

We further analysed the prevalence of trypanosomes in other domestic animals: goats, sheep, donkeys, and cattle, that are co-herded with camels by PCR. *Trypanozoon* DNA was detected in small ruminants (goats and sheep), but not in cattle (Fig. 7). *Trypanosoma vivax* was more prevalent in sheep compared to *Trypanozoon* ($\chi^2 = 13.146$, df = 1, $P = 0.0003$). In goats, *T. vivax* infections accounted for 8.2%, while the infection rate of *Trypanozoon* was 10.2%, and the difference was not significant (χ^2, $P > 0.05$). Cattle were infected with *T. vivax* (9.5%) and *T. congolense* (2.4%) with no significant difference (Fig. 7). Furthermore, *T. vivax* was detected in 14% of donkeys, but no other trypanosomes species were detected (Fig. 7). However, all these livestock were negative microscopically.

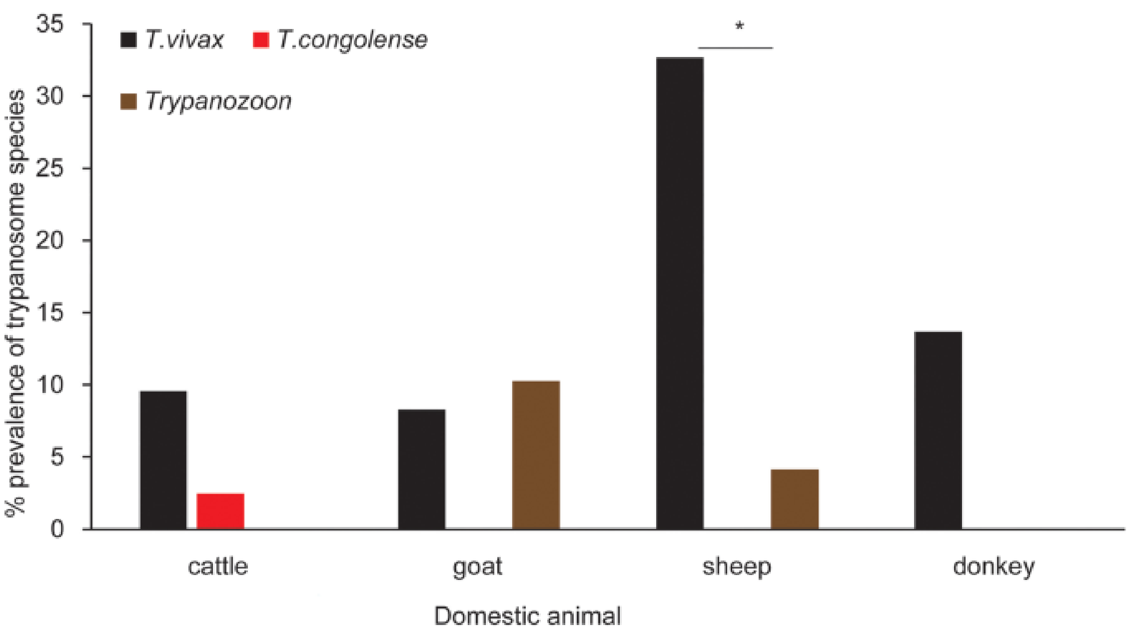

Fig. 7. Prevalence and diversity of trypanosomes in domestic animals co-herded with camels. Percent prevalence of the three trypanosomes species in four domestic animals that co-herded with camels based on PCR. Significant difference in prevalence between trypanosomes species are depicted by an asterisk. An equal number of blood samples were collected from Ngurunit and Shurr for cattle, goats, and sheep, while donkeys were exclusively sampled from Shurr

Identification of vertebrate hosts in blood meals of *H. camelina* and *S. calcitrans*

Proportion of blood-meals taken by *H. camelina* from different hosts

The majority of fed *H. camelina* collected (n = 100) using traps had fed on domestic animals as compared to human or rat ($\chi^2 = 96.04$, $P < 0.0001$). The camel host was the primary source of bloodmeals for 60% of the fed flies ($\chi^2 = 7.96$, $P = 0.005$), followed by goats (15%), sheep (14%), and rats (4%) (Fig. 8A–B). Some of the flies fed on multiple vertebrate hosts; for instance, 1% of *H. camelina* fed on both human and sheep, 3% on camel and sheep, and 3% on goats and sheep. Each household kept diverse domestic animals (Fig. 8D). Sheep and goats were the most abundant, followed by camels, and relatively few cattle. The average camel population per household was 35, while the average sheep population per household was 223 at Shurr site. The camel: sheep abundance ratio was 35/223 = 0.157. 60% of *H. camelina* fed on camels, 15% fed on sheep, thus the observed feeding rate was 60/15 = 4. A feeding index of

25.5 (4/0.157) was estimated for *H. camelina* with higher feeding preference on camels than sheep.

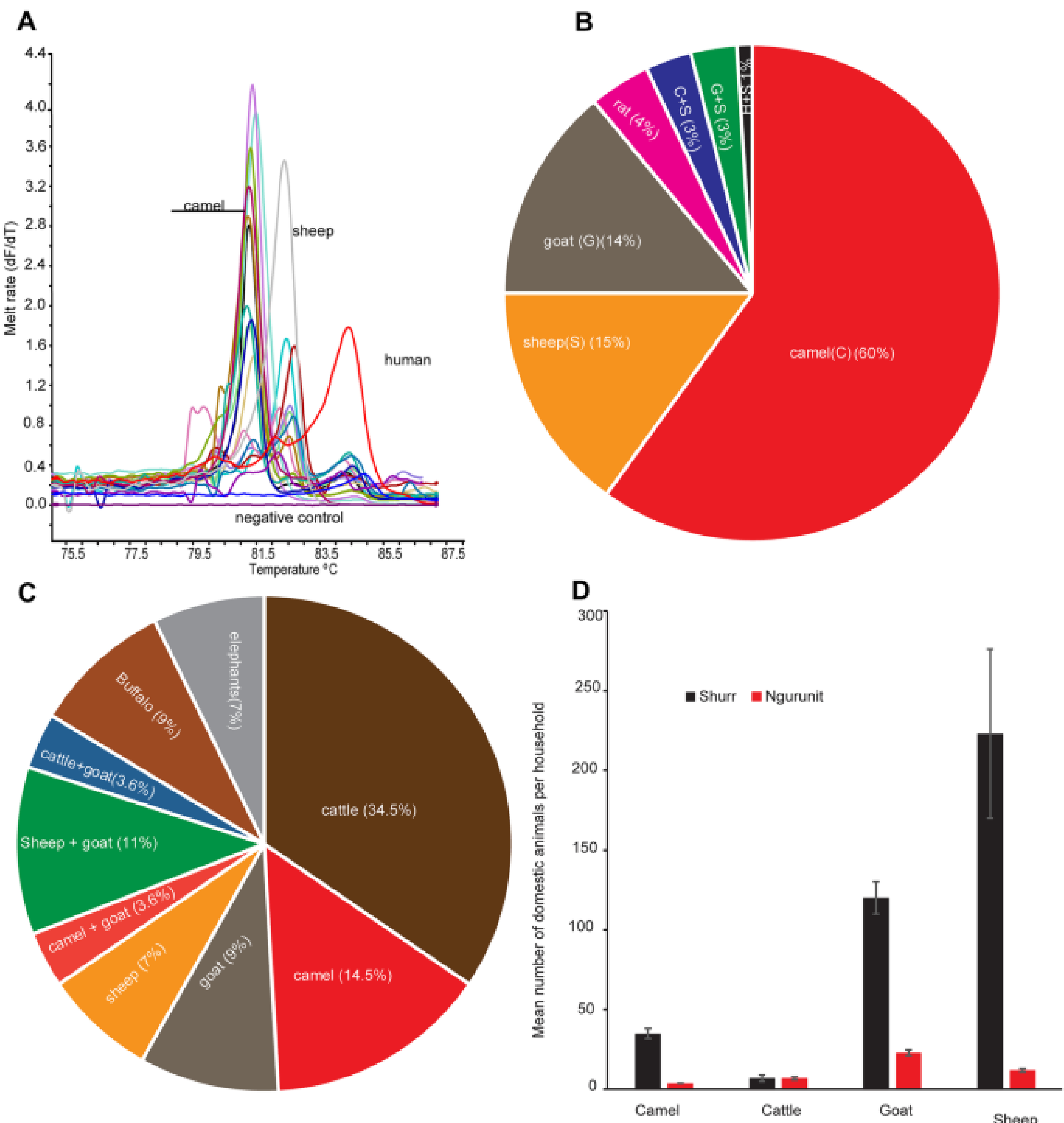

Fig. 8. Bloodmeal analysis in *H. camelina* and *S. calcitrans* to determine vertebrate hosts. (**A**) Representative HRM profiles of selected *H. camelina* bloodmeal sources analyzed using 16S rRNA gene target. Positive controls were included for comparison. (**B**) Percent of bloodmeals for *H. camelina* identified using the 16S rRNA primer (n = 100). (**C**) Percent of blood meals from various vertebrate in *S. calcitrans* identified using16S rRNA primer (n = 40). (**D**) Diversity and abundance of domestic animals in Shurr and Ngurunit. Error bar indicates standard error, n = 96 and 92 households from Shurr and Ngurunit, respectively

Proportion of blood meals taken by *S. calcitrans* from vertebrate hosts

The bloodmeals of *S. calcitrans* included mainly cattle, camels, buffaloes, and goats (Fig. 8C). About 15% of *S. calictrans* had fed on camel, 7% on sheep, translating to an estimated feeding ratio of 2 (15/7). The feeding index of 12.7 (2/0.157) was estimated for *S. calcitrans* with higher feeding preference for camel than sheep. However, *S. calcitrans* fed more on cattle than camel. The camel: cattle abundance ratio was 5 (35/7), and, the ratio of the number of feeds by *S. calcitrans* on camel and cattle, 0.428 (15/35). Thus, the feeding index for camel was 0.428/5 = 0.09, showing higher preference to feed on cattle than camel.

Discussion

Animal trypanosomosis is caused by several trypanosome species of the genus *Trypanosoma* that are transmitted cyclically by tsetse flies and mechanically by other hematophagous flies. Our results show that diverse non-tsetse hematophagous biting flies from the tsetse-free area harboured similar trypanosomes as the tsetse fly *G. pallidipes*. These flies, stable flies, *H. camelina, P. rueppellii* and tabanus could serve as mechanical vectors. Their roles in mechanical transmission of trypanosomes could be facilitated by survival of the pathogens in the proboscis and midgut as observed in *H. camelina* (Oyieke and Reid 2003), consistent with our observation of *T. congolense* and trypanozoon survival in the mid gut of *S. calcitrans* for at least 3 and 5 h, respectively (Unpublished data). Similarly, Sumba et al. (1998) demonstrated that *T. congolense* survived for 3 and half hours and *T. evansi* for 8 h in the gut of *Stomoxys niger* and *S. taeniatus*, showing there might be variation in vector competence between *Stomoxys* species that needs to be investigated. Another index of mechanical transmission relates to the time spent by a vector to find the next host after disrupted feeding which in *H. camelina* occurs within 1 h to re-locate camels translocated up to 1.5 km away (SF2). Taken together, our findings support the ability of the flies to circulate the pathogens among livestock via mechanical means, although further competence studies are needed to verify this.

The high number of *H. camelina* and *S. calcitrans,* their occurrence throughout the year, and the diversity of trypanosomes detected makes these two biting flies the most important potential vectors of trypanosome in tsetse-free ecologies. Furthermore, these two biting flies fed preferentially on camels, but also on other hosts such as cattle and goats. Nonetheless, the role of other biting flies identified, such as *Tabanus* spp. and *Pangonia* spp., should not be underestimated (Desquesnes and Dia 2003; Sumba et al. 1998; Taioe et al. 2017; Votýpka et al. 2015). These biting flies harboured similar trypanosome species as *G. pallidipes,* one of the major tsetse species in sub-Saharan Africa, due to its wider geographical distribution and economic importance (Getahun et al. 2014; Njiru et al. 2004a, b; Saini et al. 2017). The detection of diverse trypanosomes in non-tsetse infested areas shows that trypanosomes are widely spread across a broad geographic area that includes tsetse-free regions (Kimenyi et al. 2021).

The low percentage of the camels identified with active infection might be due to the low sensitivity of the microscopic technique (Mossaad et al. 2017; Njiru et al. 2005, Getahun et al. 2022). However, the molecular data showed that a significant number of camels were infected by trypanosomes, indicating that they might have subclinical infections. Thus, there is a possibility that sub-clinically infected camels can serve as a reservoir of trypanosomes and pass it to susceptible livestock via vectors bite or during pregnancy to foetus, which results in abortion (Jittapalapong et al. 2009; Kassa et al. 2011). We also documented infected camels presenting with high parasitaemia (Fig. 5B), which is a requirement for mechanical

transmission by biting flies (Toukam et al. 2011). Another reason for not detecting trypanosomes in blood might be due to trypanosomes moving to other tissues; for example, the skin is an anatomical reservoir of parasites of arthropod-borne diseases such as trypanosomes (Capewell et al. 2016). Thus, we checked the lymph node aspirate (LNA) from a few sick camels with clear clinical signs of trypanosomosis, but with negative results during blood examination. We found that camel blood samples could test negative for trypanosome infection through microscopy and PCR but be positive in LNA (SF3 A-B). This finding agrees with previous reports in other livestock that showed the presence of trypanosomes in LNA, but absence in the blood of cattle and sheep (Luckins and Gray 1979) suggesting trypanosome detection could be affected by the sample type (blood or LNA) and diagnostic technique.

Camels that showed active infection microscopically were anaemic with average PCVs below 25, as compared to those microscopically negative camels with an average PCV of 30. With regards to different clinical outcomes, we found severely anaemic haemorrhagic camels that were infected by *T. vivax* (PCV 9%) as observed previously (Mossaad et al. 2017) or by *T. evansi* type A (PCV 11%). However, certain samples with high *T. evansi* parasitaemia (5×10^8 trypanosomes/mL of blood) were from camels that were feeding well and clinically healthy (PCV 21%). This finding indicates that *T. evansi* populations could differ in their degree of virulence in camels. This could be suggestive of the presence of different strains of *T. evansi* as reflected by different virulence degrees to camels that may be due to genetic variation (Kimenyi et al. 2021) or host response, that need to be investigated in the future. However, virulence also depends on the immune status of the host, past and recent infection by complex pathogens, as well as the genetic makeup of individual hosts.

We sequenced the trypanosome ITS-1 fragment, which is the preferred target for species-specific molecular diagnostics of trypanosomes (Desquesnes et al. 2001; Njiru et al. 2005). The ITS-1 sequences produced distinct clusters for *T. vivax* and *Trypanozoon* (Fig. 6). *Trypanosoma vivax* was the most prevalent trypanosome followed by *Trypanozoon*, both in flies and domestic animals, but not in camels, in which *Trypanozoon* was the most common. We further detected maxicircle kDNA (Masiga et al. 2006) in our *Trypanozoon* samples, confirming the presence of *T. brucei* in *G. pallidipes* and three camel samples. Additionally, we characterized *T. evansi* type A by the presence of the type A-specific marker targeting the RoTat 1.2 VSG gene, but we failed to detect *T. evansi* type B. The absence of *T. evansi* type B could be due to diagnostic limitations or the small sample size analysed, but these findings are congruent with previous findings of low occurrence of the *T. evansi* B subtype in camels (Birhanu et al. 2016; Borst et al. 1987). However, for 20 of the *Trypanozoon* ITS-1 sequences obtained, we were not able to determine conclusively whether they were of *T. brucei* or *T. evansi,* showing that the ITS-1 gene cannot be used to effectively differentiate these species. This challenge of *Trypanozoon* subgenus identification and the need for more specific and sensitive diagnostics have been discussed (Büscher et al. 2019).

The detection of *T. brucei* and *T. congolense* both in biting flies and camels in the tsetse-free areas of northern Kenya might suggest the long-distance travel of camels and other domestic animals between tsetse-free and -infested areas. In our recent study we did not find genetic variation between *T. congolense* from northern Kenya and coastal Kenya (Kimenyi et al. 2021), supporting possible introduction of *T. congolense* to the region. Similarly, previous studies detected *T. brucei* from camel in tsetse free areas in northern Kenya (Gibson et al. 1983; Njiru et al. 2006). To support this claim, a study done in North Eastern Kenya showed that animals move more than 120 km from their homestead for better pasture and water, and infection of Rift Valley fever increases in herds that move than in those that remain at the homestead

(Owange et al. 2014). The other possibility is the presence of unidentified biological vectors, which will require further investigation, combined with vectorial capacity study of the identified biting flies and population genetics of trypanosomes.

Despite the variation in livestock density (Fig. 8D), *H. camelina* demonstrated high preference for camel as most of them fed on camel. The high preference of *H. camelina* to camel needs further investigation. However, *S. calcitrans* obtained blood meal from various domestic and wild animals. An earlier study by Mihok and Clausen (1996) showed *Stomoxys spp*. collected from forested areas of Kenya fed on various wild animals, demonstrating its diverse host range that could also depend on ecology of collection site and available hosts.

Conclusions

The detection of diverse trypanosomes species/strains both in various biting flies and in camels suggests that trypanosomosis in camels is not only due to surra (*T. evansi* infection), but also nagana (*T. brucei*, *T. congolense*, and *T. vivax* infections). Such knowledge helps in drug administration because one could tailor the treatment to each trypanosome species (Giordani et al. 2016). Furthermore, our analysis shows that other domestic animals could serve as a reservoir of different trypanosomes, such as *T. vivax* and *T. congolense*, which are more deadly to camels (Osório et al. 2008; Kamidi et al. 2017, 2018). Finally, the similarity of pathogens found in biting flies and their domestic animal blood-meal hosts demonstrates that these hematophagous flies could be used for xenomonitoring to track trypanosomes circulating in domestic animals as an early detection method. Our results demonstrate a high animal trypanosomosis and surra burden outside tsetse belt that alerts continental and national institutions working on animal trypanosomosis control to give equal emphasis to non-tsetse infested areas when it comes to control of animal trypanosomosis.

Data availability

All data generated or analysed in this study are included in the article and as additional files. The newly generated sequences were deposited in the NCBI Nucleotide database under the accession numbers listed in Supplementary table.

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Contributions

MNG, BT, DM, SR conceptualized and designed the experiments. MNG, JLB, AO, POA, JN, JMM generated experimental data, JV contributed in the molecular part of the study. MNG analysed the data and wrote the manuscript. All authors revised and approved the final manuscript.

Ethics approval and consent to participate

We collected blood samples within the framework of epidemiological surveillance activities, in accordance to the International Centre of Insect Physiology and Ecology*'s* Institutional Animal Care and Use Committee (IACUC) guidelines as performed during prophylaxis or diagnostic campaigns (approval number: 495 icipe-IACUC-10/2018.1). Local authorities did not require ethical statements for the research studies. We did the blood sampling of domestic animals with the authorisation of the owner. Herdsmen/women gave their consent for their animal sampling after explaining the objectives of the study. No samples other than those for routine screening and diagnostic procedures were collected. All animals sampled and found positive with trypanosomes were treated using trypanocides.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

Supplementary file1: Figure S1 PCR products were resolved 1% ethidium-bromide stained agarose gel (8V for 1.5 hrs) to check for any contamination. The DNA isolated from whole fly was amplified targeting trypanosomal ITS1 gene. Lane: M 10- bp marker, Bf (reaction buffer), wt (PCR water), TB (T. brucei ILTat 1.4) TV (T. vivax IL 2136), TC (T. congolense savannah (IL3000)), and TE (T. evansi KETRI 2479), F1- F10 DNA sample from H. camelina flies. The absence of PCR product under Bf, and wt show no contamination from extraction buffer (TIF 11846 KB)

Supplementary file 2: Figure S2 Number of H. camelina recaptured at the specified distance from pint of release. Number in parenthesis shows percentage of flies recaptured (TIF 5515 KB)

Supplementary file 3: Figure S3 (A) PCR products were resolved 1% ethidium-bromide stained agarose gel (8V for 1.5 hrs) to check for trypanosomes in blood and lymph node aspirate. The DNA isolated from blood and lymph node aspirate was amplified targeting trypanosomal ITS1 gene. Lane: M 10- bp marker, -Ve (reaction buffer), TE (T. evansi KETRI 2479) TV (T. vivax IL 2136), TC (T. congolense savannah (IL3000)), and LN_C1, LN_C2, DNA sample from two camel lymph node aspirate, B_C1 and B_C2 DNA from corresponding blood samples from the same camel. The result shows both samples of the lymph node aspirate were positive, while blood samples were negative from the same camel. (B) Five camels blood and lymph node aspirate were analysed, only camel five lymph node aspirate was positive for T.vivax but blood sample from the same camel was negative (TIF 26280 KB)