


# Genetic connectivity of trypanosomes between tsetse-infested and tsetse-free areas of Kenya

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## Research Article

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### Abstract

The prevalence rates of trypanosomes, including those that require cyclical transmission by tsetse flies, are widely distributed in Africa. *Trypanosoma brucei* and *Trypanosoma congolense* are actively maintained in regions where there are no tsetse flies although at low frequencies. Whether this could be due to an independent evolutionary origin or multiple introduction of trypanosomes due to continuous movement of livestock between tsetse-free and -infested areas is not known. Thus, the aim of the study was to carry out microsatellite genotyping to explore intra-specific genetic diversity between *T. (Trypanozoon)*, *T. congolense* and *Trypanosoma vivax* from the two regions: tsetse infested and tsetse free. Microsatellite genotyping showed geographical origin-based structuring among *T. (Trypanozoon)* isolates. There was a clear separation between isolates from the two regions signalling the potential of microsatellite markers as diagnostic markers for *T. brucei* and *Trypanosoma evansi* isolates. *Trypanosoma vivax* isolates also clustered largely based on the sampling location with a significant differentiation between the two locations. However, our results revealed that *T. congolense* isolates from Northern Kenya are not genetically separated from those from Coastal Kenya. Therefore, these isolates are likely introduced in the region through animal movement. Our results demonstrate the occurrence of both genetic connectivity as well as independent evolutionary origin, depending on the trypanosome species between the two ecologies.

## Introduction

Tsetse flies (*Glossina* sp.) transmit *Trypanosoma vivax*, *Trypanosoma b. brucei* and *Trypanosoma congolense* biologically, which involves development in the vector. *Trypanosoma vivax* develops in the vector's mouthparts (Rotureau and Van Den Abbeele, 2013), whereas *T. brucei* and *T. congolense* develops in fly's midgut. These developmental stages involve proliferation and adaptation of the parasite for infection and survival in the mammalian host (Caljon *et al.*, 2014; Ooi *et al.*, 2016). The tsetse-borne trypanosomes follow distinct developmental pathways within the fly which minimizes competition among the parasites and maximizes their transmission (Rotureau and Van Den Abbeele, 2013). Trypanosomes are also transmitted mechanically by biting flies including horseflies (*Tabanus* spp.), *Hippobosca camelina*, stable flies (*Stomoxys* spp.) and tsetse flies (Desquesnes and Dia, 2003, 2004; Duffy *et al.*, 2009). No development occurs during mechanical transmission (Wells, 1972). *Trypanosoma evansi* is thought to have evolved from *T. brucei* by completely losing kinetoplast maxicircles which are involved in morphological development and multiplication within tsetse flies. These trypanosomes are thus independent of tsetse transmission (Moreno and Nava, 2015).

*Trypanosoma b. brucei* and *T. congolense* have been isolated in tsetse-free areas of Northern Kenya from as far back as the 1980s (Gibson *et al.*, 1983; Njiru *et al.*, 2006; Getahun *et al.*, 2020). Although they can be transmitted mechanically by non-tsetse biting flies, these parasites cannot be actively maintained in tsetse-free areas for long periods (Wells, 1972). However, isolation of the parasites from tsetse-free parts of Northern Kenya is evidence that these species are actively maintained in these areas (Gibson *et al.*, 1983; Getahun *et al.*, 2020). An explanation for this may be the possibility of biological transmission by an unidentified non-tsetse vector (Gibson *et al.*, 1983). Nonetheless, the genetic connectivity between these trypanosomes from tsetse-infested and tsetse-free areas is not well understood. East African *T. vivax* strains are distinct from West African strains and genetically heterogeneous (Rodrigues *et al.*, 2008, 2017; Adams *et al.*, 2010; Garcia *et al.*, 2014). To show that the high degree of heterogeneity among African isolates is related to biological transmission, there is need for comparative analysis of *T. vivax* isolates from tsetse-free and tsetse-infested areas. Sequence analysis of rDNA *T. vivax* genes from tsetse-free and tsetse-infested areas of Ethiopia could not explain whether the isolates are genetically distinct implying that more sensitive technologies such as microsatellite genotyping are needed (Fikru *et al.*, 2016).

Thus, we compared Northern Kenyan and Coastal Kenyan *T. (Trypanozoon)* isolates (while ignoring established nomenclature and treating the group as one), *T. vivax* and *T. congolense* isolates from tsetse-free and tsetse-endemic areas of Kenya, to explore their intraspecific

population structure, genetic differentiation, diversity and gene flow rates. This is the first intra-species comparative population-genetic study on trypanosomes from tsetse-free and tsetse-endemic areas of Africa. Ribosomal DNA sequence analysis may fail to inform on interspecies genetic separation among a group of trypanosome isolates (Fikru *et al.*, 2014; Getahun *et al.*, 2020). Microsatellite analysis, which is a powerful tool for population-genetic studies (Senan *et al.*, 2014; Fikru *et al.*, 2016) has the potential for being used to study genetic separation of trypanosome spp. Therefore, we carried out microsatellite genotyping on *T. (Trypanozoon)*, *T. vivax* and *T. congolense* isolates from tsetse-free and tsetse-endemic areas of Kenya, and explored their intraspecific population structure, genetic differentiation, diversity and gene flow rates (Fig. 1).

## Materials and methods

### Study area

Trypanosome isolates used in this study were sampled from Shurr (N02°08', E038°27'), Ngurunit (N01°74', E037°29') and Laisamis (N01°23'11" E37°57'11.7") all in Marsabit County, Northern Kenya and Shimba Hills (lat. -4.243 and long. 39.403) in Kwale County, Coastal Kenya (Fig. 1). The main livestock in Shurr, Ngurunit and Laisamis are camels, cattle, goats and sheep whereas in Shimba Hills there are only cattle, goats and sheep. These livestock are the hosts and reservoirs of the trypanosome species in these areas. Shimba Hills in Kwale County falls within the Coastal tsetse fly belt; therefore, tsetse flies are abundant in the area and they act as biological and in some cases mechanical vectors of trypanosome species. Ngurunit falls at the edge of a tsetse fly belt; however, no tsetse flies have been collected in the area in previous studies and is thus considered a tsetse-free area (Oyieke and Reid, 2003; Getahun *et al.*, 2020). Laisamis and Shurr are also tsetse-free areas and vectors of trypanosomes there are camel flies, tabanids and horseflies (Getahun *et al.*, 2020).

### Trypanosome isolates

We used trypanosome isolates from camel and cattle blood collected in Shurr, Laisamis and Ngurunit, Marsabit County of Northern Kenya and cattle blood and tsetse flies collected in Shimba Hills, Kwale County. The blood and tsetse fly samples were collected between 2018 and 2020 at different months and seasons. For samples collected in 2017, DNA was extracted in 2017 and stored at -20°C at the International Center for Insect Physiology and Ecology (icipe) laboratory.

### DNA extraction and internal transcribed spacer (ITS) classification

Archived DNA samples were checked for the presence of isolates through ITS-1 region polymerase chain reaction (PCR) amplification (Njiru *et al.*, 2005). Samples with no isolates detected were freshly extracted along the blood and tsetse fly samples with no DNA available. DNA extractions were carried out with the Qiagen DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's protocol. A diagnostic ITS-based PCR test was performed on all the samples. The test involved amplification of a 480 bp fragment for *T. (Trypanozoon)* isolates, a 700 bp fragment for *T. congolense* isolates and a 250 bp fragment for *T. vivax* isolates (Njiru *et al.*, 2005). A 10- $\mu$ L PCR reaction mixture was prepared with 1  $\mu$ L of the template, 0.5  $\mu$ L each of the forward and backward ITS1 primers (100 pmol), 5  $\mu$ L Dreamtaq polymerase and 3  $\mu$ L of nuclease-free water. The amplification reactions

were carried out using the following PCR cycling profile: 95°C for 3 min for the initial denaturation step, 35 cycles of 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min (Getahun *et al.*, 2020).

### Microsatellite genotyping

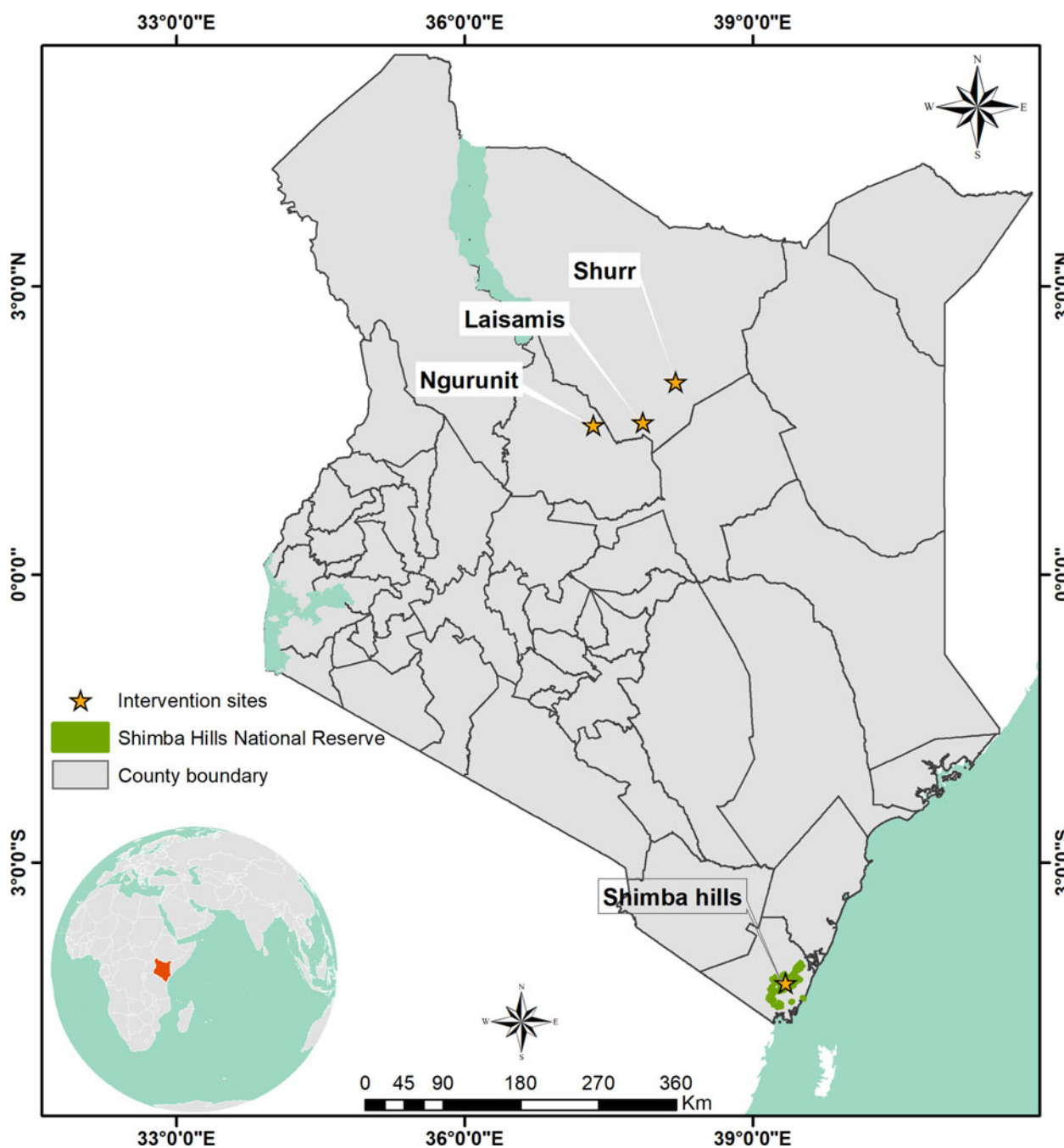
Fluorescently labelled forward primers (6-FAM, HEX and ROX) and reverse primers of previously described 14 microsatellite loci were used for microsatellite genotyping (Sistrom *et al.*, 2013; Kamidi *et al.*, 2017). The primer details of the microsatellite loci are provided in Table S1. Fourteen primer pairs were used to amplify microsatellite loci in *T. (Trypanozoon)*, six were used to amplify microsatellite loci in *T. vivax* and only five amplified microsatellite loci in *T. congolense*. A 15- $\mu$ L PCR reaction mixture was prepared with 1.5  $\mu$ L of the template, 0.75  $\mu$ L each of the forward and backward ITS1 primers (100 pmol), 3- $\mu$ L blend Taq polymerase and 9  $\mu$ L of nuclease-free water. The PCR conditions used were: initial denaturation at 95°C for 15 min, 35 amplification cycles of 95°C for 30 s, primer-specific annealing temperature for 30 s, elongation at 72°C for 30 s and final elongation at 72°C for 7 min. Genotyping by capillary electrophoresis was carried out with Applied Biosystems (Waltham, MA, USA) 3730 DNA Analyser and 500 LIZ as the size standard. Allele scoring was performed using Geneious Prime 2020.2.2 software (<https://www.geneious.com>) from where the fragment sizes were exported into an excel sheet and manually edited.

### kDNA amplification

kDNA was amplified using kDNA-specific primers kDNA-12\_JV: 5'-TTAATGCTATTAGATGGGTGTGG-3'; kDNA-13: 5'-CTCTCTGGTTCTCTGGGAAATCAA-3' (Getahun *et al.*, 2020). The PCR conditions used were: initial denaturation at 95°C for 15 min, 35 amplification cycles of 95°C for 50 s, primer-specific annealing temperature 55°C for 30 s, elongation at 72°C for 50 s and final elongation at 72°C for 7 min.

### Population structure and differentiation

STRUCTURE v2.3.4 software (Pritchard *et al.*, 2000) was used to infer the population's structures for *T. (Trypanozoon)*, *T. vivax* and *T. congolense* isolates through the Bayesian clustering method. Ten independent runs each for  $K$  (genetic clusters) = 1–7 were performed with a burnin of 50 000 and 100 000 Markov Chain Monte Carlo (MCMC) reps for 1000 iterations. The optimal value of  $K$  was determined through the *ad hoc* statistic ' $\Delta K$ ' (Evanno *et al.*, 2005) in Structure Harvester v0.6.94 (Earl and von Holdt, 2012). STRUCTURE runs for *T. (Trypanozoon)* and *T. congolense* isolates were performed with independent allele frequencies and admixture models whereas runs for *T. vivax* isolates with performed with correlated allele frequencies and no admixture models (Porrás-Hurtado *et al.*, 2013). The STRUCTURE membership coefficients ( $Q$  values) were used to assess the probability of assignment of each isolate to a specific cluster. Sub-populations within *T. (Trypanozoon)*, *T. congolense* and *T. vivax* populations were also identified through multivariate analysis using principal component analysis (PCA), in R package Adegenet (Solymos *et al.*, 2020). Multivariate analysis was performed to complement Bayesian analysis and unlike the latter, it does not make assumptions on the Hardy–Weinberg equilibrium (HWE) or linkage disequilibrium as it is not model based (Jombart *et al.*, 2010). The optimal number of clusters was determined through Bayesian information criterion (Jombart, 2008). In addition, Cavalli-Sforza genetic distances were then calculated and an unweighted pair group method with arithmetic mean (UPGMA) dendrogram



**Fig. 1.** Map of Kenya showing locations of trypanosome sampling sites: Shimba Hills (tsetse belt), Ngurunit, Laisamis and Shurr (tsetse-free areas) from where samples used in the study were collected. Laisamis, Ngurunit and Shurr are all characterized by arid and semi-arid conditions whereas Shimba Hills area has wet climatic conditions.

based on Cavalli-Sforza similarity coefficient constructed in Populations v1.2.32 (<http://bioinformatics.org/populations/>) to determine intraspecific hierarchical structuring.

Pairwise  $F_{ST}$  and their related  $P$  values for *T. (Trypanozoon)*, *T. vivax* and *T. congolense* isolates were calculated to estimate the levels of population differentiation at two levels: among the STRUCTURE-inferred clusters and among the populations derived from sampling localities. Between and within population variance at the two levels was determined through analysis of molecular variance (AMOVA) in FSTAT v2.9.4 (Goudet, 2003). We tested for isolation by distance and isolation by environment by plotting among population-genetic distances ( $(1/F_{ST} - 1)/4$ ) against between populations geographical (km) and environmental distance using a partial Mantel test (Mantel, 1967) calculated in R package vegan (Oksanen *et al.*, 2020).

### Genetic diversity

Total number of different alleles and the effective number of alleles were determined in GenALEX v6.5 (Peakall and Smouse, 2006). Allelic richness ( $A_R$ ) was calculated in the PopGenReport package in R (Adamack, 2014), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_e$ ) and Fisher's inbreeding coefficient ( $F_{IS}$ ) were estimated in GenALEX v6.5 (Peakall and Smouse, 2006). As a test to the non-random association of alleles within diploid individuals and at different loci, agreement with the HWE and linkage disequilibrium was estimated in R package Genepop (Rousset *et al.*, 2020).

*Trypanosoma congolense* alleles were rarefied for the smallest sample (Kalinowski, 2004) upon which allelic richness and private allelic richness were determined in HP-RARE (Kalinowski, 2005).

### Gene flow analysis

Gene flow ( $N_e m$ ) within the three trypanosome species was inferred indirectly from the allele frequency data based on Wright's equation  $F_{ST} = 1/(4N_e m + 1)$  ( $N_e$  is the effective population size and  $M$  is the migration rate) (Wright, 1990; Whitlock and McCauley, 1999). BayesAss Edition 3.0 software was used to estimate the migration rate between populations of the three trypanosome species based on Bayesian inference (Wilson and Rannala, 2003).

## Results

### PCR diagnostic tests and microsatellite genotyping

A total of 52 *T. (Trypanozoon)* isolates (30 from Northern Kenya and 22 from Shimba Hills, Coastal Kenya), 60 *T. vivax* isolates (30 from Northern Kenya and 30 from Coastal Kenya) and 32 *T. congolense* isolates (29 from Coastal Kenya and three from Northern Kenya) were isolated from camel, and cattle blood and tsetse fly samples. The results of the ITS1 assay are provided in Table S2. All trypanosome isolates were successfully genotyped and scored (Table S5).

### Trypanosoma (Trypanozoon)

#### Population structure

Structure harvester results indicated the best  $K$  value as  $K = 2$  for Bayesian clustering analysis with STRUCTURE v2.3.4 (Fig. 2A) thus indicating the presence of two distinct genetic clusters as the most likely hierarchical level of population structure. Cluster II (green) includes all Northern Kenyan isolates plus two Coastal Kenyan isolates. Cluster I (red) includes only Coastal Kenyan isolates. The next best fit of  $K = 3$  revealed the level of sub-structuring within isolates from Coastal Kenya (Fig. 2B). Cluster 'a' (red) includes 31 *T. (Trypanozoon)* isolates, all but one being from Northern Kenya, whereas cluster 'b' (green) consists of four isolates all from the Coastal Kenyan region. Cluster 'c' (blue) is made up of 17 isolates, all from Coastal Kenya. Assignment of the isolates to these three clusters based on  $Q$  values is provided in Table S3. Of the 52 *T. (Trypanozoon)* isolates, six Coastal Kenyan isolates and one Northern Kenyan isolate showed uncertain assignment to either of the three clusters ( $Q < 0.8$ ) and these were excluded from further STRUCTURE-based analysis.

Multivariate analysis confirmed Bayesian clustering results by revealing two distinct genetic clusters that were clearly differentiated based on geography (Fig. 2C). Each cluster was dominated by isolates from the two different sampling localities indicating the relevance of geographical isolation in clustering by multivariate analysis of these isolates. Cluster a (red) in the PCA includes two isolates from Coastal Kenya in addition to all isolates from Northern Kenya whereas cluster b (green) includes 20 isolates from Northern Kenya.

Hierarchical clustering by an UPGMA (Fig. 2D) further confirmed the results of Bayesian and multivariate analysis by revealing a population structured according to geography. Although not strong (bootstrap = 0.4023), the first level of distinction clearly separated Northern Kenyan samples from Coastal Kenyan samples. However, unlike other clustering methods where two isolates (CB13 and CB14) from Coastal Kenya grouped among Northern Kenyan isolates, all isolates from both locations grouped separately.

We further did kDNA amplification using kDNA-specific primer and found that samples from Coastal Kenya and northern Kenya and positive control for *T. brucei* and *T. evansi* showed amplification, but with different sizes (Fig. 2E). However, we used *T. vivax* and *T. congolense* as positive control, but no kDNA amplification.

### Genetic diversity

Only 4% of all the loci combinations were in linkage disequilibrium. However, no loci showed conformity to the HWE in both populations. Deviation from the HWE within the *T. (Trypanozoon)* taxa is expected due to the clonal reproduction. Among the location of origin-based populations, 171 different alleles were identified with the effective number of alleles estimated at 9.714 and 9.286 for Coastal Kenyan and Northern Kenyan populations, respectively. Shannon's information index ( $I$ ) for the two populations were 0.114 and 0.084, respectively. The average number of private alleles for the coastal and northern populations was 2.929 and 2.50, respectively.

Allelic richness detected was 5.08 and 5.33 for the Northern and Coastal Kenyan populations respectively, showing relatively greater diversity among Coastal Kenyan isolates.

Observed heterozygosity ( $H_o$ ) was 0.79 and 0.835 whereas the expected heterozygosity ( $H_e$ ) was 0.819 and 0.813 for the Northern and Coastal Kenyan populations, respectively (Table 1). The Fisher inbreeding coefficient ( $F_{IS}$ ) for the Northern Kenyan isolates was  $-0.044$  and that for the Coastal Kenyan isolates was 0.023. Among the structure-defined clusters, allelic richness ranged from 4.487 in cluster 'a' to 4.201 in cluster 'b'. Observed heterozygosity ranged from 0.836 to 0.759 whereas expected heterozygosity ranged from 0.807 to 0.772. Inbreeding coefficient ( $F_{IS}$ ) values ranged between  $-0.053$  and 0.011 (Table 1).

### Genetic differentiation and gene flow analysis

Fixation index ( $F_{ST}$ ) value between sampling localities was 0.0617 and revealed significant differentiation between the populations ( $P < 0.05$ ). The small  $F_{ST}$  value is an indication of moderate differentiation between the Northern and Coastal Kenyan populations. Among STRUCTURE-based clusters,  $F_{ST}$  values were 0.117 between clusters a (red) and b (green), 0.0965 between clusters a (red) and c (green), 0.1062 between clusters c (blue) and b (green) indicating rather moderate differentiation among all clusters. Differentiation between clusters c (blue) and a (red) was significant ( $P = 0.012$ ).

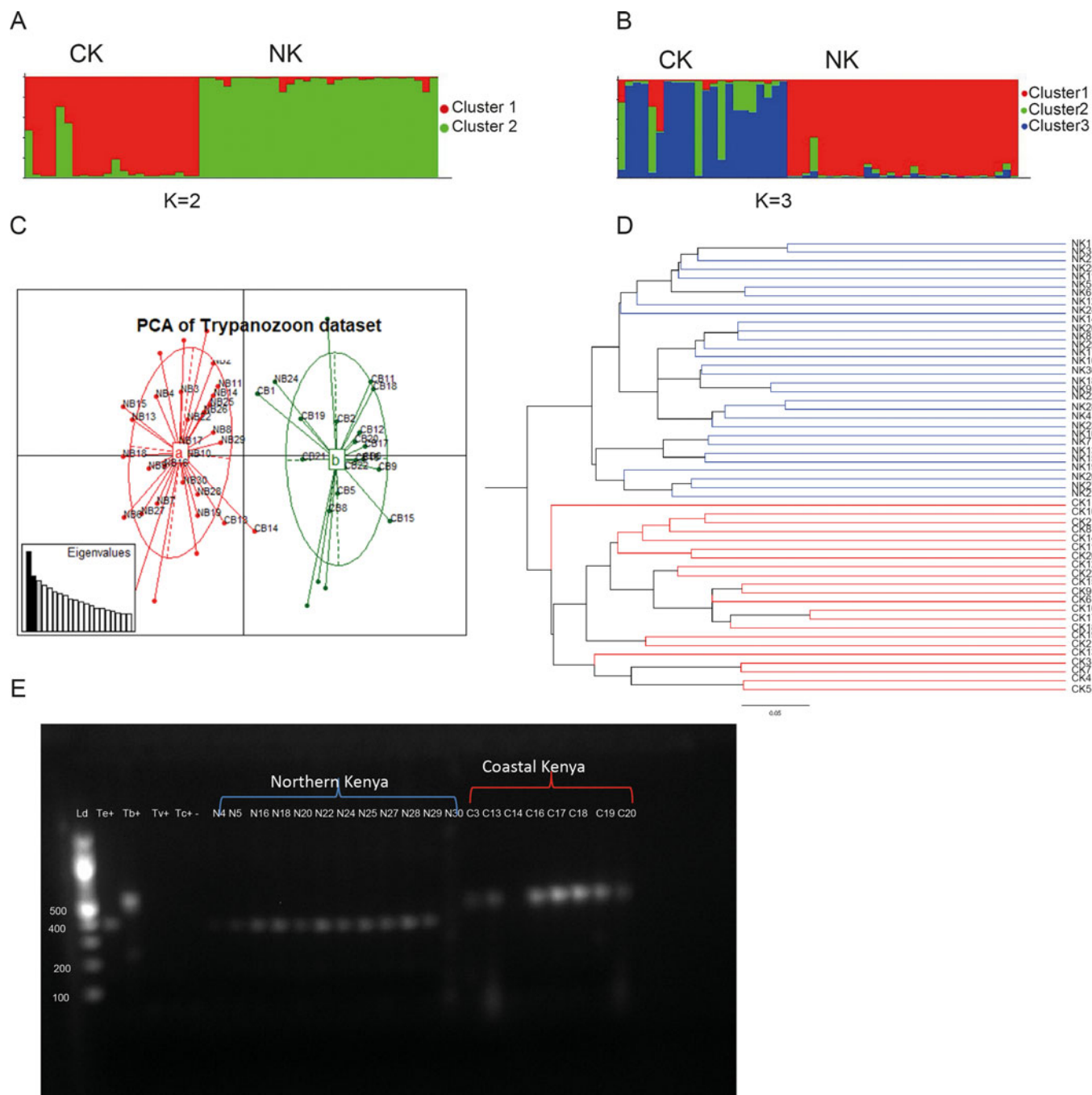
AMOVA results (Fig. 3) revealed that 99% of the variation was caused by differences in genotypes within isolates and differences between populations accounted for 1% of the variation.

The partial Mantel test results revealed a statistically significant correlation ( $r = 0.3493$ ,  $P = 1 \times 10^{-4}$ ) between pairwise genetic distances  $F_{ST}$  ( $1/1 - F_{ST}$ ) and geographical distances (Haversine distances) while controlling for environmental distances (Euclidian distances). Recent emigration and migration rates between the coastal and northern populations were 0.0113 and 0.0391, respectively. The high rate of self-distribution within both populations was an indication of asymmetric gene flow within the populations (Table S4). Gene flow ( $N_e m$ ) or the effective number of migrants in the coastal and northern populations was 3.801 and 3.803, respectively.

### Trypanosoma vivax

#### Genetic structure

A  $K$  value of 3 was suggested as the most likely level of hierarchy of population structure by Bayesian analysis (Fig. 4A). Cluster 'a' is made up of 19 *T. vivax* isolates all of which were from Northern Kenya. Cluster 'b' had 20 isolates, 17 of which were from Coastal Kenya and three from Northern Kenya. Cluster 'c' had 21 isolates, 13 of which were from Coastal Kenya and eight from Northern Kenya. Assignment of the *T. vivax* isolates to specific clusters based on the  $Q$  values is provided in Table S3. As seen from the  $Q$  values, a number of isolates from the two different sampling localities displayed variable degrees of assignment to specific



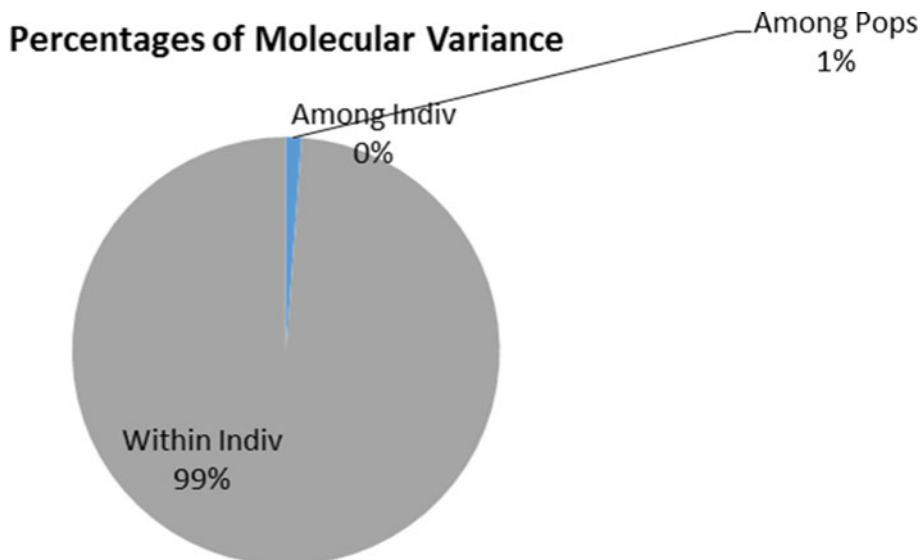
**Fig. 2.** Clustering analysis results of 52 *T. (Trypanosoon)* isolates (*Trypanosoma brucei* and *Trypanosoma evansi*) from Northern (NK) and Coastal (CK) regions in Kenya based on 14 microsatellite loci: (A, B) represent Bayesian clustering based on STRUCTURE results. (C) represents a PCA showing genetic population structure of the Trypanosoon isolates. Cluster a (red) consists mainly of Northern Kenyan isolates other than isolates CB13 and CB14, whereas cluster b (green) consists mainly of Coastal Kenya. (D) represents an UPGMA dendrogram for Trypanosoon isolates constructed based on 1000 bootstraps using Cavalli-Sforza and Edwards distances. The red branches are of Northern Kenyan isolates whereas the green branches are of Coastal Kenyan isolates. (E) Agarose gel electrophoresis (1.2%) performed on kDNA PCR amplicon PCR products were resolved through 1% ethidium-bromide stained agarose gel at 80 V for 1.5 h. Lane Kd M 100-bp marker (Thermo Scientific, USA) Te+, *T. evansi* (KETRI 2479) positive control; Tb+, *T. brucei* ILTat 1.4 positive control; Tv+, *Trypanosoma vivax* IL 2136 positive control; Tc+, *Trypanosoma congolense* savannah (IL3000) positive control; –, negative control; the rest *T. (Trypanosoon)* samples from Northern Kenya and Coastal Kenya.

clusters, which may be evidence of long-range dispersal between the sampling localities.

Multivariate analysis for *T. vivax* isolates confirmed the presence of three distinct genetic clusters (Fig. 4B). None of the three clusters were in the same multivariate space. PC axis 1 separated cluster a from clusters b and c whereas PC axis 2 separated cluster b from cluster c. Cluster a had 18 isolates from Northern Kenya, cluster b had 11 isolates from Northern Kenya and 17 isolates from Coastal Kenya. Cluster c had only one isolate from Northern Kenya and

13 from Coastal Kenya. Multivariate analyses such as Bayesian analysis revealed the separation of a group of Northern Kenyan isolates from Coastal Kenyan isolates (Fig. 4B and C).

Furthermore, an UPGMA dendrogram was constructed to show the relationship between *T. vivax* isolates from tsetse-free areas and isolates from tsetse-endemic areas (Fig. 4C). The dendrogram largely confirmed results from Bayesian and multivariate analyses. It revealed the presence of three distinct clusters, one with a group of NK isolates similar to in cluster 'a' in



**Fig. 3.** AMOVA results on Trypanozoon isolates based on 14 microsatellite markers.

**Table 1.** Genetic diversity among *T. (Trypanozoon)* isolates from both sampling origin-based populations and STRUCTURE

Pop	<i>N</i>	<i>N<sub>a</sub></i>	<i>N<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>uH<sub>e</sub></i>	<i>F</i>	<i>I</i>	<i>A<sub>r</sub></i>
a (red)	29	9.0	5.292	0.836	0.807	-0.053	1.804	4.487
b (green)	2	2.571	2.4	0.75	0.726	-0.444	0.882	-
c (blue)	14	7.286	4.604	0.759	0.772	0.011	1.625	4.201
Overall	52	6.286	4.099	0.809	0.815	-0.148	1.625	4.344
Pop	<i>N</i>	<i>N<sub>a</sub></i>	<i>N<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>uH<sub>e</sub></i>	<i>F</i>	<i>I</i>	<i>A<sub>r</sub></i>
CK	22	8.429	5.082	0.79	0.819	0.023	2.026	5.33
NK	30	10.857	6.701	0.835	0.813	-0.044	1.752	5.08
Overall	52	9.643	5.892	0.812	0.816	-0.01	1.889	5.205

Parameters *N* (sample size), *N<sub>a</sub>* (number of different alleles), *N<sub>e</sub>* (number of effective alleles), *H<sub>o</sub>* (observed heterozygosity), *uH<sub>e</sub>* (unbiased expected heterozygosity), *F* (fixation index) and *I* (Shannon's information index). Allelic richness for cluster 'b' with *N* below 4 could not be calculated.

STRUCTURE and PCA with all isolates from Northern Kenya. One other cluster had a majority of isolates from CK and the third cluster had isolates from both localities.

#### Genetic diversity and HWE

Only one (TB8/11 and TB1/8) of 30 loci combinations was in linkage disequilibrium ( $P < 0.05$ ). Yet, all but loci TB1/8 showed deviation from the HWE in at least one of the sampling localities. There were 69 different alleles identified with the effective allele size estimated at 3.635. Within sampling localities, allelic richness was 6.094 and 6.011 for Northern Kenyan and Coastal Kenyan populations, respectively. The average number of private alleles for Northern Kenyan and Coastal Kenyan populations was 3.33 and 3.667, respectively. Shannon's information index (*I*) of the populations was estimated at 1.502 and 1.531, respectively. Observed heterozygosities were 0.734 and 0.732 and the expected heterozygosities (Nei's gene diversity) were 0.698 and 0.718 for the northern and coastal populations, respectively. Inbreeding coefficient ( $F_{IS}$ ) values were -0.082 and -0.065. Among STRUCTURE-defined clusters, allelic richness ranged from 5.21 in cluster 'b' to 3.49 in cluster 'c'. Observed heterozygosity ranged from 0.667 in cluster c (blue) to 0.832 in cluster b (green) and expected heterozygosity ranged from 0.662 in cluster c (blue) to 0.806 in cluster b (green) (Table 2). The average numbers of private alleles were 2.167, 2.833 and 1.167 and the average numbers of different alleles and 5.33, 6.667 and 4.167 for clusters a (red), b (green) and c (blue), respectively (Table 2).

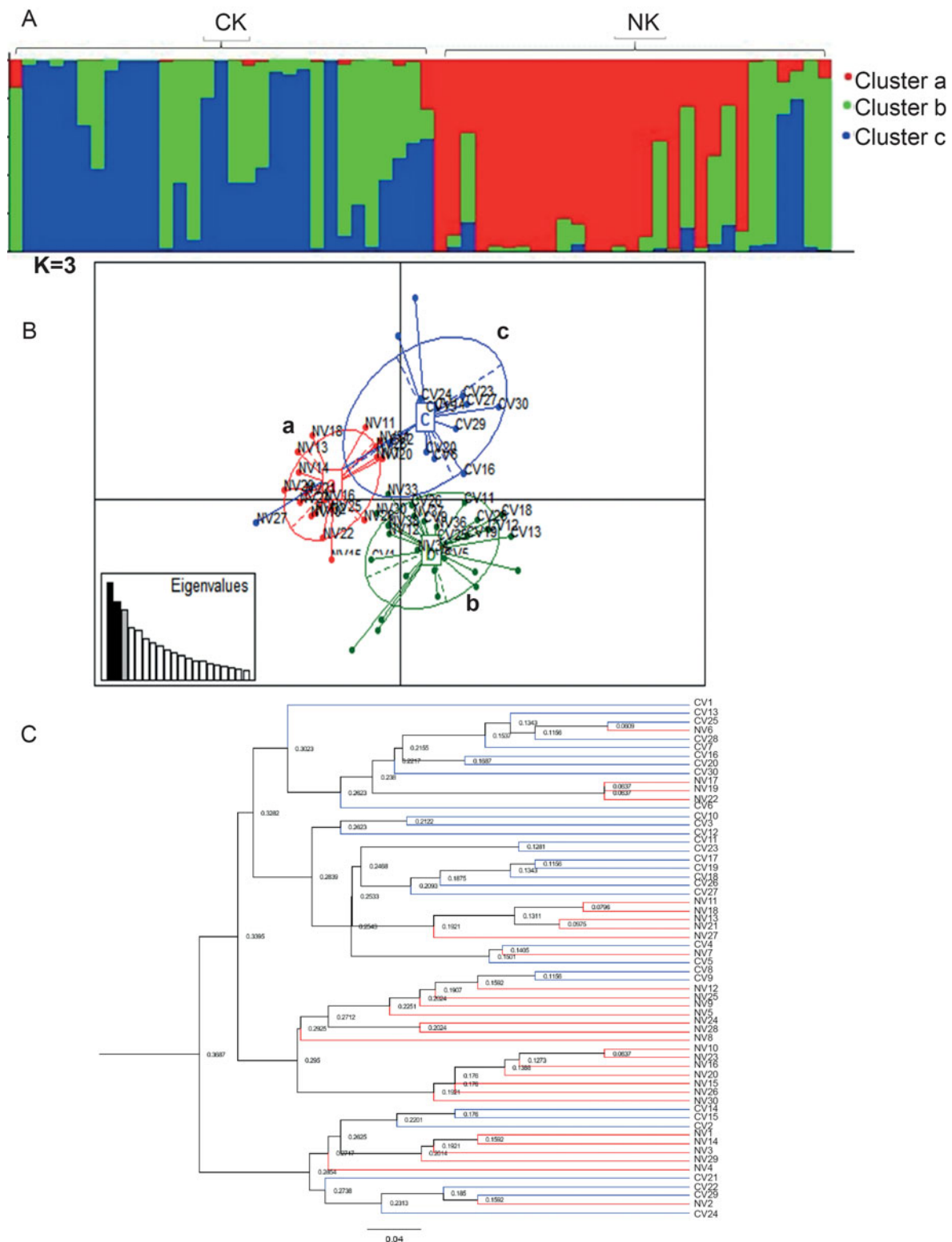
#### Genetic differentiation and gene flow analysis

Genetic differentiation between the sampling localities-based populations was significantly differentiated ( $F_{ST} = 0.099$ ,  $P = 0.005$ ). Between structure-defined clusters,  $F_{ST}$  values were 0.182 between clusters 'a' and 'c', 0.102 between clusters 'b' and 'c' and 0.053 between clusters 'a' and 'b' with significant differentiation in all cluster pairs ( $P < 0.05$ ). AMOVA results for *T. vivax* showed that 91% of the variation was due to differences in genotypes within individuals while differences between the sampling localities-based populations accounted for 6% of the variance (Fig. 4C). Differences between isolates in a population only accounted for 3% of the total variance. These results indicate that genetic differentiation among *T. vivax* isolates was not explained by sampling location but largely by differences in genotypes within the isolates (Fig. 5).

According to the partial Mantel test results, there was significant correlation between genetic distances and geographical distances while controlling for environmental conditions ( $r = 0.3846$ ,  $P = 1 \times 10^{-4}$ ).

Emigration and immigration rates between the Coastal Kenyan and Northern Kenyan populations were 0.0238 and 0.0356, respectively. Asymmetric gene flow within both populations was evident due to the high self-distribution recorded (Table S4). The effective number of migrants ( $N_{em}$ ) was equal in both populations at 2.275. Being that  $1 < N_{em} < 4$ , the two populations are genetically close but gene flow between them is limited.

These results suggest moderate gene flow between the populations regardless of isolation by distance. This may be due to the



**Fig. 4.** Clustering analysis results on 60 *T. vivax* isolates from Northern (NK) and Coastal (CK) regions in Kenya based on six microsatellite loci. (A) STRUCTURE results image of best fit at  $K$  ( $K=3$ ) based on the ad hoc statistic. (B) PCA image showing how *T. vivax* samples from both sampling locations are sub-structured. Cluster a is made up of isolates from Northern Kenya, cluster b has samples from both locations while cluster c has samples from only Coastal Kenya. (C) An UPGMA tree based on six microsatellite markers, constructed using Cavalli-Sforza and Edwards distances showing hierarchical structuring of *T. vivax* isolates. The blue branches represent isolates from Coastal Kenya whereas the red branches are of isolates from Northern Kenya.

introduction of *T. vivax* in one population from the other due to animal movement, from tsetse-infested areas of Kenya or from the region. Migration rate results indicate that migration is low and non-directional. Therefore, the parasites are introduced in both populations from the adjacent population.

### *Trypanosoma congolense*

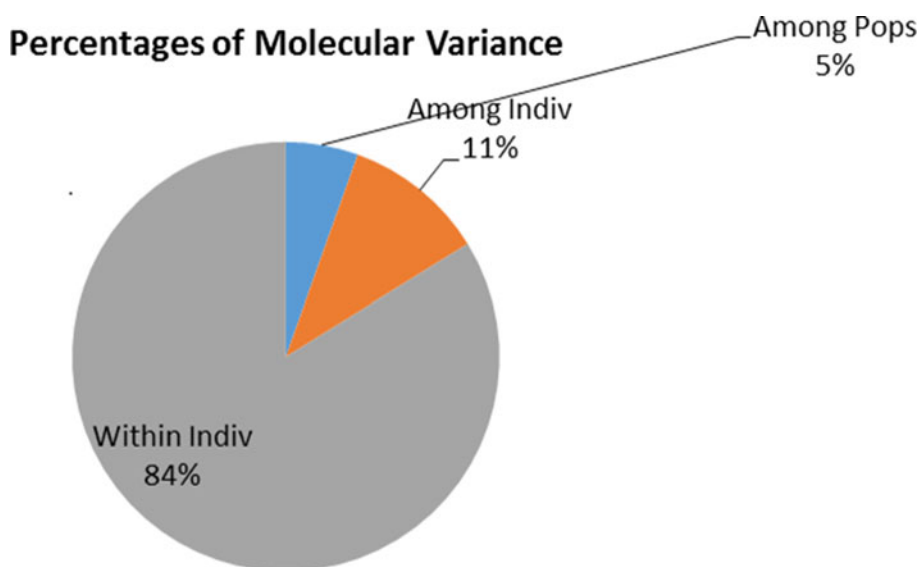
#### Population structure

Bayesian clustering analysis (STRUCTURE results) was consistent with hierarchical clustering results on the presence of two distinct genetic clusters (Fig. 6A). All NK isolates grouped together with

**Table 2.** Genetic diversity of *Trypanosoma vivax* isolates within sampling origin-based populations and STRUCTURE-based populations subject of six microsatellite loci

Pop	<i>N</i>	<i>N<sub>a</sub></i>	<i>N<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>uH<sub>e</sub></i>	<i>F</i>	<i>I</i>	<i>A<sub>r</sub></i>
CK	30	8.167	3.673	0.732	0.718	−0.065	1.531	6.011
NK	30	7.833	3.597	0.734	0.698	−0.082	1.502	6.094
Overall	60	8	3.635	0.733	0.708	−0.073	1.52	6.053
a (red)	18	5.333	3.11	0.763	0.652	−0.211	1.253	3.872
b (green)	10	6.667	4.941	0.789	0.798	−0.067	1.639	5.21
c (blue)	13	4.167	2.776	0.61	0.634	−0.109	1.14	3.499
Overall	41	5.389	3.61	0.744	0.695	−0.129	1.344	4.145

*N*, sample size; *N<sub>a</sub>*, number of different alleles; *N<sub>e</sub>*, number of effective alleles; *H<sub>o</sub>*, observed heterozygosity; *uH<sub>e</sub>*, unbiased expected heterozygosity; *F*, fixation index; *I*, Shannon's information index.

**Fig. 5.** AMOVA analyses results based on six microsatellite loci on *T. vivax* isolates from Northern and Coastal Kenyan regions

CK isolates indicating no separation. To determine the level of population structure based on the genetic distance matrix, an UPGMA dendrogram of similarity based on Cavalli-Sforza and Edwards, pairwise genetic distances was constructed (Fig. 6C). The results showed no separation between the Northern Kenyan and the Coastal Kenyan isolates and were consistent with Bayesian analysis. In addition, the dendrogram revealed the presence of two clusters and two isolates (C10 and C1) sharing a multilocus genotype (MLG). However, the PCA data disputed the hierarchical and Bayesian clustering results and revealed three clusters but agreed with both on no separation between NK and CK isolates.

### Genetic diversity

Hardy–Weinberg proportions from allele frequency data showed deviation from the HWE in at least one population of all loci. One reason for deviation from the HWE is limited genetic exchange between isolates due to predominant clonal reproduction. Linkage disequilibrium analysis revealed that only one (TB8/11 and TB6/7) of ten loci combinations was in linkage disequilibrium. This is an indication that loci used in this analysis are evenly distributed within the genome, which is favourable. However, results of linkage disequilibrium among loci within Northern Kenyan isolates were inconclusive due to the limited number of isolates in the population

Genetic diversity analysis revealed the presence of 42 alleles across the two populations. Allelic richness (with rarefaction) for the northern and coastal populations was 3.56 and 2.80,

respectively, thus indicating greater genetic diversity within the coastal population. The observed mean heterozygosities were 0.668 and 0.667 whereas the expected heterozygosities were 0.710 and 0.653 for the northern and coastal populations, respectively. Inbreeding coefficient ( $F_{IS}$ ) was −0.195 and 0.009, respectively, as displayed in Table 3.

### Differentiation and gene flow analysis

We estimated fixation index ( $F_{ST}$ ) between the coastal and northern populations at −0.028 ( $P = 0.713$ ). Negative  $F_{ST}$  value is statistically equal to 0 and the negative value is a result of sampling bias within the populations. A fixation index of zero is an indication of no differentiation between Coastal and Northern Kenyan isolates.

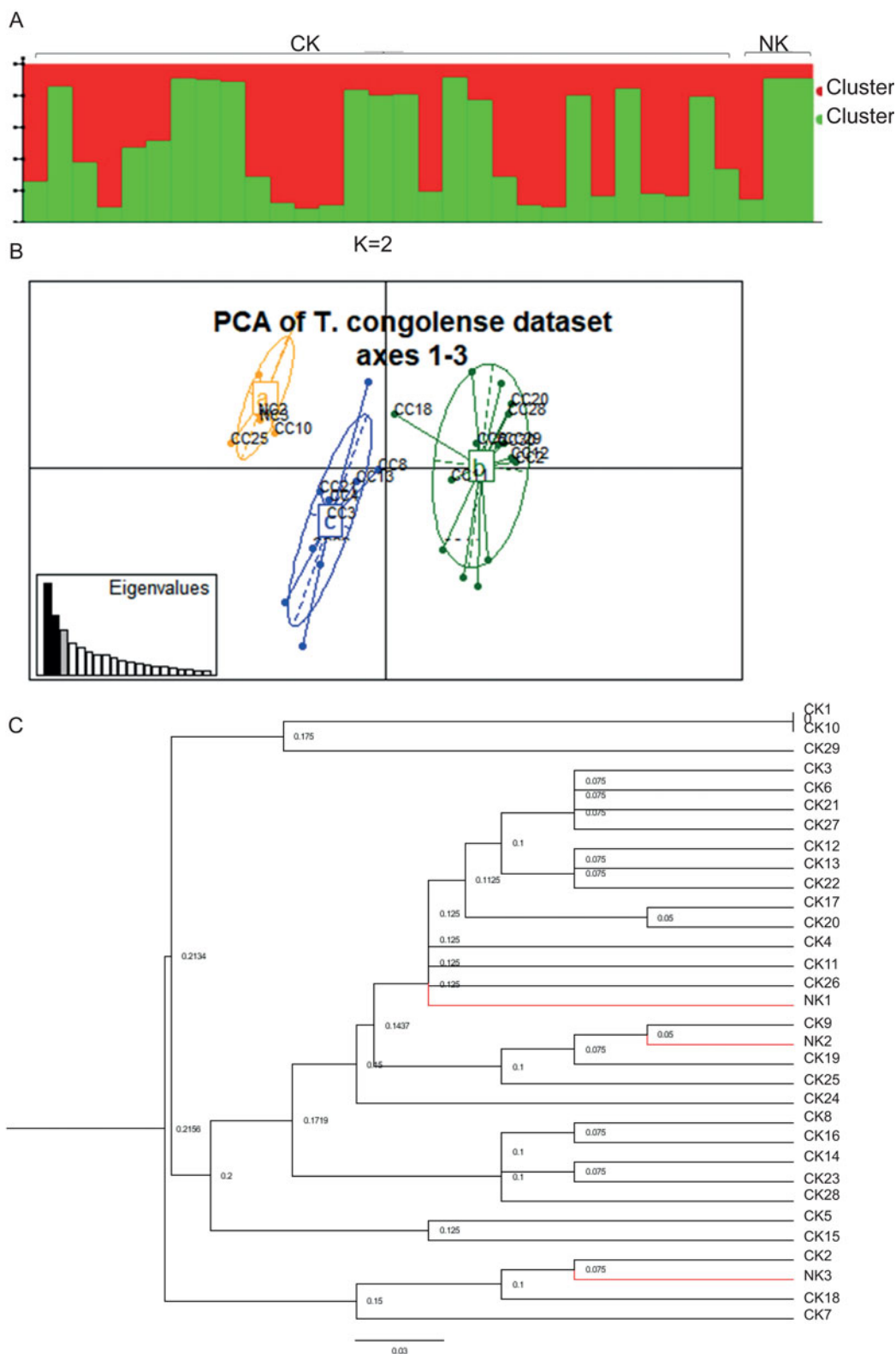
Partial Mantel test results revealed a positive but not significant correlation between geographical and genetic distances while controlling for environmental conditions ( $r = 0.311$ ,  $P = 0.3219$ ). This means that genetic structuring among the isolates was independent of sampling location.

Immigration and emigration rates from the coastal to the northern populations were 0.2612 and 0.033, respectively. Self-distribution rate within the coastal and northern populations was at 0.9667 and 0.788.

### Discussion

This study comprising population-genetic differentiation of three veterinary important trypanosome species from two distinct





**Fig. 6.** Clustering analysis results on 32 *T. congolense* isolates from Northern (NK) and Coastal (CK) regions in Kenya based on five microsatellite loci. (A) STRUCTURE HARVESTER results based on the ad hoc statistics indicated  $K=2$  as the best  $K$  value. (B) A PCA showing structuring of *T. congolense* isolates. The clusters are clearly separated and in different PC components. Isolates from Northern Kenya are however not genetically separated from isolates from Coastal Kenya. (C) An UPGMA dendrogram based on Cavalli-Sforza distances showing no separation of *T. congolense* between Coastal Kenyan and Northern Kenyan isolates.

localities, i.e. from tsetse-infested and non-tsetse-infested area has demonstrated close interspecies phylogenetic relationships, for example among *T. congolense* isolates regardless of the variations in the mode of transmission, climate and vectors present. However, we have also seen that some trypanosomes such as *T.*

(*Trypanozoon*) are distinct between the two sites demonstrating local adaptation and evolution. Individual trypanosome species were found to be distributed across different ecological settings, spanning from wet Coastal Kenya infested with tsetse flies to dry Northern Kenya, where there are no tsetse flies. Most host-

**Table 3.** Sample size and genetic diversity of *Trypanosoma congolense* isolates within sampling origin-based populations

Pop	<i>N</i>	<i>N<sub>a</sub></i>	<i>N<sub>e</sub></i>	<i>H<sub>o</sub></i>	<i>uH<sub>e</sub></i>	<i>F</i>	<i>I</i>	<i>A<sub>R</sub>*</i>
CK	28.6	8.6	3.617	0.668	0.711	0.01	1.562	2.8
NK	3	2.6	2.354	0.667	0.653	-0.195	0.861	3.56
Overall	15.8	5.6	2.986	0.667	0.682	-0.093	1.212	3.18

*N*, sample size; *N<sub>a</sub>*, number of different alleles; *N<sub>e</sub>*, number of effective alleles; *H<sub>o</sub>*, observed heterozygosity; *uH<sub>e</sub>*, unbiased expected heterozygosity; *F*, fixation index; *I*, Shannon's information index calculated in GenALEX v6.5 (Peakall and Smouse, 2006); *A<sub>R</sub>\**, allelic richness calculated in HP-RARE (Kalinowski, 2005).

parasite systems exhibit remarkable heterogeneity in the contribution of transmission of certain individuals, locations, host infectious states or parasite strains. It was noted in this study that both sites have domestic animals and blood feeding insects, the only differences being the absence of camels in the coastal region, which are abundant in Northern Kenya and the absence of tsetse flies in Northern Kenya which are abundant in Coastal Kenya. All three trypanosomes: *T. (Trypanozoon)*, *T. congolense* and *T. vivax* were encountered in both regions.

### T. (Trypanozoon)

*Trypanosoma (Trypanozoon)* isolates clustered into two genetic clusters by Bayesian clustering in STRUCTURE (Fig. 2A). A majority of isolates in each cluster were from one of the two sampling locations indicating genetic distinctness of isolates from each population. These results are consistent with both multivariate and hierarchical clustering results, both of which grouped the isolates into two groups each dominated by isolates from each sampling location. Clustering of these isolates mainly by geographical origin is due to the presumed presence of different *T. (Trypanozoon)* species: *T. evansi* and *T. brucei* in the two geographical regions. *Trypanosoma evansi*, which is exclusively transmitted mechanically, is mainly found in tsetse-free Northern Kenya, whereas *T. brucei* is present in the coastal region where tsetse flies are abundant (Getahun *et al.*, 2020). Because of its diskinetoplastidy, *T. evansi* can no longer complete cyclical development in the tsetse flies (Lai *et al.*, 2008). However, we cannot rule out its circulation in tsetse-infested areas as tsetse flies do transmit *T. evansi* mechanically (Roditi and Lehane, 2008) and other domestic and wild animals are hosts and reservoirs of the parasite. Nonetheless, previous genomic analyses of *T. evansi* and *T. brucei* isolates have shown that the species are not always distinguishable even with sequencing (Büscher *et al.*, 2019; Getahun *et al.*, 2020).

The next best fit of *K* (*K* = 3) in STRUCTURE revealed a level of sub-structuring within Coastal Kenyan isolates (*T. brucei*) and genetic homogeneity among Northern Kenyan *T. (Trypanozoon)* (*T. evansi*). These, together with greater genetic diversity among coastal *T. (Trypanozoon)* (*T. brucei*) evidenced by greater allelic richness, Shannon's information index (*I*), and average private allele values (Table 1) compared to northern *T. (Trypanozoon)* (*T. evansi*) isolates is consistent with findings from previous studies on microsatellite genotyping of the *T. (Trypanozoon)* group which revealed that *T. evansi* and *T. brucei* are closely genetically related to each other (Claes *et al.*, 2005; Carnes *et al.*, 2015; Wen *et al.*, 2016; Kamidi *et al.*, 2017) and that *T. brucei* displays significant sub-structuring and genetic diversity within the species (Echodu *et al.*, 2015; Kamidi *et al.*, 2017). Isolates with *Q* values below 0.8 revealed ambiguous assignment into either of the three clusters. Ambiguous assignment may be evidence of occurrence of genetic admixture, migration and subsequent mating between local and immigrant strains or shared ancestry. In addition, it may be an indication of the limitation of the microsatellite markers used to separate recently diverged taxa into distinct clusters (Pritchard *et al.*, 2000).

Ninety-six percent of loci were in the linkage equilibrium. This means that genotypes at majority of the loci were independent of genotypes on other loci. Overall, *H<sub>o</sub>* values were lower than *H<sub>e</sub>* values among all isolates. This may be credited to deviation from HWE, Wahlund effect and null alleles (Li *et al.*, 2019). Negative inbreeding coefficient (*F<sub>IS</sub>*) among the Northern Kenyan (*T. evansi*) isolates may be explained by clonal or non-sexual reproduction. This is because, *F<sub>IS</sub>* is the proportion of the variance in the subpopulation contained in an individual and its calculation is dependent on observed and expected heterozygosities. Although clonal reproduction does not affect the observed heterozygosity, it does reduce the expected heterozygosity among isolates (Prugnolle and De Meeûs, 2008). *Trypanosoma evansi* strains are thought to reproduce clonally because the species does not transform into developmental stages that colonize the gut and salivary glands in tsetse flies where sexual reproduction occurs. However, there were no MLGs in the population and this casts doubt on the idea of exclusive of clonal reproduction among *T. evansi* isolates as did results by (Salim *et al.*, 2011).

Wright's fixation index (*F<sub>ST</sub>*) between the location of origin-based populations was statistically significant thus revealing genetic differentiation between Northern Kenyan (*T. evansi*) and Coastal Kenyan (*T. brucei*) *T. (Trypanozoon)* isolates. Furthermore, the low gene flow rate (*N<sub>e</sub>m* < 4) is a clear indication that the two populations are not panmictic, thus there exist barriers to gene flow. However, the effective number of migrants is greater than one in both populations, which is more evidence of the close genetic relationship and low-genetic differentiation between the two species. Nonetheless, isolation by distance analysis revealed that structuring and genetic differentiation within the *T. (Trypanozoon)* isolates was strongly dependent on the location of origin. In addition, AMOVA results showed that although the isolates are mainly structured based on location of origin, differences in genotypes within isolates was the greatest contributing factor to genetic differentiation and sub-structuring. This indicates that although they are very closely related genetically, the two species are fundamentally genetically distinct.

This may be evidence against the need for revision of the entire taxonomic unit *T. (Trypanozoon)* as proposed by Büscher *et al.* (2019). Classification of Trypanosome DNA is mainly based on ribosomal DNA genetic markers (Njiru *et al.*, 2004, 2005, 2006). Telling apart *T. brucei* and *T. evansi* with these markers is impossible with gel electrophoresis and at times difficult after sequencing (Büscher *et al.*, 2019; Getahun *et al.*, 2020). However, since a large number of microsatellite loci that amplify *T. (Trypanozoon)* genotypes have been identified (Balmer *et al.*, 2006; Salim *et al.*, 2011; Siström *et al.*, 2013), identification of private microsatellite marker bands (gel electrophoresis bands unique in each taxa) within the *T. (Trypanozoon)* group may present a potential for application of microsatellite loci as species identification markers to remove the problem of precise species identification. The amplification of kDNA in *T. (Trypanozoon)* samples obtained from both regions agrees with the limitation of kDNA to differentiate these two subspecies, as some *T. evansi*

are dyskinetoplastic (Borst *et al.*, 1987; Schnauffer, 2010; Büscher *et al.*, 2019).

### *Trypanosoma vivax*

From this study, STRUCTURE and PCA results suggest that *T. vivax* isolates are clustered into three distinct genetic groups. The results are consistent with previous phylogenetic studies on *T. vivax* which indicated genetic heterogeneity within the species especially in East Africa (Fasogbon *et al.*, 1990; Duffy *et al.*, 2009). These results also suggest that *T. vivax* isolates within clusters were more similar in geographical origin which might be affected by local adaptation such as mode of transmission, vertebrate hosts and environmental factors. The clustering of a group of Northern Kenyan isolates separately shows an independent evolution due to differences in geographical factors, hosts and vectors which may be a driving force of genetic separation. However, the identification of two distinct clusters in Coastal Kenya in the same ecosystem indicates that the existence of different *T. vivax* strains demonstrating genetic differentiation can result in the same habitat, which may be an indication that other factors, other than geographical variation, can contribute to genetic heterogeneity. In addition, the possibility of strains specific to different livestock host species cannot be ruled out.

Five out of six loci showed deviation from the HWE which may be due to predominant clonal reproduction of *T. vivax* populations. Genetic diversity in the Northern Kenyan population was relatively lower compared to the Coastal Kenyan population. This was inferred from results of genetic diversity measures: average number of alleles, Shannon's information index, Nei's gene diversity ( $H_E$ ) and allelic richness, all of which had lower values of the Northern Kenyan population. Among STRUCTURE-defined clusters (Fig. 3), cluster a (red) with all isolates from Northern Kenya had higher genetic diversity compared to cluster c (blue) with the highest number of Coastal Kenyan isolates which may be attributed to disparity in population size which leads to bias in allele frequencies. Cluster b (green) with isolates from both sampling locations however revealed the greatest genetic diversity. These results of greater genetic diversity among tsetse-borne *T. vivax* agree with findings from previous studies of greater diversity among *T. vivax* from tsetse-endemic areas (Rodrigues *et al.*, 2008). Greater observed heterozygosities, deviation from the HWE and negative  $F_{IS}$  among Coastal Kenyan isolates suggests that the population is clonal. These results suggest that the *T. vivax* populations studied here are predominantly clonal and agree with previous studies that revealed clonal reproduction *T. vivax* including in tsetse-borne *T. vivax* (Duffy *et al.*, 2009). However, previous studies on *T. vivax* isolates have revealed that meiosis-associated genes in *T. brucei* are highly conserved in *T. vivax* and therefore we cannot completely eliminate the possibility of genetic recombination among *T. vivax* strains (El-Sayed *et al.*, 2005). Given that these results suggest that *T. vivax* populations studied here are clonal, the high number of unique genotypes in the populations may be due to amplification failure, null alleles or dropout alleles.

The Northern Kenyan isolates are significantly differentiated from the Coastal Kenyan isolates based on Wright's  $F_{ST}$ . Among STRUCTURE-defined clusters, the greatest differentiation observed was between cluster a (red), with all Northern Kenyan isolates and cluster b (green) with a mixture of isolates from both populations and the least differentiation was between clusters a (red) and c (blue) with a majority of isolates from the Coastal Kenyan population. This revealed that cluster b (green) is significantly differentiated from other clusters. These results agree with AMOVA results that revealed that differences between isolates in a subpopulation accounted for more variance than did

differences between subpopulations. In addition, isolation by distance results exposed further evidence that the structuring of the *T. vivax* isolates was dependent on geographical origin. However, the findings suggest moderate gene flow between the populations regardless of isolation by distance. This is an indication that the populations are genetically close with the limited gene flow likely due to the barrier caused by the geographical distance between them.

However, a group of Northern Kenyan isolates cluster together with a group of Coastal Kenyan isolates in STRUCTURE analysis and this cluster (b – green) shows a significant differentiation from other clusters dominated by isolates from both sampling locations. Therefore, it is likely that the isolates in this subpopulation have undergone independent evolution and therefore they cluster together. These results therefore agree with the idea that heterogeneity among African *T. vivax* isolates is linked to biological transmission by tsetse flies. *Trypanosoma vivax* isolates from tsetse-free Northern Kenya are genetically separated from isolates from the tsetse-endemic Coastal Kenyan region. These observations therefore further dispute sequence analysis results of the rDNA of *T. vivax* strains from tsetse-free and tsetse-infested areas of Ethiopia that indicated that genetic heterogeneity among the strains is not linked to their geographical origin. This then means that greater genetic heterogeneity among African *T. vivax* strains compared to Latin American strains is associated with mode of transmission.

### *Trypanosoma congolense*

All the three clustering analysis methods used failed to separate *T. congolense* isolates from NK from *T. congolense* isolates from the CK region. Wright's fixation index  $F_{ST}$  results also show that the former are not genetically differentiated from the latter. In addition, isolation by distance analysis revealed that genetic structuring within the population was independent of sample's location of origin. Trypanosomes undergo genetic adaptation events that allow them to utilize different energy sources in vectors and hosts (Ooi *et al.*, 2016; Szöör *et al.*, 2020). *Trypanosoma brucei*, for example, has mitochondrial genes that allows it to utilize  $\alpha$ -ketoglutarate as an energy source in the tsetse midgut (Szöör *et al.*, 2020). *Trypanosoma evansi* lacks these genes and thus it has lost its ability for biological transmission. Therefore, genetic data would reflect intra-species differences in isolates with different transmission mechanisms. With these considerations in mind, our findings do not support the hypothesis that *T. congolense* from tsetse-free areas are being maintained by unknown biological vectors first proposed by Gibson *et al.* (1983). However, the small number of samples in the Northern Kenyan population makes the findings indicative and not conclusive and therefore, there is need for more population-genetic studies on *T. congolense* from tsetse-free and tsetse-infested areas with a large number of samples.

*Trypanosoma congolense* isolates have, however, been isolated in livestock blood and biting flies from tsetse-free areas of Northern Kenya for a long period of time (Gibson *et al.*, 1983; Getahun *et al.*, 2020) Yet, these results show no genetic separation between Northern Kenyan and Coastal Kenyan (tsetse-borne) isolates which suggests that the parasite is probably introduced in these areas from livestock that travels to tsetse-inhabited areas for pasture and water. Also, these results may suggest the presence of tsetse flies in Northern Kenyan regions but at very low densities hard to detect densities that need detailed wide survey of tsetse flies.

Our data provide novel insights into the population structure and genetic range of the three trypanosome species from the two ecological settings. The major findings of this work confirm that genetic heterogeneity among African *T. vivax* strains is linked

to biological transmission. *T. (Trypanozoon)* microsatellite analysis results show clear separation of Coastal Kenyan and Northern Kenyan isolates. These results also reveal the need to explore application of microsatellite loci in *T. (Trypanozoon)* taxa identification. Lastly, the results reveal no genetic separation of *T. congolense* strains from tsetse-free and tsetse-endemic areas, even though we have a small sample size from Northern Kenya.

## Conclusion

All three trypanosome species were found to be widely distributed across different ecological settings, spanning from wet Coastal Kenya infested with tsetse flies to dry Northern Kenya, where there are no tsetse flies. With regards to this, we found genetic diversity and a clear separation of *T. vivax* and *T. (Trypanozoon)* isolates between the two ecosystems but not between *T. congolense* isolates. In future studies, optimizing microsatellite-based markers to differentiate *T. brucei* and *T. evansi* needs to be investigated. Furthermore, trypanosomes from more livestock host and vectors need to be analysed to find out if there is specific adaptation of trypanosomes to a given host.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182021001815>.

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**Author contributions.** NNK conceived and performed the experiments, analysed data and wrote the manuscript. KMK analysed data. NOA and TM reviewed the experimental design and manuscript. MNG envisioned the research idea, mobilized resources, generated data, supervise the research and reviewed the manuscript.

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**Conflict of interest.** None.

**Ethical standards.** We collected blood samples within the framework of epidemiological surveillance activities, in accordance with the International Centre of Insect Physiology and Ecology's Institutional Animal Care and Use Committee (IACUC) guidelines, in accordance with protocols approved by the International Centre of Insect Physiology and Ecology's Institutional Animal Care and Use Committee (IACUC) guidelines (approval number: icipe-IACUC-10/2018.1).

**Data availability.** All data generated or analysed in this study are included in the article and as Supplementary files.

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