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Michael Frederic Mengual
Yale University, michael.mengual@yale.edu

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Title
Spatial and Temporal Dynamics of *Glossina pallidipes* in Nguruman National Park (Kenya).

Thesis Advisor
Dr. Adalgisa Caccone.

Second Reader
Dr. James Childs.

Keywords

Glossina pallidipes, Nguruman, Kenya, tsetse, vector, trypanosomiasis

Acknowledgements

Dr. Adalgisa Caccone, Dr. Norah Saarman, Kirstin Dion, Carol Mariani, Robert Opiro,

and Dr. Augustine Dunn.

Abstract:

Background: *Glossina pallidipes* is a significant vector of trypanosomiasis for humans and livestock across Sub-Saharan Africa. Nguruman national park in Kenya is regarded as a potential location for targeted vector control activity. We screened flies from three locations in Nguruman in 2003, 2009, and 2015 using 13 microsatellite loci to assess population structures of *G. pallidipes*.

Methods: We acquired Tsetse samples from three sites across Nguruman in Kenya at three time points. We then performed genetic analyses on 267 flies using 13 microsatellite loci and evaluated genetic structure of Tsetses across a geographic distance of about 22 km and changes associated with sampling at 3 different time points separated by a maximum of 12 years.

Results: We found that samples collected in different locations separated by up to 22 km within the same year were not significantly differentiated from one another. However, samples separated temporally were significantly differentiated.

Conclusion: Previous studies on the population structure of *G.pallidipes* in East-Africa have found population structure at variable geographic scales, with population structure across just 5 km in some cases. Our study shows that in Nguruman, the genetic structure of *G.pallidipes* is dominated by a temporal signal in samples collected across 12 years, but do not display population structure at the small spatial scale investigated.

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Specific Aims and Hypotheses

This study aims at evaluating the shifts in genetic structure of *G. pallidipes* across both space and time in Nguruman National Park (Kenya). We hope to use genetic structure information of this vector species to better inform future vector control efforts to decrease the burden of animal African trypanosomiasis (AAT or Nagana) in Kenya.

Introduction

Nguruman is a national park located in Southwestern Kenya. Kenya is a developing country that still suffers disproportionately from the burden of infectious diseases (Feikin et al. 2011). Furthermore, it has been shown that rural areas in Kenya suffered a greater burden from infectious diseases compared to urban areas (Feikin et al. 2011). African trypanosomiasis is a disease of both humans and animals that mainly impacts rural areas (WHO . 2008). The World Health Organization (WHO) reported that there were no reported cases of human African trypanosomiasis (HAT) in Kenya in 2014 (WHO. 2016). However, the burden of AAT as well as the potential re-emergence of HAT remain significant issues.

The burden of AAT as well as the potential re-emergence of HAT in Kenya may be significantly impacted by targeted interventions to control *G.pallidipes* in Nguruman. AAT is a major impediment to the development of animal husbandry in Nguruman with the trypanosome being recovered from the blood as much as 40% of cattle tested (Tarimo-Nesbitt et al. 1999). Accordingly, the cattle of Nguruman's pastoralist Maasai community are negatively affected by AAT (Tarimo-Nesbitt et al. 1999). *G. pallidipes*

and *G. longipennis* are the main vectors for Nagana in Nguruman with *G. pallidipes* accounting for most of the infections (Tarimo-Nesbitt et al. 1999).

Tsetse flies of the morsitans goup such as *G. pallidipes* have been shown to be the main vectors for the trypanosomes which cause Nagana in cattle, mainly: *T. b. brucei* and *T. congolense* (Geiger et al. 2015). Furthermore, the movement of cattle infected with *T. b. rhodesiense* has been implicated in new outbreaks of HAT in Uganda (Fevre et al. 2001). AAT is prevalent in some of the poorest areas in Sub-Saharan Africa and imposes a significant burden on development in this region (WHO . 1998). Cattle account for 70% of the ruminant biomass in affected areas with costs estimated between $0.7 and $4.5 billion (Shaw et al. 2013). It has also been estimated that a successful control of AAT could result in an additional income of $2.5 billion in yearly agricultural development (Shaw et al. 2013).

Trypanosomes are transmitted through the bites of Tsetse flies during their blood meal. There are currently thirty-two known species and sub-species of Tsetse and all are capable of transmitting human infectious trypanosomes (Wamwiri et al. 2015).
Tsetse flies are known to belong to three distinct groups: the savannah species (*G. morsitans),* the riverine species (*G. palpalis)*, and the primary forest species (*G. fusca)* (WHO . 1998). *G. pallidipes* is a member of the *G. morsitans* group and is considered as the most economically important specie of Tsetse (Krafsur et al. 2002). Information derived from blood meals has shown that Tsetse flies can feed on a wide variety of hosts (Allsopp et al. 1972). Additionally, Tsetse flies exhibit highly specific host preferences in different contexts (Allsopp et al. 1972).

G. pallidipes has been shown to feed overwhelmingly on a few species of wild game and also on domestic cattle and humans but at far lower rates (Allsopp et al. 1972). All Tsetse fly belts across Kenya have shown to be dominated by *G. pallidipes* carrying pathogenic trypanosomes (Njiru et al. 2004b). *G. pallidipes* in Kenya was shown to be predominantly infected with *T. congolense* and *T. vivax* with multiple co-infections being common in the flies (Njiru et al. 2004b).

G. pallidipes is one of the main vectors that transmit the trypanosomes to humans (Simarro et al., 2012). *G. pallidipes* is also believed to play an important role in the maintenance and transmission of the trypanosomes in their animal reservoirs as they have a preference for these hosts (Allsopp et al. 1972). Crucially, *G. pallidipes* is shown to take blood meals from humans when the host is available (Allsopp et al. 1972). It is therefore believed that *G. pallidipes* plays a central role in the disease burden of both HAT and AAT (Roger et al, 2004).

AAT is caused by several species of trypanosomes, including: *Trypanosoma brucei rhodesiense, T. b. brucei, T. b. gambiense, T. vivax, T. congolense, T. simiae, and T. godfreyi* (Krafsur et al. 2002). Both *T. b. brucei,* and *T. congolense* have been shown to be the main causative agents of AAT in livestock in sub-Saharan Africa (Geiger et al. 2015). Testing of the cattle in Nguruman showed that *T. vivax* and *T. brucei* were the most prevalent trypanosomes (Tarimo-Nesbitt et al. 1999). It has also been shown that both *T. b. brucei* and *T. b. rhodesiense* are dependent on an animal reservoir for their survival (Fevre et al. 2001). *T. b. rhodesiense* is of particular interest as it causes an acute version of HAT with a mean progression to disease of six months (Geiger et al. 2015).

T. b. rhodesiense infection has been discovered in a variety of domestic animals in Kenya, including: cattle, sheep, pigs, and dogs (Nijiru et al. 2004). The increasing popularity of pigs in Kenya is further becoming a concern due to their increasing use and their potential as a reservoir for *T. b. rhodesiense* (Nijiru et al. 2004). It has further been shown that flies may feed on cattle at five times the rate that they feed on humans in certain settings (Fevre et al. 2001). Accordingly, it was found that infections with certain trypanosomes were more common in cattle than humans (Fevre et al. 2001).

Vector control programs including aerial spraying of pesticides, and the usage of traps and screens have been shown to have significantly contributed in decreasing the disease burden of African sleeping sickness (Allsopp, 2001). For example, a combination of case detection, treatment, and vector control has resulted in a decrease of 87.9% in *T. b. rhodesiense* infections between 2000 and 2012 *(*Wamwiri et al. 2015). Furthermore, vector control programs have been greatly improved by the tool of population genetics in identifying populations that are isolated from one another and therefore vulnerable to vector control programs (Krafsur et al. 2002).

Population genetics can help identify shifts in incidence of disease across a region that can be traced to changes in the composition of vector populations (Solano et al. 2010). Additionally, population genetics information can help understand which Tsetse populations outside an area of interest are responsible for seeding new epidemics through migration (Aksoy et al. 2013). Previous vector control programs to control human trypanosomiasis have shown remarkable effectiveness (Allsopp, 2001). However, certain instances have shown that areas that underwent recent vector control could be quickly re-colonized (Grant et al. 2009). Furthermore, these re-colonization events into treated areas

could be traced back to the immigration of flies from areas where a control program had not been implemented (Dransfield et al. 1991).

Populations of *Glossina* spp. have been shown to exist as separated foci across sub-Saharan Africa (Geiger et al. 2015). It is clear that the population structure of *Glossina* spp. has been impacted by the expansion of human habitat along with the development of agriculture (WHO . 1998). Some populations of *Glossina* spp. have been found to be sufficiently isolated from others in order to warrant efforts at elimination/eradication (Echodu et al. 2011). The measurement of population isolation depends on a measurement of the relative importance of genetic drift and gene flow in determining population structure and diversity. Therefore, isolated populations would be the least likely to be successfully recolonized following vector control programs.

The study presented here aims to identify the spatial and temporal dynamics of *G. pallidipes* within Nguruman (Kenya). Nguruman populations have been shown to be highly differentiated from surrounding foci which suggests that highly targeted control efforts may be successful (Ouma et al. 2011). Our study aims to further study the temporal and spatial dynamics of *G. pallidipes* within Nguruman which previous work has found to be temporally and seasonally stable (Ouma et al. 2006).

Additionally, it has been found that *G. pallidipes* populations formed local substructures over distances over 4 km in Nguruman (Odulaja et al. 2001). New knowledge concerning the spatial and temporal population dynamics of *G. pallidipes* within Nguruman may increase the impact of future vector control programs while maximizing their cost-effectiveness. This project also aims to build on the existing data

available for *G. pallidipes* in order to inform better models of vector distribution and spread.

Materials and Methods

Sampling

Samples of *G.pallidipes* from the years 2003, 2009, and 2015 collected in Nguruman were used for this study (Figure 1). The 2003 and 2009 samples were procured from Dr. Krafsur and Dr. Ouma and were both in the form of extracted DNA and *G. pallidipes* fly specimens including (wings, torso, and legs). The 2003 samples were matched with locations close to the sample collections for 2015 and were grouped under LEN for Lengongu, PAK for Pakase, and MOK for Mukinyo. An additional sampling location known as Camp Site (CAM) was grouped with Mukinyo (MOK) due to how close the sites are to one another. The 2009 samples were all collected from a site falling within the Lengongu area and were therefore considered as being a part of a single area (Ouma et al., 2011).

Data Collection

The DNA was extracted from the samples using PrepGEM insect DNA extraction kit (ZYGEM Corp Ltd, Hamilton, New Zealand). We used the Promega GoTaq kit (9PIM300) for our PCR reactions using: 1ul of DNA extraction, 6ul dH_20, 2.6ul Promega PCR Buffer (5X), 0.1 ul BSA(100X), 0.5 ul Primer (For) (10uM), 0.5 ul Primer (Rev) (10uM), 1.1ul $MgCl_2$, 1.1ul dNTP, and 0.1ul GoTaq DNA polymerase.

PCRs for specific microsatellite markers was initiated once samples had been identified (Table: 3). This was done to determine which markers identified from previous studies could be used to characterize *G. pallidipes* populations in Nguruman. We therefore tested 24 samples selecting from a pool of 36 potential markers. All DNA samples underwent PCR amplification for one of the 13 microsatellite markers used in this study. The markers used in this study have been validated in previous studies, namely: D05: (Abila et al. 2008), GmK22, GmA06, GmC17, and GmL11: (Hyseni et al. 2011), Gmm8, and GpCAG133: (Baker et al. 2001), GpB20b, GpC26b, GpA19a, GpB6b, GpC5b, and GpC10b: (Ouma et al 2003). (All detailed in table 2).

Genetic Analyses

Detecting population structure

Samples were then analyzed for deviation from Hardy-Weinberg Equilibrium as well as Linkage disequilibrium using *Genepop v4.2* to evaluate the Hardy-Weinberg equilibrium, as well as linkage disequilibrium (Rousset, 2008). Samples were then analyzed for allelic frequencies, allelic richness, both observed and expected heterozygosity, and allelic richness using the programs *GenAlEx v6.502* and *FSTAT v2.9.3* (Hyseni et al., 2012). Samples were then analyzed for genetic structure using the program STRUCTURE *v2.3.4* (Pritchard, Stephens, & Donnelly, 2000). STRUCTURE relies on a Bayesian clustering approach as well as a two-tiered multivariate ordination analysis (Hyseni et al., 2012).

Samples were then subjected to an analysis of molecular variance (AMOVA), and pairwise F_{st} using the program *Arlequin v3.5* (Excoffier & Lischer, 2010). The samples

were then analyzed for discriminant analysis of principal components as an alternative to the Bayesian clustering approach (T Jombart, Devillard, & Balloux, 2010) using the R package *adagenet v.1.4-2* (Thibaut Jombart, 2008) in R *v 3.0.2* (R Development Core Team).

Estimates of effective population size (Ne) and population bottlenecks

We estimated contemporary Ne, microsatellite data were analyzed with NeESTIMATOR v2 (Do et al. 2014), using the linkage disequilibrium-based method. Samples were also analyzed for recent bottlenecks using the program *BOTTLENECK 1.2.02* (Piry, Luikart, & Cornuet, 1999). We further investigated the possibility of bottlenecks with the M-Ratio test (Garza and Williamson, 2001), which uses the ratio of the number of alleles to range in allele size to detect reductions in effective population size. We used Pg = 0.2 and $\Delta g = 3.5$, following Garza and Williamson (2001), and examined a range of Θ-values ($4Ne\mu$ for diploid autosomal genes, where Ne is the pre-bottleneck effective population size and μ is the microsatellite mutation rate), corresponding to Ne = 50, 500, 1000, and 1500. We also explored slow and fast microsatellite mutation rates (i.e., $\mu = 1.5x10^{-4}$ and $1.5x10^{-3}$) (Do et al. 2014).

Results

Population structure

Our initial analysis of our microsatellite structure using DAPC (Figure 2) indicates three clusters defined by the years of collection. We ran a cross validation with one hundred replicates and got a PC value of 55. There is a clear break between the sites sampled in 2015 and the same sites sampled in 2003 and 2009.

STRUCTURE runs with the Evanno method indicated that the rate of change of K (ΔK) was fastest at a K value of 5, but that there was no clear structure at this K value. Running CLUMPP for several K values, we found a pattern of visible structure by year of sampling at a K value of 2 (Figure 4).

F_{ST} estimates (Table 4) show differentiation between three groups, the 2003 samples, the 2009 samples, and the 2015 samples. We do not however see any significant differentiation between sites within the same year. With the combination of pairwise FST estimates (Table 4) and geographic distance matrix (Table 5), we produced a Mantel test with a one tailed p-value of 0.63700. The Mantel test therefore failed to show isolation by distance.

AMOVA tests for genetic structure based strictly on location (LEN, MOK, and PAK) indicated non-significant structure among geographic localities (Table 6). On the other hand, AMOVA tests for genetic structure based year of sampling (LEN-2015, PAK-2015, and MOK-2015 versus LEN-2009 versus LEN-2003, MOK-2003, and PAK-2003) indicated significant structure among temporal groups (Table 6).

Effective population size and bottlenecks

Estimates of Ne ranged from 45 to infinity (Table 7). We ran two analyses to look for bottlenecks. The first analysis looked at evidence of bottlenecks separating each site by year. The second analysis grouped together samples based on their year of sampling. There was a signal of a bottleneck in LEN-2015, and the M-ratio test indicated a likely bottleneck with a pre-bottleneck Ne of anything smaller than 500 and 50, or with a slow mutation rate of $1.15X10^{-4}$ (Table 8).

Discussion

It has been shown that *G. pallidipes* populations were significantly differentiated from one another when comparing populations in both Uganda and Western Kenya (Ouma et al. 2011). Additionally, populations of *G .pallidipes* separated by 240km in Nguruman and Lambwe have been found to form distinct populations (Ouma et al. 2006). This, and other evidence, suggests that gene flow between *G. pallidipes* populations separated by distances of as little as several hundred meters might be low (Odulaja et al. 2001).

Indeed, it was found that *G. pallidipes* populations in Nguruman and Lambwe shared little of their microsatellite variations with other sites thereby undermining the theory that significant immigrations of flies occurred between sites as a result of control activities (Brightwell et al. 1997). The lack of similarity between Nguruman and other sites such as Lambwe, Kodera, and Serengeti undermines the theory that Nguruman might have been re-colonized by immigrants as opposed to residual populations having survived control activities (Ouma et al. 2006).

The Lambwe and Nguruman sites in Kenya were significantly different from other sites and different from each other in a study by Ouma and colleagues (2006). This study investigated 1388 samples that were collected at seasonal intervals between 2000 and 2003 at sites separated by 200 meters to 14 kilometers (Ouma et al. 2006). Additionally, significant population structure has been found in *G. pallidipes* at sites separated by just several kilometers at several sites across Kenya including Nguruman (Ouma et al. 2005, Odulaja et al. 2001). Surprisingly, separate work specific to *G. pallidipes* in Nguruman has found a homogenous population structure for populations with a radius of 15-30km (Krafsur et al. 1997).

Our results did not show significant population structure of *G. pallidipes* in Nguruman for sites sampled within the same year. Additionally, our sites were separated from one another by distances of 14.15 to 22.29 km, which is consistent with studies that have found structure at this scale (Ouma et al. 2005, Odulaja et al. 2001). However, the neighboring sites that showed structure in previous studies may differ with the sites in our study in important ways due to the timing of sampling.

Temporal sampling of *G. pallidipes* in Nguruman has shown population substructures between sites separated by distances of the order of several kilometers (Odulaja et al. 2001, Ouma et al. 2006). However, it should be noted if a study of the population substructure of *G. pallidipes* came after several years of vector control activities. Our current study covers a time lapse that has not seen recent control activities in Nguruman. This difference between the timing of previous control activities and the sampling that took place might help explain the difference in substructure observed

across different studies. Additionally, our study looked at three different time points and did not consider the timing of seasons.

Our current study undertook sampling at three time points as opposed to other studies that account for differences in distribution of the flies between different seasons within the same year. We cannot rule out that seasonality might have affected the result of our sampling and could therefore be a limitation of our study. However, previous work has shown that the seasonality of *G. pallidipes* in Nguruman only accounted for a small fraction of the genetic variation and therefore suggested the relative stability of these populations across seasons (Ouma et al. 2006).

Our study clearly showed the spatial homogeneity of the populations in Nguruman as well as their temporal heterogeneity. These findings suggest that a targeted control strategy choosing a particular location in the park is likely to fail. The high level of homogeneity suggests that there is significant migration of flies across the different areas of the park. These migrations might be aided by a potential high level of connectivity in the habitat of the flies across the park. Therefore, any control activity that did not address the park in its entirety would likely predispose the park to re-colonization events. Indeed, Tsetse not affected by the control efforts could survive and repopulate the park.

The specific reason for the temporal structure observed in this study remains unknown. We investigated the possible reasons for the shift in population structure between 2003 and 2015 with local officials. The only event of significance for *G. pallidipes* that came to our attention was the implementation of a new irrigation program in areas that previously supported high vector densities. Cattle in Nguruman experience

higher rates of infection two months following the rainy season which is correlated with increasing Tsetse densities (Tarimo-Nesbitt et al. 1999). These irrigation canals may have provided new avenues of movement that facilitated migrations within Nguruman.

Further studies would be needed to definitively determine the importance and magnitude of gene flow into the park. Barriers to migration may be necessary if gene flow into the park were to be found to be strong (Echodu et al. 2011). Overall, the homogeneity of *G. pallidipes* populations in Nguruman we uncovered shows that gene flow within Nguruman is likely to be high. This study provides important information as to the population structure of *G. pallidipes* over the course of several years within a limited spatial area. Further studies will be needed to determine if this population structure is also present at this spatial scale elsewhere in East-Africa. If duplicated, our findings could have significant implications for the control of *G. pallidipes* in infested areas.

Current evidence points to the emergence of drug resistant form of trypanosomiasis (Solano et al. 2010). This is very concerning as little progress has been made in the development of new drugs due to the absence of financial incentives (Solano et al. 2010). The possibility that trypanosomes may become resistant to currently used drugs could potentially result in a major public health crisis in Sub-Saharan Africa. Additionally, vector control programs are vulnerable to depressions in funding which are historically linked to renewed epidemics of HAT in formerly controlled areas (Wamwiri et al. 2015). It is therefore crucial that we see a renewal in interest in developing both new drugs and vector control programs if both HAT and AAT are to be successfully controlled.

Climate modeling has shown that higher temperatures due to climate change are likely to help decrease the range of *Glossina* .spp thereby positively impacting both human and animal health (Terblanche et al. 2006). However, members of the *G. palpalis* group such as *G. f. fuscipes* have shown themselves to be able to adapt to urban environments (Solano et al. 2010). This finding is highly concerning as human populations are both expanding and becoming increasingly urbanized (Bruckner. 2011). The result of the interplay between increasing temperatures and changes in human development on the burden of trypanosomiasis remains to be determined.

Human actions in the past have been associated with new outbreaks of African sleeping sickness (Fevre et al. 2001). The movement of cattle across trypanosomiasis endemic areas has shown to be a significant risk factor in the spread of trypanosomiasis (Fevre et al. 2001). We therefore recommend that cattle moved within the context of agricultural development programs be screened and treated appropriately before being transposed into a new area. Such practices have the potential to significantly lower the spread of both trypanosomiasis and its vectors. It should however be pointed out that the prospect of treating all wild and domestic hosts of trypanosomes would not be feasible in a resource limited setting (Solano et al. 2010).

Resource limited settings could benefit from relatively inexpensive interventions such as shifting pastoralism. Shifting pastoralism is a strategy that has shown some success in limiting the infections of cattle and should be further explored as a strategy to limit the burden of trypanosomiasis in Nguruman (Tarimo-Nesbitt et al. 1999).

Our work did not show significant spatial population structure for *G. pallidipes* in Nguruman. However, we were able to show population structure across our time points.

We therefore recommend that programs that aim at controlling *G. pallidipes* in Nguruman take into consideration both the temporal and spatial components of *G. pallidipes* population structure.

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Tables and Figures

Table 1. Marker validation and summary statistics based on 13 microsatellite loci. For each locality, we report latitude, longitude, collection date, number of samples analyzed (N), mean allelic richness across all loci (AR), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient (Fis). Bold Fis values indicate significant HW disequilibrium after Benjamini-Hochberg correction.

Locations	Latitude	Longitude	Collection-Date	N	AR	Ho	He	Fis
LEN-2003	-1.9576	36.1199	2003	30	3.345	0.544	0.501	-0.076
LEN-2009	-1.9582	36.1196	2009	29	3.78	0.432	0.519	**0.172**
LEN-2015	-1.9769	36.1167	2015	48	3.262	0.515	0.517	0.002
MOK-2003	-1.8246	36.0966	2003	59	3.511	0.576	0.539	**-0.088**
MOK-2015	-1.8316	36.0893	2015	53	3.681	0.501	0.518	0.037
PAK-2003	-2.0000	36.0000	2003	29	3.482	0.555	0.529	**-0.066**
PAK-2015	-1.9804	35.9825	2015	19	3.279	0.469	0.481	0.054

Table 2. Microsatellite amplification parameters. We report locus name, dye used, primer sequences, size range, repeat motif, and the original source of the primer.

Gp Msats						
Locus	Dye	F Primer	R Primer	Size Range	Repeat Motif	Original Source of Primer
D05	FAM	TTTCCTTCCAGACGAACTG	CTTGGTATGGTCGTACATGG	Missing	Missing	Abila et al. 2008
GmK22	TAM	ACGCTTACGTTTCCGTTACAC	AAGCTAACCGAACCAGCAC	192-198	$(GTT)_{19}$	Hyseni et al. 2011
GmA06	TAM	ACTTCCATGTTATGCCTTAGTTGAGAAACTCTGCTGTTCGTTGC	Missing	154-166	$(AC)_{31}$	Hyseni et al. 2011
Gmm8	HEX	CGCGCTTCAATGTTTGCTTTC	TGCAGATGCAATGCGGAGAG	125-131	$(GA)_{11}$	Baker et al. 2001
GmC17	HEX	TGCGCTTTGAACGGAACG	CTATGCCGCCTGGCTTATC	190-202	$(ATGT)_{14}$	Hyseni et al. 2011
GmL11	HEX	CCACCACTAACAACGACAGC	TGGCTGGTTACAAGATTGCAC	250-252	$(AT)_{27}$	Hyseni et al. 2011
GpB20b	FAM	AGTTTGCTTCTCAACGCAGTAG	GTTTCGGCAGTAGATGGCAA	139-200	$(GA)_{29}$	Ouma et al. 2003
GpC26b	FAM	GGATCACCCTTCTTGAATG	GTTTGGACGTTATTTGTTCGTGTAA	168-201	$(CAT)_3CGT(CAT)_{12}$	Ouma et al. 2003
GpA19a	FAM	CATATCCACACCCACATACAT	GTTTGCGATTATGGCTAGAGGTTT	142-189	$(CA)_7GA(CA)_7$	Ouma et al. 2003
GpCAG133	HEX	ATTTTTGCGTCAACGTGA	GTTTATGAGGATGTTGTCCAGTTT	185-209	$(CAG)_{12}$	Baker et al. 2001
GpB6b	HEX	GTAAACCGCCTGTCACATC	GTTTAGGGAGAGAGCCGTAAGAG	187-224	$(CT)_{15}$	Ouma et al. 2003
GpC5b	NED	GTTGTTTTCTGCTCCTCAATA	GTTTCAAGGGTGTGTCGTCTTC	187-239	$(TGA)_{11}$	Ouma et al. 2003
GpC10b	NED	GTTGATGTTGTGATGGTAATGA	GTTTGCTGGCAAAGAAACTAATGA	283-314	$(CAT)_9$	Ouma et al. 2003

Table 3. Thermocycler settings.

Step	Time	Temperature
Step 1: Initiation Denaturation	5m	95˚C
Step 2: (12 Cycles):		
Denature	30s	95˚C
Annealing	25s	62-50˚C
Extension	30s	72˚C
Step 3: (40 Cycles):		
Denature	30s	95˚C
Annealing	25s	50˚C
Extension	30s	72˚C
Step 4: Final Extension:	20m	72˚C

Table 4. Pairwise Fst values. Bold indicates significant p values after Benjamini Hochberg correction.

	LEN-2015	MOK-2015	PAK-2015	LEN-2009	LEN-2003	MOK-2003	PAK-2003
LEN-2015	0						
MOK-2015	0.00027	0					
PAK-2015	0.00005	0.00057	0				
LEN-2009	**0.03937**	**0.03805**	**0.04758**	0			
LEN-2003	**0.03337**	**0.04544**	**0.05278**	**0.01284**	0		
MOK-2003	**0.03762**	**0.04434**	**0.05141**	**0.01061**	-0.0013	0	
PAK-2003	**0.04371**	**0.05331**	**0.058**	**0.01084**	0.00667	-0.00161	0

Table 5. Spatial distance of sampled localities. The Mantel test failed to display isolation by distance, with a one-tailed p-value of 0.63700.

	LEN-2015	MOK-2015	PAK-2015	LEN-2009	LEN-2003	MOK-2003	PAK-2003
LEN-2015	0						
MOK-2015	16.46	0					
PAK-2015	14.94	20.38	0				
LEN-2009	2.11	14.49	15.45	0			
LEN-2003	2.18	14.43	15.5	0.07	0		
MOK-2003	17.11	1.13	21.5	15.1	15.04	0	
PAK-2003	13.24	21.21	2.92	14.1	14.15	22.29	0

Table 6. AMOVA by geographic origin and by year of sampling. We report the definition of groups, the level of the test, degrees of freedom (d.f.), percent variation explained (% variation), p-value of the test (p-value), and fixation indices (F-index). Bold p-value indicates statistical significance of population structure at the level specified.

By geographic origin:	d.f.	% variation	P-values	F-index
3 groups (LEN, MOK, PAK)				
Among geographic groups	2	-1.76	0.939	F_{CT}: -0.017
Among populations within groups	4	4.09	**0.000**	F_{SC}: 0.040
Among individuals	260	-0.5	0.602	F_{IS}: -0.005
Within individuals	267	98.17	0.112	F_{IT}: 0.018
By year of sampling:	**d.f.**	**% variation**	**P-values**	**Fixation Indices**
3 groups (2003, 2009, 2015)				
Among temporal groups	2	3.83	**0.01662**	F_{CT}: 0.038
Among populations within groups	4	0.03	0.3695	F_{SC}: 0.000
Among individuals	260	-0.49	0.61193	F_{IS}: -0.005
Within individuals	267	96.64	0.12903	F_{IT}: 0.033

Table 7. Effective population size estimates (Ne), and 95% confidence interval (CI) using the linkage disequilibrium method in NeEstimator v.4.2, and tests for population bottlenecks using the TPM method and mode-shift method implemented in BOTTLENECK 1.2.02. Significant signal of population bottleneck in bold.

	Ne	**CI**	**TPM p-value**	**Mode Shift**
LEN-2015	58	34-127	**0.020**	normal L-shape
MOK-2015	Infinite	232-Infinite	0.554	normal L-shape
PAK-2015	200	25-Infinite	0.227	normal L-shape
LEN-2003	Infinite	129-Infinite	0.170	normal L-shape
MOK-2003	149	75-826	0.342	normal L-shape
PAK-2003	Infinite	70-Infinite	0.040	normal L-shape
LEN-2009	45	24-137	0.473	normal L-shape
2003	315	171-1187	0.446	normal L-shape
2009	45	24-137	0.473	normal L-shape
2015	507	224-Infinite	0.446	normal L-shape

Table 8. Results from M-ratio test for a recent bottleneck in LEN-2015. Pre-bottleneck theta was calculated as 4*(effective population size)*(mutation rate), and we Pg = 0.2 and Δg = 3.5. The M-ratio for LEN-2015 was 0.646345. Bold indicates a p-value showing significant evidence of a recent bottleneck.

Fast mutation rates:

Ne	μ	Pre-bottleneck theta	p-value
3000	0.0015	18	0.3794
1500	0.0015	9	0.1804
1000	0.0015	6	0.1178
500	0.0015	3	**0.0494**
50	0.0015	0.3	**0.0008**

Slow mutation rates:

Ne	μ	Pre-bottleneck theta	p-value
3000	0.00015	1.8	**0.0204**
1500	0.00015	0.9	**0.0052**
1000	0.00015	0.6	**0.0030**
500	0.00015	0.3	**0.0008**
50	0.00015	0.03	**0.0002**

Figure 1. Map of Sampling areas located in Nguruman Kenya; **(A)** indicates region of Kenya sampled, **(B)** indicates area of detail (ESRI).

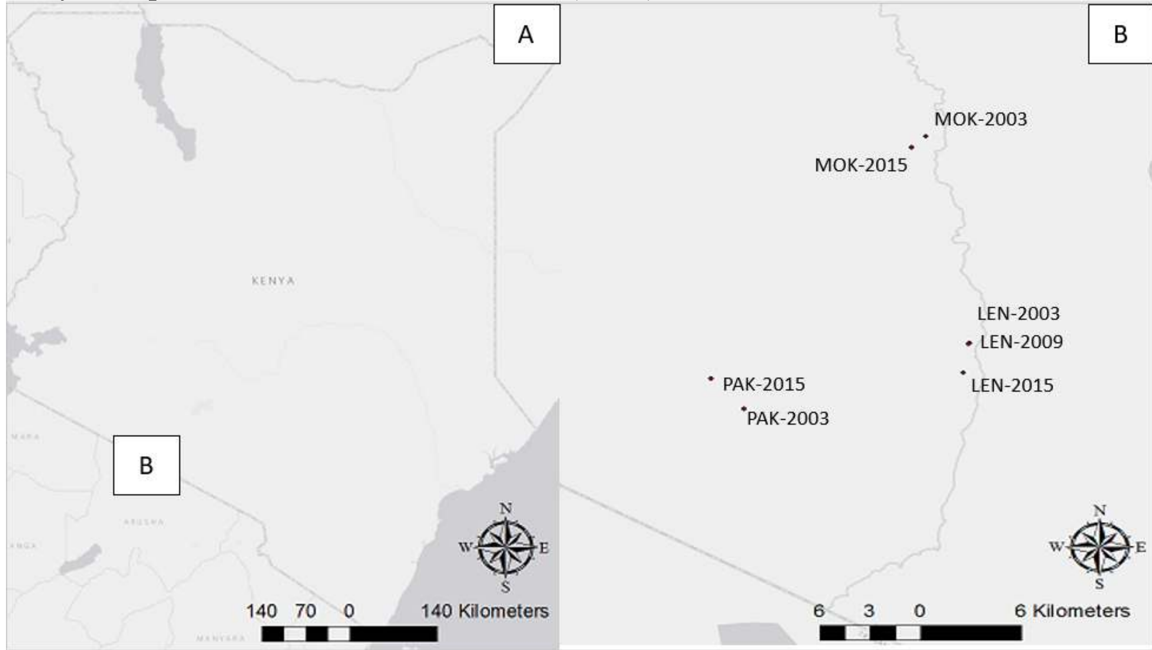

Figure 2. Discriminant Analysis of Principle Component.

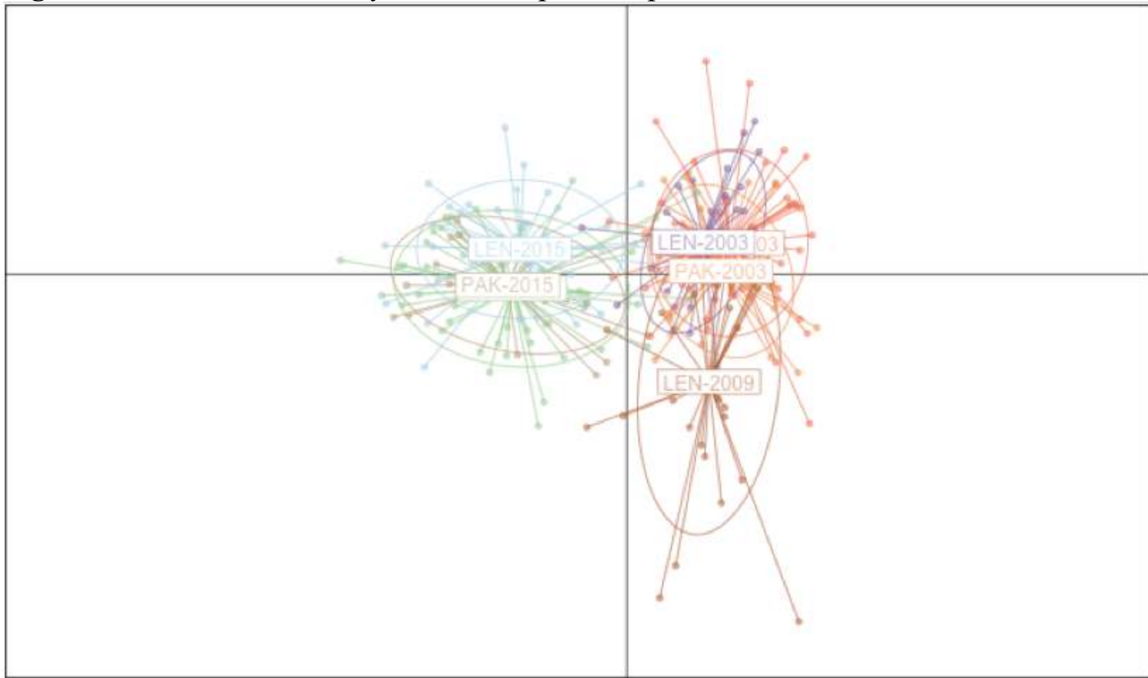

Figure 3. Genotype frequency of 13 microsatellite loci at all sampled time points.

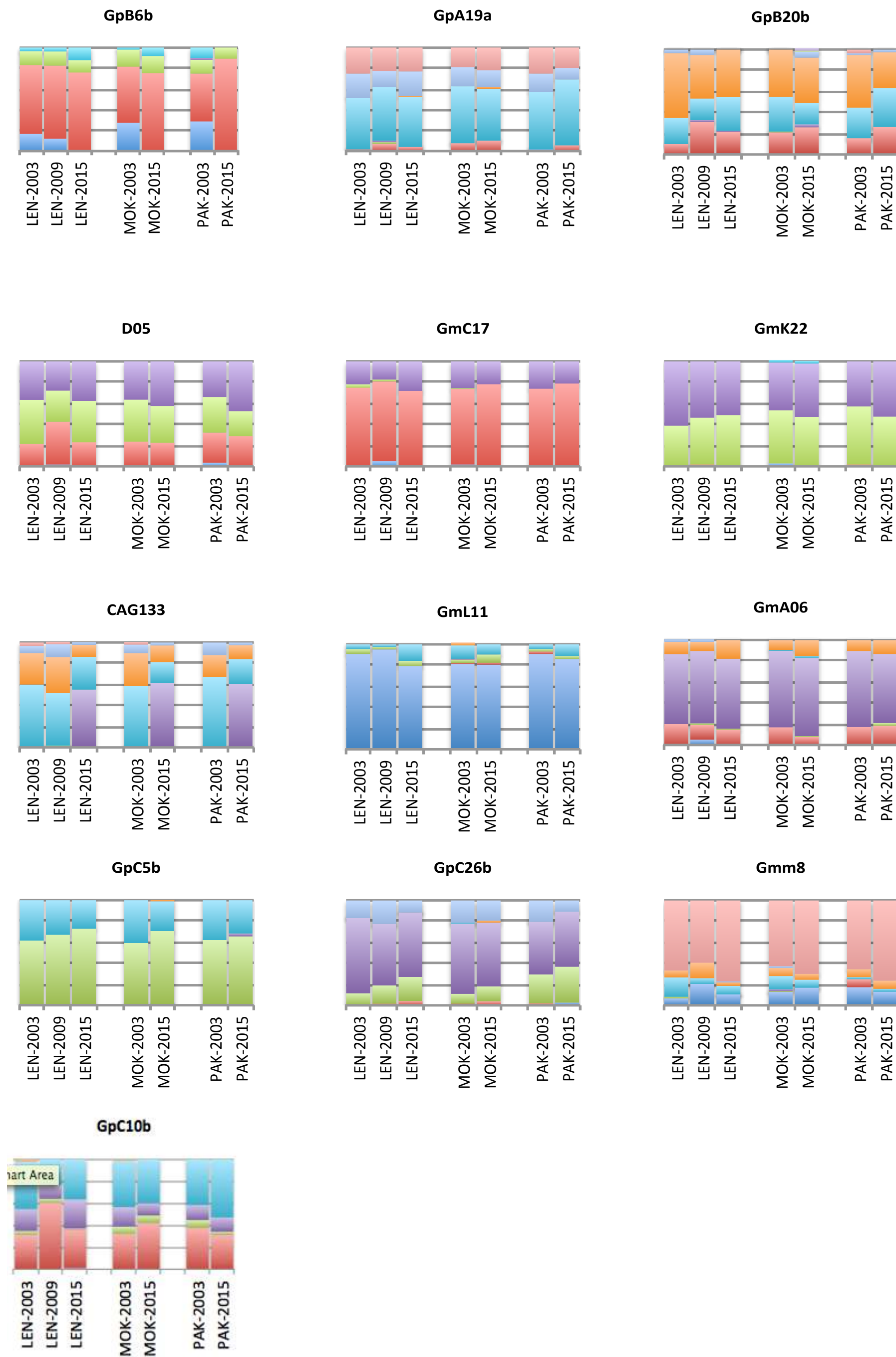

Figure 4. STRUCTURE plot with K=2.