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The fine-scale genetic structure of the malaria vectors *Anopheles funestus* and *Anopheles gambiae* (Diptera: Culicidae) in the north-eastern part of Tanzania

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Abstract. Understanding the impact of altitude and ecological heterogeneity at a fine scale on the populations of malaria vectors is essential to better understand and anticipate eventual epidemiological changes. It could help to evaluate the spread of alleles conferring resistance to insecticides and also determine any increased entomological risk of transmission in highlands due to global warming. We used microsatellite markers to measure the effect of altitude and distance on the population genetic structure of *Anopheles funestus* and *Anopheles gambiae s.s.* in the Muheza area in the north-eastern part of Tanzania (seven loci for each species). Our analysis reveals strong gene flow between the different populations of *An. funestus* from lowland and highland areas, as well as between populations of *An. gambiae* sampled in the lowland area. These results highlight for *An. funestus* the absence of a significant spatial subpopulation structuring at small-scale, despite a steep ecological and altitudinal cline. Our findings are important in the understanding of the possible spread of alleles conferring insecticide resistance through mosquito populations. Such information is essential for vector control programmes to avoid the rapid spread and fixation of resistance in mosquito populations.

Key words: *Anopheles funestus*, *Anopheles gambiae*, altitude, malaria, microsatellite, population genetics, Tanzania

Introduction

Environmental heterogeneity is known to be a driver of population structure (Temunović *et al.*, 2012; Stein *et al.*, 2014), and malaria mosquitoes are no exception (Touré *et al.*, 1994). Without doubt, altitude is a major parameter affecting it because of the abiotic (temperature and humidity) as well as the biotic conditions, including the lower presence of pathogens such as *Plasmodium* spp., as shown in

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bird populations (van Rooyen *et al.*, 2013). As global warming is significantly affecting species distribution as well as their mutual interactions, altitude can be considered as a proxy to understand its impact on the structure of populations of terrestrial insects (Hodkinson, 2005), including mosquitoes. In the case of malaria, the large increase in household ownership of long-lasting insecticidal nets (LLINs) has contributed to an important decrease in the number of malaria cases in the last decade (WHO, 2014), but at the same time, this is associated with a greater risk of the spread of resistance to pyrethroids, the sole insecticidal class used for LLIN treatment (Ranson *et al.*, 2011). Understanding, at a fine scale, how resistance can spread in mosquito populations is of critical importance to optimize current and future vector control. Genetic data can, not only help obtain this information, but can also provide a better understanding of the role of connectivity between populations on the dynamics of disease transmission (Donnelly *et al.*, 2004). Finally, and on a more hypothetical aspect of the control of vector-borne diseases, it is a prerequisite for any release of genetically modified mosquitoes or sterile insects aimed to control the disease at a large scale.

Along the coast of Tanzania, the major malaria vectors are *Anopheles funestus* and *Anopheles gambiae s.s.* (Magesa *et al.*, 1991; Temu *et al.*, 1998; Mboera and Magesa, 2001; Maliti *et al.*, 2014). Here, we present a comparative study of the population genetics of these two major vectors from the Muheza district in the north-eastern part of Tanzania, to investigate the spatial population structure of these two anopheline species. This region is a rural area known for its holoendemic transmission of malaria in the lowland area (Mboera and Magesa, 2001; Alilio *et al.* 2004), and it also presents a steep ecological cline with the presence of highland areas close to the town of Muheza and in the vicinity of the Amani Nature Reserve in the Tanga Region of Tanzania, East Africa.

Materials and methods

Collection site

Adult mosquitoes were collected in Muheza district, Tanga Region in the north-eastern part of Tanzania. Six sites were chosen that could be organized in two clusters according to their altitudes: the lowland cluster (mean altitude = 236 m ± 11.8 m) included three villages (Mamboleo, Songa Kibaoni, Zeneth), and the highland area (mean altitude = 952 m ± 63.2 m) with three villages (Mikwinini, Mlesa, Ndola) (Fig. 1 and Table 1). Pairwise distances between villages inside each cluster ranged from 5 to 10 km (Table 2).

Mosquito sampling, species identification and oocysts collection

The collections were performed between May 2005 and August 2005 where indoor-resting blood-fed anopheline females were collected with aspirators in the morning regularly during this period, i.e. between 15 and 19 times in the lowland and the highland areas, respectively. At each visit, the same four houses were visited for 15 to 20 min in each village. Mosquitoes were brought to the insectary in Muheza, fed with a sugar solution *ad libitum*, and maintained for 7 days in small pots. On the seventh day after capture, midguts of the surviving mosquitoes were dissected with tweezers under a light microscope and the presence/absence of *Plasmodium falciparum* oocysts recorded. All the collected mosquitoes did not survive the period of 7 days in the insectary. Performing an analysis of the mortality rate neither revealed any significant differences between areas and species, nor their interactions (see Additional File 1). This reveals the absence of any bias in the mortality. Similarly, a number of mosquitoes survived the period of 7 days in the insectary, but they were untested for genotyping (conservation issues). Again the statistical analysis (see Additional File 2) revealed no significant difference between the groups, and this did not affect the survey.

Morphological identification based on the taxonomic keys for the identification of Afrotropical mosquitoes (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987) was done to identify *An. funestus* group and *An. gambiae* s.l. DNA extraction from whole insects was performed using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions and DNA concentration was measured with a Nanodrop® spectrophotometer. Identification of subspecies inside each complex was performed by polymerase chain reaction (PCR) according to a protocol based on Koekemoer *et al.* (2002) within the *An. funestus* group and Wilkins *et al.* (2006) within the *An. gambiae* complex. Sampling details are summarized in Table 1.

Microsatellite genotyping

A total of 142 *An. funestus* were genotyped using 10 microsatellite loci: AF2, AF3, AF5, AF19, AF20 (Sinkins *et al.*, 2000), FunF, FunG, FunL, FunO, FunR (Cohuet *et al.*, 2002). A total of 181 *An. gambiae* individuals were genotyped using 13 microsatellite loci: 11 loci from Zheng et al. (1996) [H29, H46, H88, H131, H143, H197, H249, H555, H675, H678, H1D1 (abbreviated names from Midega *et al.* (2010)] and two microsatellite loci from the NOS and cecropin 3′ UTR genes (Luckhart *et al.*, 2003), which function in the innate immune system of insects.

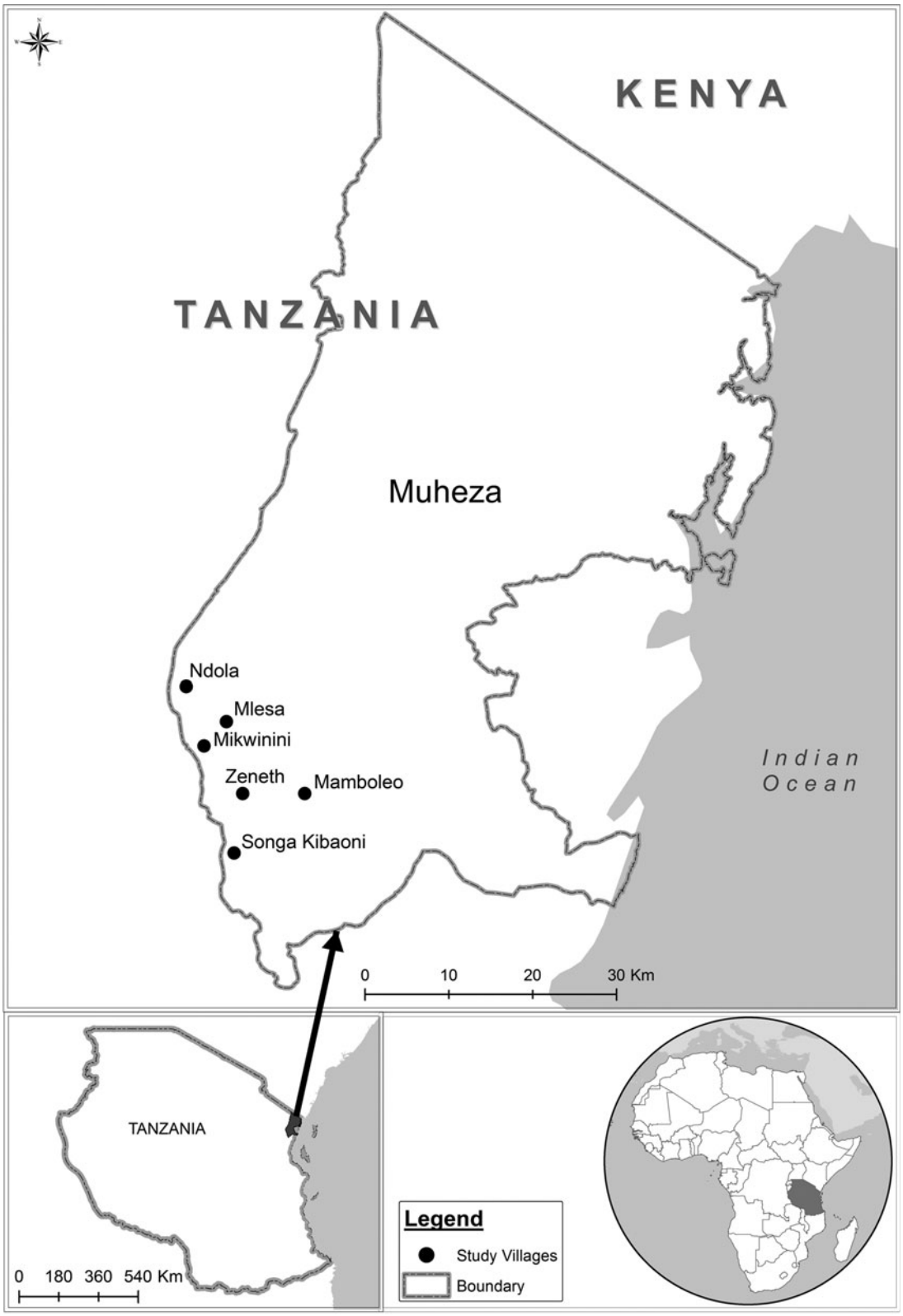

Fig. 1. Map of the area where collections of malaria mosquitoes were performed.

Table 1. Number of collected adult (*Anopheles funestus* and *An. gambiae*) surviving 7 days after collection and their infection status

Species	Village	Latitude	Longitude	Altitude (m)	Area of collection (altitude)	Number of collected mosquitoes	Number of surviving mosquitoes after 7 days	Number of mosquitoes used in the genetic analysis
An. funestus	Ndola	S 05°06.400′	E 038°34.325′	1060 m	Highland	129	40	28
	Mlesa	S 05°07.921′	E 038°37.365′	845 m	Highland	16	9	4
	Mikwinini	S 05°08.432′	E 038°35.164′	989 m	Highland	1	0	–
	Mamboleo	S 05°13.123′	E 038°43.244′	220 m	Lowland	8	3	3
	Songa Kibaoni	S 05°15.204′	E 038°38.639′	259 m	Lowland	20	6	4
	Zeneth	S 05°13.492′	E 038°39.620′	229 m	Lowland	616	176	103
An. gambiae	Ndola	S 05°06.400′	E 038°34.325′	1060 m	Highland	42	14	–
	Mlesa	S 05°07.921′	E 038°37.365′	845 m	Highland	11	8	–
	Mamboleo	S 05°13.123′	E 038°43.244′	220 m	Lowland	126	66	43
	Songa Kibaoni	S 05°15.204′	E 038°38.639′	259 m	Lowland	66	34	27
	Zeneth	S 05°13.492′	E 038°39.620′	229 m	Lowland	366	215	102

Table 2. Matrix of pairwise geographic distance amongst sampled sites. Distances are expressed in km

	Highland		Lowland		
	Ndola	Mlesa	Zeneth	Songa Kibaoni	Mamboleo
Mlesa	6.0	–			
Zeneth	16.4	11.3	–		
Songa Kibaoni	17.9	13.5	3.5	–	
Mamboleo	20.5	15.5	6.5	9.2	–

For each species, the forward primer of each locus was labelled with a fluorescent dye using a M13 labelled tail added to the 5′-end of the oligonucleotide (four colours: FAM, VIC, NED, PET). PCR were performed in a volume of 10 µl with 5 µl of MasterMix 2x (Applied Biosystems™; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), 0.25 µl of each primer (10 µM) and 0.25 µl of labelled M13 tail (10 µM), 2 µl of DNA template (10 ng/µl) using the following cycling: 2 min at 95 °C; followed by 30 cycles of 30 sec at 95°C, 30 sec at 55 °C and 30 sec at 72 °C); then 5 min at 95 °C. Mixes of PCR products were then made according to amplicon size and dye, and genotyping was performed using an ABI PRISM 3730 XL DNA sequencer (Applied Biosystems™). Results were analysed with GeneMapper™ v. 4.0 software (Applied Biosystems™).

Data analyses

Estimates of linkage disequilibrium were performed using FSTAT v. 2.9.3 (Goudet, 1995). The presence of null alleles was assessed using MICRO-CHECKER v. 2.2.3 (Van Oosterhout *et al.*, 2004).

Genetic diversity of mosquito populations was assessed by the number of alleles (*Na*), observed heterozygosity (H_O), and expected heterozygosity (H_E) using Arlequin v. 3.5.1.2 (Excoffier *et al.*, 2005). Tests of deviation from Hardy–Weinberg equilibrium (HWE) were performed using FSTAT v. 2.9.3 (Goudet, 1995).

The significance of genetic differentiation between populations based on allelic distribution across populations was examined using a Fisher exact test with FSTAT v. 2.9.3 (Goudet, 1995). The pairwise F_{ST} statistic between populations was calculated after Weir and Cockerham (1984) using FSTAT v. 2.9.3 (Goudet, 1995) and Arlequin v. 3.5.1.2 (Excoffier *et al.*, 2005).

For each species, without taking into account their geographic origin, *An. funestus* and *An. gambiae* individuals were clustered on the basis of their genetic relatedness using the Bayesian clustering approach implemented in STRUCTURE v. 2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). Simulations were performed using the admixture and correlated frequencies model. We estimated the number K of genetic clusters (here between $K = 1$ and $K = 4$) to which the individuals should be assigned. For all simulations, we did not force the model with predefined allele frequencies for source clusters. Five independent runs were conducted to assess the consistency of the results across runs,

Table 3. Summary statistics of *Anopheles funestus* populations

Population	N	N_a	H_O	H_E	F_{IS}	r
Highland						
AF2	30	9	0.600	0.785	0.239	0.11 (yes)
AF19	31	6	0.645	0.555	− 0.165	− 0.14 (no)
AF20	29	3	0.552	0.639	0.138	0.07 (no)
FunF	28	4	0.429	0.442	0.031	− 0.01 (no)
FunG	30	4	0.733	0.629	− 0.170**	− 0.13 (no)
FunO	31	5	0.484	0.526	0.081	0.03 (no)
FunR	32	4	0.406	0.496	0.183	0.08 (no)
All	**32**	**5 ± 2**	**0.550 + 0.120**	**0.583 + 0.110**	**0.056**	
Lowland						
AF2	110	11	0.718	0.824	0.129**	0.06 (yes)
AF19	107	6	0.411	0.503	0.183**	0.10 (yes)
AF20	106	4	0.396	0.542	0.268***	0.12 (yes)
FunF	107	5	0.579	0.603	0.040	0.01 (no)
FunG	106	6	0.651	0.725	0.102***	0.04 (no)
FunO	108	6	0.565	0.569	0.008	0.00 (no)
FunR	105	4	0.305	0.505	0.400***	0.18 (yes)
All	**110**	**6 ± 2.38**	**0.518 + 0.150**	**0.610 + 0.121**	**0.151****	

N: number of amplified individuals; N_A: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}: inbreeding coefficient. Next to the F_{IS} is indicated the significance of the P-values for deviation to Hardy–Weinberg equilibrium: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; r: Null allele frequencies (presence or absence is indicated next by yes or no).

and all runs were based on 10^6 iterations after a burn-in period of 10^5 iterations. We then identified the number of genetically homogeneous clusters as described by Evanno *et al.* (2005).

As analyses using STRUCTURE are based on strong assumptions (Panmixia, no linkage disequilibrium), we performed a dissimilarity analysis calculating the Shared Allele Distance (DAS) between pairs of genotypes and drawing a neighbour-joining tree with DARwin v 5.0 (Perrier and Jacquemoud-Collet, 2006).

Results

Species identification

Of the 142 *An. funestus* individuals, all were identified as *An. funestus sensu stricto* following PCR identification tests. Amongst 181 *An. gambiae* individuals, 172 were identified as *An. gambiae sensu stricto* and nine as *An. arabiensis*. *Anopheles arabiensis* were excluded from further analyses.

Linkage disequilibrium and test for Hardy–Weinberg equilibrium

Due to a large number of missing data for some of the loci, which may be due to null alleles, data from seven microsatellite loci from 10 were retained for further analyses for *An. funestus* (AF2, AF19, AF20, FunF, FunG, FunO and FunL). Amongst 21 linkage equilibrium tests, none was significant at the 5% level after Bonferroni correction for multiple testing. As such, these seven loci were considered as independent and kept for further analyses. Because of small sampling size for some locations and low *Plasmodium* prevalence over the dates of collection, all individuals were pooled into two populations: 'Highland' ($n = 32$) and 'Lowland' ($n = 110$).

For *An. gambiae*, due to a large number of missing data for some of the loci, which may also be due to null alleles, data from seven microsatellite loci from 13 were retained for further analyses (H29, H88, H131, H249, H678, H1D, NOS). Amongst 21 linkage equilibrium tests, none was significant at the 5% level after Bonferroni correction for multiple testing. As such, these seven loci were considered as independent and kept for further analyses. Due to low *Plasmodium* prevalence, infected individuals were pooled with non-infected for each village. For *An. gambiae*, the term 'population' referred to individuals sampled in the same village during the time of the experiment.

Regarding the two *An. funestus* populations, the highland population did not show deviation from Hardy–Weinberg equilibrium over all loci, whilst the lowland population showed a significant excess of homozygotes (F_{IS} = 0.151**; Table 3). Additionally, the three populations of *An. gambiae* showed significant excess of homozygotes over all loci (F_{IS} ranged from 0.162** to 0.179**; Table 4).

Table 4. Summary statistics of *Anopheles gambiae* populations

Population	N	N_a	H_O	H_E	F_{IS}	r
Zeneth						
H29	98	6	0.255	0.320	0.205**	0.08 (yes)
H88	99	7	0.364	0.656	0.436***	0.20 (yes)
H249	100	7	0.630	0.686	0.077**	0.03 (no)
H1D1	101	2	0.465	0.487	0.044	0.02 (no)
H131	98	6	0.633	0.728	0.130*	0.06 (yes)
H678	100	11	0.790	0.806	0.021	0.01 (no)
NOS	100	9	0.300	0.511	0.414***	0.19 (yes)
All	**102**	**6.8 ± 2.8**	**0.491 ± 0.200**	**0.598 ± 0.167**	**0.178****	
Songa Kibaoni						
H29	26	3	0.308	0.370	0.172	0.07 (no)
H88	22	6	0.455	0.644	0.285*	0.13 (no)
H249	24	7	0.583	0.682	0.136	0.06 (no)
H1D1	27	2	0.444	0.453	0.019	0.00 (no)
H131	21	5	0.571	0.723	0.223	0.11 (no)
H678	23	9	0.739	0.778	0.051	0.00 (no)
NOS	24	6	0.250	0.430	0.424*	0.16 (yes)
All	**27**	**5.4 ± 2.4**	**0.479 ± 0.169**	**0.583 ± 0.162**	**0.179****	
Mamboleo						
H29	43	3	0.302	0.285	− 0.062	− 0.02 (no)
H88	43	7	0.372	0.554	0.331**	0.14 (yes)
H249	41	7	0.585	0.778	0.248	0.12 (yes)
H1D1	43	2	0.488	0.502	0.026	0.01 (no)
H131	40	7	0.525	0.682	0.242	0.11 (yes)
H678	41	8	0.756	0.813	0.071	0.03 (no)
NOS	41	5	0.341	0.396	0.140	0.06 (no)
All	**43**	**5.6 ± 2.3**	**0.482 ± 0.159**	**0.573 ± 0.196**	**0.162****	

N: number of amplified individuals; N_A: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS} : inbreeding coefficient. Next to the F_{IS} is indicated the significance of the P-values for deviation to Hardy–Weinberg equilibrium: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; r: Null allele frequencies (presence or absence is indicated next by yes or no).

Genetic diversity and population differentiation

The number of alleles per locus ranged from four (AF20) to 12 (AF2) for *An. funestus* and from two (H1D1) to 11 (H678) for *An. gambiae* (Tables 3 and 4, respectively).

Tests of genetic differentiation between populations were performed for each species. For *An. funestus*, the highland population was undifferentiated from the lowland population ($F_{ST} = 0.012$, $P = 0.05$; Fisher exact test not significant). Moreover, allelic richness of the highland population was not significantly different from the lowland population (permutation test, $P = 0.67$).

For *An. gambiae*, the allelic richness of the three populations was not significantly different from each other (permutation test, all $P > 0.05$). F_{ST} estimates were not significantly different from 0 (Table 5). Thus, no genetic differentiation was found between the three populations, nor when considering only the NOS locus implied in the immune response (F_{ST} ranged from 0.001 to 0.018, all $P > 0.05$).

Table 5. Pairwise multilocus estimates of F_{ST} for *Anopheles gambiae* populations. All comparisons for Fisher exact tests were not significant at the 0.05 level

	Mamboleo	Songa Kibaoni
Songa Kibaoni	0.003^{NS}	–
Zeneth	0.001^{NS}	0.009^{NS}

Assignment tests

A Bayesian clustering analysis was performed using STRUCTURE. For both *An. funestus* and *An. gambiae*, the clustering analysis indicated that the posterior distribution of the allele frequencies amongst clusters was best explained with a grouping into

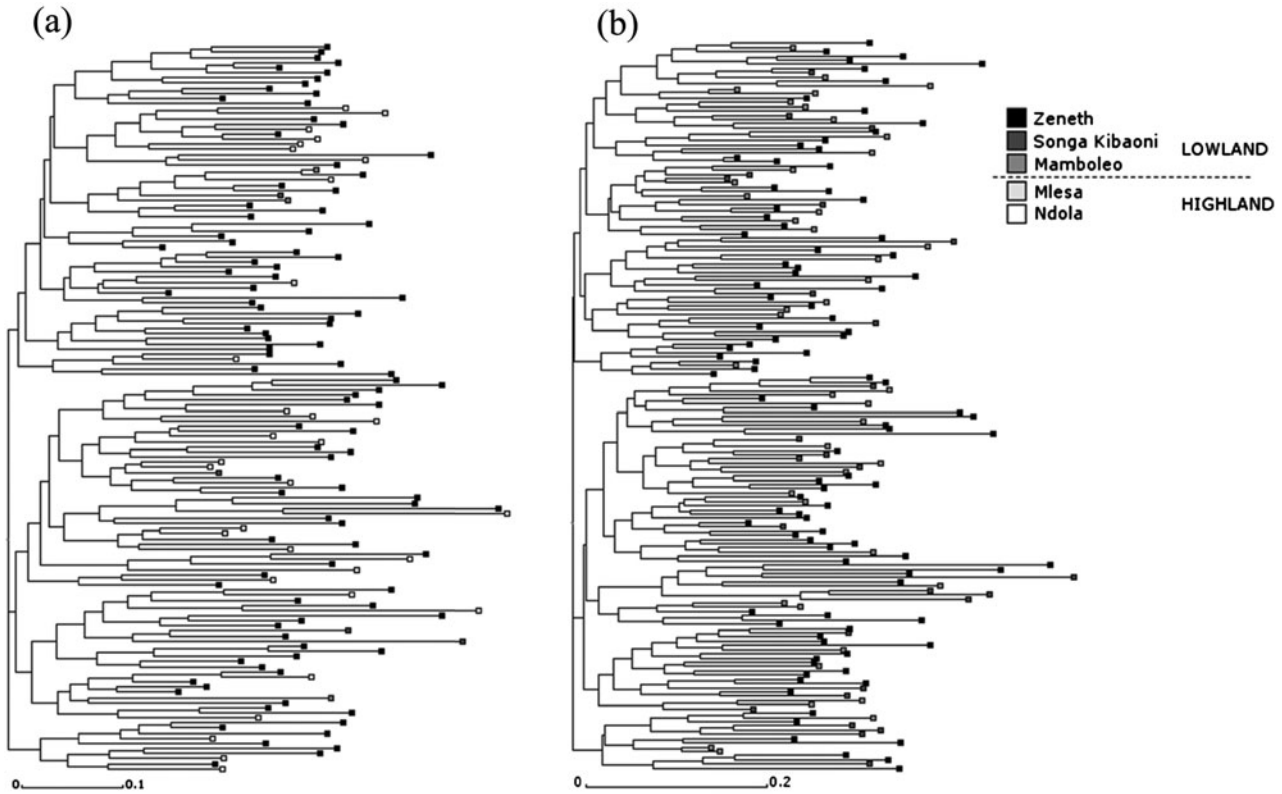

Fig. 2. Neighbour joining trees based on shared allele distance (DAS) for each species. (a) *Anopheles funestus* and (b) *Anopheles gambiae*.

$K = 1$ genetic cluster (data not shown). This is confirmed by the construction of a neighbour-joining tree based on DAS between pairs of multilocus genotypes for each species. Indeed, neither different genetic clusters nor spatial clustering for both species could be observed (Fig. 2a and b).

Discussion

Low prevalence of Plasmodium

We could not perform tests to assess genetic differentiation between 'infected' and 'uninfected' individuals due to the low prevalence found, but it is interesting to note that all infected mosquitoes (10 *An. gambiae* and six *An. funestus*) were caught in the lowland, suggesting that altitude could be a structuring factor for immunity gene. We should, however, remain cautious as temperature in altitude could also preclude *Plasmodium* infection. This would then release the pressure favouring a stronger immune response against malaria parasites. Obviously, we have no information about a potential higher mortality in the infected mosquitoes that were collected but died before dissection 7 days after capture.

High gene flow between populations

Our results showed that the highland population of *An. funestus* was not genetically differentiated from the lowland population. We had hypothesized that differences, both in altitude and in ecological factors, could lead to significant genetic differentiation, but this does not appear to be true despite a steep ecological cline. This is important because it shows that altitude does not act as a strong barrier to gene flow.

Likewise, for *An. gambiae*, the populations were not significantly differentiated from each other in the lowland area (pairwise distances ranged from 3 to 9 km; Table 2). This is consistent with previous studies that have revealed a deme size greater than 50 km for *An. gambiae* (Lehmann *et al.*, 1996; Kamau *et al.*, 1998). The similarity between topography and climate of the different locations probably explains this lack of differentiation.

Furthermore, the high positive F_{IS} found for both species could be explained by the presence of null alleles or by the Wahlund effect, either spatial (pool of individuals originated from different houses or different foci within houses) and/or temporal (pool of successive cohorts over a 4-month sampling period). One could suggest that we pooled partially

diverging clades from *An. funestus* (Michel *et al.*, 2005), but this hypothesis can be rejected as this should have been revealed by the Bayesian assignment tests. The hypothesis of possible local scale and/or temporal genetic heterogeneity cannot be ruled out. This is, however, not in contradiction with our result of an absence of genetic differentiation at a larger scale, as already found in other organisms (Torda *et al.*, 2013; Gorospe *et al.*, 2013, 2015). To test for local structure, the sampling and genotyping efforts (number of individuals and number of loci) should be enhanced for the different houses and foci to allow fine-scale structuring analyses.

Nevertheless, for both *An. funestus* and *An. gambiae*, this is a major point of concern when monitoring the spread of resistance against insecticides, as well as the resistance against drugs in *Plasmodium*. In a more hypothetical way, such high gene flow between locations presenting different ecological conditions would facilitate the spread of a genetic mutation. The lack of ecological barriers could be an advantage in the use of genetically modified mosquitoes for malaria control (Boëte, 2006; Levy, 2007; Wang and Jacobs-Lorena, 2013).

Conclusion

Our study found evidence of high gene flow between populations of malaria vectors in north-eastern Tanzania. In the case of *An. funestus*, the genetic structure is unaffected by altitude, at least in this part of East Africa. However, it might be a factor influencing *An. gambiae* distribution with a decrease of mosquito number as altitude increases. Moreover, and given the importance of the spatio-temporal dynamics of vectors in disease transmission (Chaves *et al.*, 2015), there is a crucial need to understand such vector population dynamics in areas with contrasted patterns of transmission (Kamugisha, 2005), to better design tailor-made solutions to control disease transmission.

Ethics

The study received ethical approval by the Tanzanian Commission for Science and Technology (COSTECH) with research permit number 2005-51-NA-2004-53 granted to C.B. All anopheline collections were undertaken following verbal consent of householders.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1742758416000175.

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