POPULATION GENETIC STRUCTURE OF THE AFRICAN MALARIA MOSQUITO
ANOPHELES FUNESTUS IN KENYA

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Abstract. Anopheles funestus Giles is a major malaria vector in Africa, but little is known about the genetic structure of natural populations. In this study, microsatellite markers were used to investigate the genetic structure of A. funestus populations from Kenya. Two populations from western Kenya 80 km apart and two from coastal Kenya 50 km apart were collected and examined for allelic variation at five trinucleotide microsatellite loci. We found A. funestus Giles was the predominant species (>98%) in the A. funestus group in these populations. The western Kenya populations exhibited higher genetic diversity than the coastal populations. No significant genetic structure for populations within the coastal or western Kenya regions was detected. However, population genetic differentiation between the two regions was high ($F_{ST} = 0.208$, $R_{ST} = 0.158$), approximately two-fold higher than A. gambiae populations from the same area. The results suggest that the minimum area associated with a deme of A. funestus in western or coastal Kenya is larger than 50 km in diameter. The Great Rift Valley in east Africa, high-elevation mountains in western Kenya, and the vast arid area east to the Great Rift Valley may all play a role in restricting A. funestus gene flow between coastal and western Kenya.

INTRODUCTION

Malaria, which is transmitted by the Anopheles gambiae species complex and A. funestus, is a major public health problem in sub-Saharan Africa. Anopheles funestus Giles occupies a wide range of ecologic niches, is highly anthropophilic, and is susceptible to human malaria parasites. The vectorial capacity of A. funestus can often exceed that of A. gambiae in some localities. Because the larvae of A. funestus develop in permanent or semi-permanent swamps or in pools along streams and river systems, as opposed to those of the A. gambiae species complex, which prefer temporary aquatic habitats, A. funestus are less dependent on rains and become abundant during the dry seasons when A. gambiae densities are low. Thus, A. funestus is often considered a vector species that bridges malaria transmission during the dry season. Knowledge of vector population genetic structure is essential for management of insecticide resistance and for developing novel malaria control strategies. While population genetic structure of A. gambiae species complex in Kenya has been studied extensively, the genetic structure of A. funestus remains poorly understood. Anopheles funestus represents a group of nine species with slight morphologic differences, including A. funestus Giles (or A. funestus sensu stricto, hereafter referred to as A. funestus), A. rivulorum Leeson, A. parensis Gillies, A. vaneedi Gillies and Couetzee, A. leesoni Evans, A. fascivenosus Leeson, A. aruni Sobti, A. brucei Service, and A. confusus Evans and Leeson. Anopheles funestus and A. rivulorum are the only two species within the A. funestus group that have been incriminated as malaria vectors. Anopheles vaneedi can be infected with Plasmodium falciparum under laboratory conditions, but has not been shown to be a malaria vector in nature. Anopheles funestus generally exhibits patchy distribution patterns because its larvae require long-standing aquatic habitats, such as swamps; since these habitats are limited to lower valleys, they are generally discontinuous in their distribution. The patchy distribution of A. funestus may lead to significant population genetic structure and behavioral differentiation. For example, A. funestus from western Senegal are highly anthropophilic, whereas the mosquitoes from eastern Senegal are much more zoophilic. Polytene chromosome analysis suggests that A. funestus mosquitoes in Senegal consist of two chromosomally differentiated populations. In Burkina Faso, significant and temporally stable deviations from Hardy-Weinberg equilibrium with a deficit of heterokaryotypes have been reported, suggesting the presence of two or more genetically differentiated populations. In east Africa, cytogenetic studies have also found substantial genetic differentiation between A. funestus populations from western and coastal Kenya. However, the mitochondrial DNA cytochrome b gene and the second internal transcribed spacer of ribosomal DNA (rDNA) sequence data did not reveal significant genetic structure for A. funestus populations from west Africa (Burkina Faso and Senegal) and Kenya. The conflict between chromosomal inversions and molecular markers may be caused by non-neutrality of chromosomal inversions and/or lack of sufficient polymorphisms in mitochondrial DNA and rDNA sequence data.

Microsatellites are powerful markers for resolving the population structure of A. gambiae and A. arabiensis because they are highly polymorphic, codominant, and abundant throughout the genome. Polymorphic microsatellite markers have been developed for A. funestus. In this paper, we report very distinct genetic structure of A. funestus populations from coastal and western Kenya as revealed by microsatellite markers.

MATERIALS AND METHODS

Study sites and mosquito collection. Adult mosquitoes were collected from two regions: coastal Kenya and western Kenya. The two regions are separated by about 700 km and span the Great Rift Valley, arid areas to the east of the Great Rift Valley, and high-elevation mountains in western Kenya. Two sites in coastal Kenya were sampled: Majajani (39°47.2'E, 3°39.5'S; elevation = 50 meters above sea level) in the Kilifi district and Magaoni (39°28.4'E, 4°23.0'S; elevation = 80 meters above sea level) in the Kwale district. The two sites in western Kenya in the basin of Lake Victoria were Udhororo (34°8.5'E, 0°0.3'S; elevation = 1.250 meters above...
Extraction of DNA and species identification. DNA from *A. funestus* sensu lato mosquitoes was extracted individually as previously described. We used the molecular method developed by Koekemoer and others to identify individual species within the *A. funestus* group with a slight modification so that single-strand conformational polymorphism (SSCP) gels could be examined in an automated DNA analyzer. This assay is based on the SSCP profile of the 28S rDNA D3 region. The method can distinguish *A. funestus*, *A. rivulorum*, *A. leesoni*, and *A. parensis/A. vaneedeni*. The polymerase chain reaction (PCR) and gel electrophoresis protocols used were similar to those of Koekemoer and others, except that one primer was labeled with a fluorescent dye. Previously identified specimens of *A. funestus*, *A. vaneedeni*, and *A. rivulorum*, kindly provided by Lizette L. Koekemoer (South African Institute for Medical Research, Johannesburg, South Africa), were used as positive controls.

Microsatellite genotyping. Because *A. funestus* was the predominant species within the four populations studied, our genotyping focused on *A. funestus*. Seven trinucleotide microsatellite markers were screened (AFUB2, AFUB3, AFUB6, AFUB8, AFUB10, AFUB11, and AFUB12): two of them (AFUB2 and AFUB8) yielded multiband, nonspecific amplification, and were not used in population genotyping. The primer sequences of the markers, genotyping procedure, and allele scoring were published previously. A total of 42–62 individuals per population were genotyped.

Data analysis. Tests of deviation from Hardy-Weinberg equilibrium for each loci and linkage disequilibrium between all pairs of loci were performed using the GENEPOP (version 3.1) computer software for each population. We further tested whether distortion from Hardy-Weinberg equilibrium resulted from deficient or excessive heterozygosity, using the FIS statistic. Variation in heterozygosity between the two regions (western and coastal Kenya) was analyzed using nested analysis of variance (ANOVA) with regions and subpopulations as factors. Genetic differentiation (FST) between populations and between coastal and western Kenya regions was examined using the FSTAT and TFPGA computer programs. Standard deviations of the F-statistics were obtained for each locus by a jackknife procedure over all alleles, and were used to test for significance. RST statistics, analogous to FST but specifically for microsatellite markers, were calculated using the FSTAT computer software with the number of tandem repeats as input.

### RESULTS

#### Species composition. A total of 221 mosquitoes within the *A. funestus* group (n = 57 for Mbita, 43 for Uhoro, 63 for Majajani, 58 for Magaoni) were tested by the PCR for species identification. Only one *A. leesoni* specimen was found in Majajani and one in Uhoro. Overall, 98.6% of the specimens were *A. funestus* and 0.9% were *A. leesoni*. One specimen showed an SSCP profile that had not been described by Koekemoer and others, and was thus scored as unidentified.

#### Genetic diversity. Population genetic diversity can be measured by the number of alleles and observed heterozygosity. On average, 4–4.4 alleles per locus were observed for the two populations from the Kenya coast (Majajani and Magaoni), but the two populations from western Kenya (Mbita and Uhoro) had an average of 7–7.4 alleles (Table 1). The average observed heterozygosity for the two populations from the Kenyan coast was 0.40–0.41, which was significantly lower than the two populations from western Kenya (0.52–0.53; F = 16.83, degrees of freedom = 1, 1093, P < 0.001, by ANOVA). Thus, genetic diversity of *A. funestus* populations from western Kenya was significantly higher than that from coastal Kenya populations.

#### Test for Hardy-Weinberg equilibrium and linkage disequilibrium. Within each population, deviations from Hardy-Weinberg expectation were found in six of 20 tests (Table 1). Locus AFUB12 showed substantially large, positive FIS values...
ues for three of four populations, suggesting that null alleles may be present in this marker. Null alleles arise when mutations in the primer annealing sites prevent primers from annealing, and generally result in a heterozygosity deficiency. The frequencies of null allele for this locus were estimated to be 0.209 for Majajani, 0.310 for Mbita, and 0.325 for Udhoro. Exact tests found that two of 40 pairs of loci (AFUB6-AFUB10 for Mgaoni and AFUB3-AFUB11 for Mbita) showed linkage disequilibrium, suggesting independent segregation of these five loci.

**Allele frequency and population genetic structure.** Distributions of microsatellite allele frequencies are shown in Figure 1. Within a region, the two populations exhibited a remarkably similar distribution profile; however, there were considerable differences between the two regions. For example, the frequency of the 176-basepair allele at locus AFUB3 in the two coastal Kenya populations was >0.38, but <0.05 in the two western Kenya populations. Highly distinct distribution patterns of allele frequency occurred in four (AFUB3, AFUB6, AFUB10, and AFUB12) of five loci (Figure 1). Population genetic differentiation indexes ($F_{ST}$ or $R_{ST}$) were not significantly different from 0 for populations within either the coastal Kenya or western Kenya regions (Table 2). Thus, *A. funestus* populations from the coastal or western Kenya regions did not exhibit significant genetic structure. In contrast, the $F_{ST}$ and $R_{ST}$ between the two regions were 0.208 ($P < 0.001$) and 0.158 ($P < 0.001$), respectively. Because null alleles were likely present at locus AFUB12 and may cause a biased estimation of population genetic structure, we excluded this locus and recalculated $F_{ST}$. The $F_{ST}$ were $-0.001$ ($P > 0.5$) and $-0.004$ ($P > 0.5$) for populations within western Kenya and coastal Kenya, respectively. Between these two regions, $F_{ST}$ was 0.190 ($P < 0.001$). Therefore, the likely presence of null alleles at locus AFUB12 did not have a substantial effect on the estimation of *A. funestus* population genetic structure.

**FIGURE 1.** Allele frequency of four *Anopheles funestus* populations from Kenya at five microsatellite loci. Alleles are denoted by their actual sizes in basepairs (bp). Majajani and Mgaoni are sites in coastal Kenya and Mbita and Udhoro are sites in western Kenya.
DISCUSSION

In this study, we found that *A. funestus* was the predominant species (>98%) in the *A. funestus* group in western and coastal Kenya populations. This is not surprising because the specimens we used were collected inside human dwellings, and *A. funestus* is highly anthropophilic and endophilic. Kamau and others found similar species compositions in the western and coastal Kenya regions using cytogenetic analysis. We further demonstrated that the genetic diversity measured by the number of alleles and observed heterozygosity of *A. funestus* populations from western Kenya were significantly higher than for the populations from coastal Kenya. We did not detect significant genetic structure for populations within the coastal or western Kenya regions, but did find a high degree of genetic differentiation between the two regions.

*Anopheles funestus* populations within western Kenya or coastal Kenya regions exhibited similar levels of heterozygosity. The greater number of alleles and higher observed heterozygosity of *A. funestus* populations from western Kenya, compared with coastal Kenya, likely reflect the difference in the effective population sizes between the two regions. Lehmann and others found similar patterns of microsatellite allele numbers and heterozygosity distribution in *A. gambiae* in Kenya, and the effective population size of *A. gambiae* in western Kenya populations was estimated to be 1.5–2.9-fold higher than the coastal Kenya populations. Thus, differences in mosquito population genetic diversity between the Kenyan coast and western Kenya may be a common phenomenon for *A. gambiae* and *A. funestus*. In coastal Kenya, habitats suitable for anopheline mosquitoes include a long narrow area (approximately 40 km wide and 200 km long) along the coast, and *A. funestus* are primarily distributed in the southern area near the Tanzanian border. However, *A. funestus* was found in western Kenya over very large areas (several thousand square kilometers). Thus, the absolute population size of *A. funestus* in western Kenya may be much larger than in coastal Kenya. Another possibility is that mosquito populations in Kenyan may have suffered recent population bottlenecks due to historical events, such as periodic droughts.

*Anopheles funestus* populations within western or coastal Kenya regions that are 50–80 km apart did not exhibit significant genetic differentiation for the five loci studied, despite the fact that *A. funestus* distribution is extremely patchy due to larval requirements for permanent or semi-permanent aquatic habitats. Therefore, similar to *A. gambiae*, the minimum area associated for a deme of *A. funestus* in western or coastal Kenya is larger than 50 km in diameter. Large *F*<sub>ST</sub> (0.208) and *R*<sub>ST</sub> (0.158) values were obtained for populations across the two regions, suggesting that gene flow between the two regions is very restricted. Our *F*<sub>ST</sub> estimate with microsatellite markers is slightly lower than that based on chromosomal inversion data (*F*<sub>ST</sub> = 0.275). Lower *F*<sub>ST</sub> estimates based on microsatellite markers may be a consequence of high mutation rates of microsatellite markers and allele size homoplasies. In addition, mutations in microsatellite flanking regions can cause null alleles (e.g., AFUB12 in this study) that may inflate or underestimate population genetic structure. Therefore, a thorough evaluation of population genetic structure using a variety of genetic markers, including microsatellites, mitochondrial gene sequences, and chromosomal inversions, would provide more complete information on the population genetic structure of *A. funestus*.

High levels of genetic differentiation for *A. gambiae* populations between western and coastal Kenya and lack of *A. gambiae* in the hot and arid Great Rift Valley suggest that the Great Rift Valley may be a major gene flow barrier for *A. gambiae*. Estimations of genetic differentiation for *A. funestus* populations between western and coastal Kenya regions are about two-fold larger than for *A. gambiae* (*F*<sub>ST</sub> = 0.104, *R*<sub>ST</sub> = 0.032). Three, not mutually exclusive, factors may have contributed to the higher genetic differentiation between coastal Kenya and western Kenya *A. funestus* populations. The first is geographic barriers. The Great Rift Valley in east Africa may limit *A. funestus* gene flow, but to what extent is unknown because *A. funestus* have been found in the Great Rift Valley, although at a low density. The high-elevation mountains in western Kenya and the vast arid area east to the Great Rift Valley may also play a role in restricting *A. funestus* gene flow. The second factor is physical distance between the populations. It is unknown whether the genetic structure of *A. funestus* is consistent with the isolation-by-distance model. The third factor is the difference in effective population sizes among the populations, as suggested by the present study. Differentiating among these factors requires further experimentation.

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