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Conspecific Sharing of Breeding Sites by Anopheline Female Mosquitoes (Diptera: Culicidae) Inferred from Microsatellite Markers

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Abstract The number of *Anopheles gambiae* and *Anopheles arabiensis* females that used each of the 33 sampled breeding sites in west Kenya was estimated by microsatellite markers and related statistics to test the hypothesis that conspecific females share aquatic sites. Totally, 166 *An. gambiae* and 168 *An. arabiensis* larvae were identified and were genotyped. The mean number of larvae per breeding site was 8.3 for *An. gambiae* and 8.4 for *An. arabiensis*. The likelihood method estimated that, for *An. gambiae*, the mean number of females that would have laid eggs per breeding site was 5.2 and ranged from 2 to 9, and for *An. arabiensis*, the mean was 5.0 with a range of 2–10. The clustering method estimated that the mean number of females laying eggs per breeding site was 6.8 for *An. gambiae*. The results provide molecular evidence that females of one or both species share breeding sites.

Keywords *Anopheles gambiae* · *Anopheles arabiensis* · microsatellites · oviposition behavior

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Introduction

Anopheles gambiae and *Anopheles arabiensis* are members of the *An. gambiae* species complex, and are the two principal malaria vectors across sub-Saharan Africa. Many aspects of behavior of the mosquitoes have been studied and well documented (Gillies and Coetzee 1987; Huang et al. 2005, 2006), but little is known about their oviposition behavior under field conditions (Chen et al. 2006a). Better understanding of breeding behavior of mosquitoes may provide novel avenues for ecological control of mosquitoes and mosquito-borne disease (Pates and Curtis 2005).

Although the two vectors are sibling species and morphologically indistinguishable, there are several obvious differences in their behavior and ecology. First, *An. gambiae* is largely anthrophilic while *An. arabiensis* is partially zoophilic and its feeding behavior varies geographically (Gwadz and Collins 1996). Second, *An. gambiae* is generally more susceptible to the malaria parasite, *Plasmodium falciparum*, than *An. arabiensis* (Vaughan et al. 1994). Third, the two mosquito species have different distribution patterns (Lindsay et al. 1998; Coetzee et al. 2000). Although the two species are sympatric in many areas of sub-Saharan Africa, *An. gambiae* tends to inhabit humid areas while *An. arabiensis* is more abundant in relatively dry areas.

Gravid females of *An. gambiae sensu lato* preferred black-bottomed water dishes to white-bottomed ones for oviposition and most eggs were laid in pools with muddy and unvegetated edges (McCrae 1983, 1984). *An. arabiensis* larvae adapted to relatively still and shallow areas of bigger ponds and streams in the Rift Valley and central region in Kenya because smaller water bodies could not exist for long in the sandy soil and dry weather (Chen, unpubl.). However, in western Kenya, *An. gambiae s. l.* mainly adopted temporary aquatic sites such as animal footprints and puddles to breed their larvae (Minakawa et al. 1999). In nature, the abundance of anopheline larvae varies between aquatic sites. Some sites contain large amounts of anopheline larvae, while many others are vacant despite high densities of mosquito adults in surrounding houses (Minakawa et al. 1999, 2001). This phenomenon leads to speculation that some aquatic sites are more suitable or attractive for oviposition (McCrae 1984; Huang et al. 2005, 2006) and/or more favourable for larval development than others (Minakawa et al. 1999). If this is true, individual aquatic sites that contain anopheline larvae may be the result of oviposition by multiple females, especially during a dry season when suitable breeding habitats are limited. The previous studies (Minakawa et al. 1999, 2001; Chen et al. 2006a) showed that *An. gambiae* and *An. arabiensis* co-habited some aquatic sites in western Kenya, but it is not known if conspecific female mosquitoes share a breeding site.

Direct observation of mosquito oviposition behavior is not feasible because individual mosquitoes cannot be tracked in the field. Since the majority (>95%) of *An. gambiae* females mate only once (Tripet et al. 2001), the larvae in a particular site should belong to one or more full-sibling families (A full-sibling family are the larvae from the same parents). Therefore, with the aid of molecular markers (Blouin et al. 1996; Goodnight 2001; Queller and Goodnight 1989), this hypothesis of breeding site sharing by conspecific female mosquitoes can be tested.

Randomly amplified polymorphic DNA (RAPD) markers (Apostol et al. 1993, 1994) and restriction fragment length polymorphism (RFLP) markers (Colton et al.

2003) were used to estimate the number of full-sibling families of the yellow fever mosquito, *Aedes aegypti*, in individual containers. Apostol et al. (1993, 1994) found that the average family size of mosquito larvae per container was 11, and that family size distribution among the containers was skewed, with an excess of containers having 1 to 2 families. Colton et al. (2003) inferred that individual *Ae. aegypti* females oviposited in more than one container, with an average of 6.2 families per container. *Ae. aegypti* primarily develops in containers, and its larval density tends to be high. However, *An. gambiae* and *An. arabiensis* often occur in temporary aquatic habitats in the area of this study, and their larval densities in individual breeding sites are lower than those of *Ae. aegypti* in containers (Minakawa et al. 2001; Wagbatsoma and Ogbeide 1995). Using microsatellite markers, Chen et al. (2006a) inferred that one *A. gamibae* female used multiple breeding sites around a hut in west Kenya.

The objective of this study was to use microsatellite markers to determine the relatedness, kinship and full-sibling family size of anopheline mosquito larvae in each of sampled breeding sites. Because microsatellite markers are codominant, highly polymorphic, and usually neutral, they should be more powerful in determining the kinship of larval samples than the dominant RAPD markers (Blouin et al. 1996). In *An. gambiae*, more than 100 microsatellite markers have been isolated and genetically or cytogenetically mapped (Zheng et al. 1996). The majority of the microsatellite markers isolated from *An. gambiae* can also yield reliable amplification for *An. arabiensis* (Kamau et al. 1998, 1999; Simard et al. 1999; Donnelly and Townson 2000; Nyanjom et al. 2003). Thus, these microsatellite markers represent excellent molecular tools for determining genetic relationships of anopheline larvae in a breeding site and for studying some aspects of oviposition behavior (Lehmann et al. 2003; Chen et al. 2006a). Two statistical methods, the likelihood method (Queller and Goodnight 1989) and clustering analysis (Apostol et al. 1993, 1994; Blouin et al. 1996), have been used for determining kinship among individuals within a population and their full-sibling family size. This study also tested whether these two methods yield comparable results.

Materials and Methods

Study Area and Species

The mosquito specimens used in this study were a random selected subset of samples that had been previously collected for larval habitat characterization (Minakawa et al. 1999). Mosquito larvae were collected from aquatic breeding sites in Suba District, Nyanza Province of western Kenya (Fig. 1), during the dry season (February–March) of 1998. An aquatic site was first inspected for the presence of mosquito larvae. When mosquito larvae were present, they were collected with a standard mosquito dipper (350 ml) at each site. All larvae were examined microscopically, and anopheline larvae were further separated from culicine larvae. *Anopheles gambiae s.l.* larvae were preserved in 100% ethanol for subsequent DNA analysis. The water surface area (m^2) of each site was measured (Minakawa et al. 1999). The coordinates of each sampling site were recorded with a hand-held GPS.

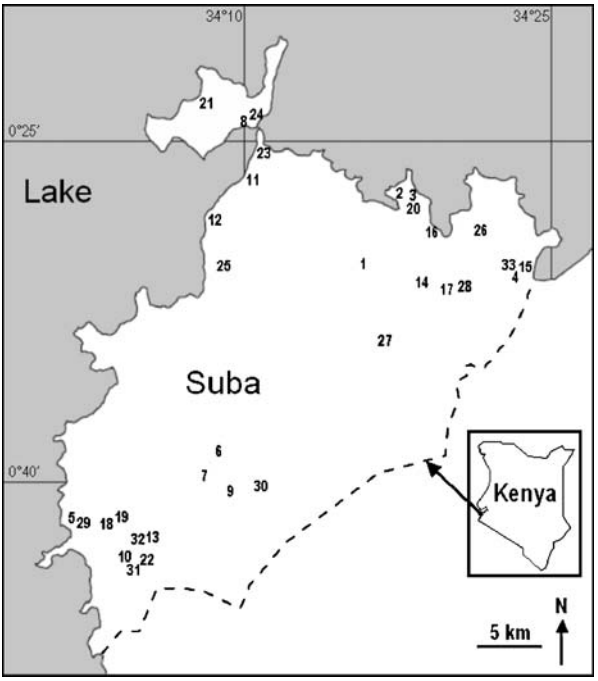

Fig. 1 Location of breeding sites sampled in Suba district, western Kenya. The *site numbers* correspond to those in Table 1.

Species identification for the sampled larvae within the *An. gambiae* species complex was conducted using the rDNA-PCR method (Scott and Collins 1993; Chen et al. 2006a, b). The present study examined 33 breeding sites out of the 128 sites previously studied by Minakawa et al. (1999) and analyzed a total of 166 *An. gambiae* larvae and 168 *An. arabiensis* larvae.

Microsatellite Genotyping

To establish kinship among mosquito larvae in a breeding site, each larva was genotyped with nine microsatellite markers, AGXH1D1, AGXH131 and AGXH503 on Chromosome X, AG2H46, AG2H79 and AG2H117 on Chromosome 2, and AG3H29C, AG3H33C and AG3H158 on Chromosome 3 (Chen et al. 2006a). The microsatellite markers were readily amplified using mosquito genomic DNA as the template (Lehmann et al. 1996; Zheng et al. 1996). Due to the high frequencies of null allele at Locus AG2H46, alternative primers were used for this locus (Nyanjom et al. 2003). Microsatellite genotyping and allelic scoring were conducted following Nyanjom et al. (2003) and Chen et al. (2004).

Data Analyses

To estimate the number of full-sibling families for conspecific larvae from each site, two methods were used. The first method was the likelihood algorithm (Queller and Goodnight 1989) programmed in the Kinship software (Goodnight 2001). The

hypothesized kinship between a pair of individuals was specified by two variables r_p and r_m, which defined the probabilities that individuals in the pair shared an allele by direct descent from their father and mother, respectively. Because the hypothesized relationship was diploid full siblings, both r_p and r_m were 0.5. The null hypothesis was that the pair was not genetically related (the paired larvae did not share any parents), and thus r_p and r_m values should be 0. The likelihood ratio for the two hypotheses was calculated, using the designated r values, population allelic frequencies and genotypes of the individuals under consideration. The population allelic frequencies were obtained from adult populations for *An. gambiae* and *An. arabiensis* collected in the same area at the same time (Chen et al. 2004). The statistical significance of the likelihood ratio was tested using the permutation test (100,000 simulated pairs). The critical likelihood values ($P<0.05$) were set up as 2.36 for *An. gambiae* and 3.06 for *An. arabiensis*, respectively. The Kinship computer program calculated a likelihood ratio matrix for pairs of larvae from the same site. Any pair of larvae from one breeding site with an estimated likelihood ratio higher than the critical value was considered full siblings.

The second method used clustering analysis modified from Apostol et al. (1993, 1994) and Blouin et al. (1996). Based on the number of alleles shared between pairs of individuals, a genetic similarity tree was generated for all larvae in a site using the unweighted pair group method with arithmetic mean (UPGMA). Briefly, a value of 0 was given if a pair of larvae shared no alleles at one locus, 1 was assigned if the pair shared one allele, and 2 if the pair shared two alleles at one locus. The allele-sharing value between a pair of larvae (M) was averaged for the six loci considered. Thus, a matrix of averaged allele-sharing values, defined as pairwise genetic similarity, was produced for all possible pairs of larvae in a site. A dendrogram was drawn based on the matrix of pairwise genetic similarity for all larvae in a site using the NTSYSpc computer program (Rohlf 2000). To determine the critical value of genetic similarity that can discriminate full siblings from non-full siblings, five bloodfed *An. gambiae* females from the field were collected and their larvae were genotyped with the same six microsatellite markers. Because more than 95% of *An. gambiae* females mate with only one male in nature (Tripet et al. 2001), the offspring from each of the five females could be considered as full siblings. The pairwise allele-sharing values were calculated for all pairs of the larvae from one female, supposed to be a full-sibling family, and the average of pairwise allele-sharing values for the six markers in the five families was used as the critical discriminating value (M_c). The estimates of full sibling families by both methods were compared using a paired t-test. The clustering analysis was conducted only for *An. gambiae* because full-sibling larvae were not available for *An. arabiensis*.

To determine whether female egg-laying behavior is related to the site size, linear regression analysis was conducted to examine the relationship between the estimated number of full-sibling families and the number of larvae in a site or the water surface area of the sites. Due to the skewness of the variable, the site sizes were $\log_{10}$ transformed before the regression analyses.

To test if the number of full-sibling families of one species is affected by the presence of the other at a site, two-sample t-test on the conspecific numbers of full-sibling families was performed between single species and co-inhabited sites. The above regression analysis and t-test were done using Minitab (Minitab 1996).

Results and Discussion

The numbers of *An. gambiae* and *An. arabiensis* in breeding sites and the estimated number of full-sibling families are shown in Table 1. Among the 33 sites, seven were inhabited by larvae of both species, and 26 were occupied by larvae of only one species. The average number of larvae per site was 8.3 for *An. gambiae* and 8.4 for *An. arabiensis*.

The likelihood method estimated that the mean number of full-sibling families was 5.2 (95% confidence interval: 4.2–6.1) and ranged from 2 to 9 for *An. gambiae*, and 5.0 (95% CI: 4.0–5.9) with a range of 2–10 for *An. arabiensis*.

The clustering analyses produced a dendrogram for all *An. gambiae* larvae from each site based on allele-sharing values between pairs of individuals (Fig. 2). The

Table 1 Sample Size and Estimated Number of Full-sibling Families of *Anopheles gambiae* and *An. arabiensis* at the Breeding Sites in Western Kenya

Site		*Anopheles gambiae*			*Anopheles arabiensis*	
Number	Area (m^2)	No. larvae	Likelihood method	Clustering method	No. larvae	Likelihood method
1	0.11	10	5	9	–	–
2	0.13	8	7	7	–	–
3	0.21	11	7	8	–	–
4	0.10	12	6	11	–	–
5	80.17	12	8	11	–	–
6	0.19	12	9	10	–	–
7	0.16	8	4	4	–	–
8	1.70	6	4	4	–	–
9	0.15	9	5	8	–	–
10	0.17	8	3	6	–	–
11	0.13	7	4	5	–	–
12	0.35	5	4	4	–	–
13	0.14	8	2	7	–	–
14	0.13	–	–	–	5	2
15	0.10	–	–	–	5	2
16	80.60	–	–	–	13	6
17	0.11	–	–	–	10	6
18	1.70	–	–	–	5	5
19	0.17	–	–	–	11	10
20	0.14	–	–	–	7	5
21	0.23	–	–	–	10	6
22	0.10	–	–	–	22	8
23	1.17	–	–	–	5	4
24	0.11	–	–	–	5	4
25	0.12	–	–	–	12	5
26	0.11	–	–	–	11	4
27	0.10	6	5	5	6	4
28	0.11	5	2	3	6	4
29	0.11	6	5	5	5	4
30	0.10	9	7	8	11	5
31	0.10	9	8	8	8	8
32	0.18	10	4	9	6	3
33	0.10	5	4	4	5	4
Average	5.12	8.3	5.2	6.8	8.4	5.0

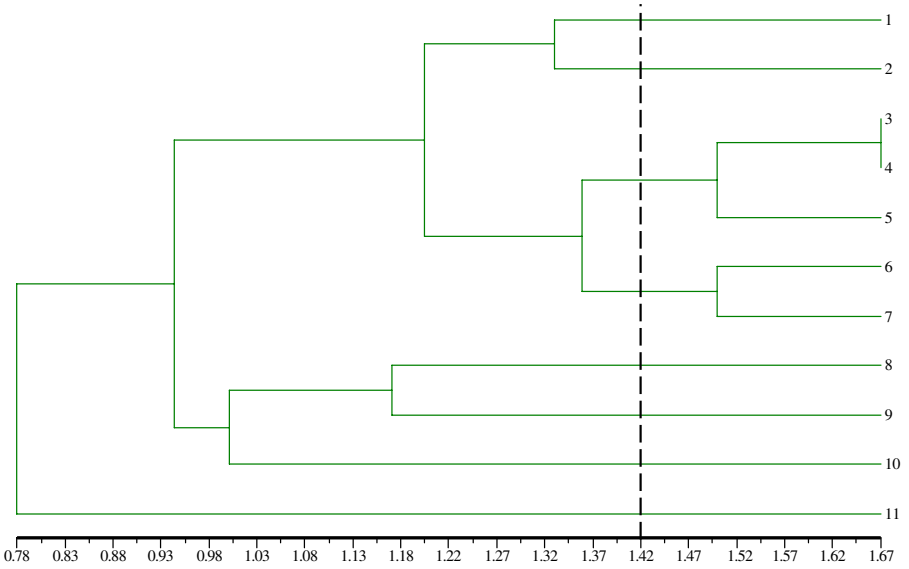

Fig. 2 An UPGMA dendrogram showing the genetic similarity among *An. gambiae* larvae from Site 3. The *numbers on the right* represent larval identity. The *vertical dashed line* shows the critical value (M_c) that discriminates full-sibling larvae from non-full siblings.

average pairwise allele-sharing value from the five *An. gambiae* larval families, each supposed to be a full-sibling one, was 1.42 (95% CI: 1.36–1.48). Therefore, 1.42 was used as the critical value (M_c) to discriminate full siblings from non-full siblings. A cluster with an allele-sharing value above 1.42 was considered a full-sibling family. The clustering analysis estimated that the average number of full-sibling families in a breeding site was 6.8 (95% CI: 5.6–8.0) for *An. gambiae*. The numbers of full-sibling families estimated by the clustering analysis was significantly higher than the numbers estimated by the likelihood method (paired *t*-test, $t=3.9$, $df=19$, $P<0.05$). Besides the different algorithms in the likelihood and clustering methods, different data sets were used, e.g. the population allelic frequencies in the likelihood and the five full-sibling families' data in the clustering method. All these could contribute to the variance between the estimates by the two methods.

Composition of full-sibling larvae among the breeding sites provides vital information on understanding the female oviposition behavior and larva control. The estimated number of full-sibling families was positively correlated with number of larvae in a site (*An. gambiae*: $F=13.38$, $df=1$, $P<0.01$ for likelihood estimates; $F=144.39$, $df=1$, $P<0.01$ for clustering estimates. *An. arabiensis*: $F=11.89$, $df=1$, $P<0.01$ for likelihood estimates. Both species, $F=33.72$, $df=1$, $P<0.01$ for likelihood estimates). This positive correlation indicated that more females used the sites with higher numbers of larvae. However, the estimated number of full sibling families was not correlated with size of breeding site (*An. gambiae*: $F=1.24$, $df=1$, $P>0.05$ for likelihood estimates; $F=1.36$, $df=1$, $P>0.05$ for clustering estimates. *An. arabiensis*: $F=0.20$, $df=1$, $P>0.05$ for likelihood estimates. Both species, $F=0.05$, $df=1$, $P>0.05$ for likelihood estimates). Furthermore, due to the observation that the two species often occur in small and temporary aquatic sites in the study area, the

two larger sites (Sites 5 and 16, >80 m^2) could be omitted from the analyses. Without using the data for the two larger sites, the correlation was not significant either (*An. gambiae*: $F=0.32$, $df=1$, $P>0.05$ for likelihood estimates; $F=1.15$, $df=1$, $P>0.05$ for clustering estimates. *An. arabiensis*: $F=0.00$, $df=1$, $P>0.05$ for likelihood estimates. Both species, $F=1.80$, $df=1$, $P>0.05$ for likelihood estimates). These non-significant correlations showed that the size of a breeding site did not affect the numbers of females laying eggs at the site. Characterizing the physicochemical and ecological features of these favorable breeding sites may lead to better control effects by environmental improvement (including to remove and destroy the breeding sites) and larvicide application targeting the breeding sites with high larval densities (Chen et al. 2006a; Minakawa et al. 1999).

There were no significant differences in the estimated numbers of conspecific full-sibling families between the sites with single species present and those with both species (*An. gambiae*: 5.2 vs. 5.0, $P>0.05$ for likelihood estimates; 5.0 vs. 5.0, $P>0.05$ for likelihood estimates omitted Site 5; 7.2 vs. 6.0, $P>0.05$ for clustering estimates; 6.9 vs. 6.0, $P>0.05$ for clustering estimates omitted Site 5; *An. arabiensis*: 5.2 vs. 4.6, $P>0.05$ for likelihood estimates; 5.1 vs. 4.6, $P>0.05$ for likelihood estimates omitted Site 16), indicating no inter-specific effect on female's choice of oviposition site at the presence of the other species. However, a competitive disadvantage was revealed for *An. arabiensis* in mixed larval populations under laboratory conditions (Schneider et al. 2000). Inter-specific larval dynamics would affect the species composition of adult mosquito populations and so might affect human malaria incidence, due to the different vectorial capacity of the two mosquito species.

The above results demonstrated that multiple females of both anopheline species laid eggs in a given aquatic site in the study area. As many as 16 females may have laid eggs in a site containing both *An. gambiae* and *An. arabiensis* (Site 31, Table 1). Therefore, the results support the hypothesis that one breeding site is utilized by multiple females of one or both species in the study area. Because the samples were collected in a dry season when suitable sites were limited, it is possible that aquatic site sharing by female mosquitoes in a dry season may be more common than in a rainy season. Population bottleneck effect was not found in both *An. arabiensis* by a genetic estimation (Taylor et al. 1993) and *An. gambiae* by a field survey (Minakawa et al. 2001) during dry seasons despite extremely low densities of adult mosquitoes. Breeding site sharing and adaptation to various sizes of available aquatic sites, both conspecifically and inter-specifically, may play an important role in maintaining mosquito genetic diversity.

An anopheline female usually produces 50–200 eggs after a blood meal (Gwadz and Collins 1996). Two hypotheses may help to explain the egg-laying behavior of anopheline females. The first is that a female lays all her eggs in one site, but egg hatchability and larval survivorship are extremely low due to competition as observed indoor (Schneider et al. 2000), predation or other factors. The second is that a gravid female lays eggs in more than one aquatic sites per gonotrophic cycle, but deposits only a few eggs per site, a phenomenon referred as the "skip-oviposition" behavior in *Ae. aegypti* mosquitoes (Chadee and Corbet 1987; Chadee et al. 1990; Harrington and Edman 2001). This study inferred 2–11 full-sibling families, each with 2–10 larvae per breeding site, but can not conclusively demonstrate "skip-oviposition" behavior in the two anopheline species because of

the inexclusively sampling method employed in this study and questions pertaining to larval survivorship in nature. These hypotheses will be tested directly in future through paternity and kinship analyses using adults collected in houses and larvae sampled in sites surrounding the houses.

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