

Ecological Zones Rather Than Molecular Forms Predict Genetic Differentiation in the Malaria Vector *Anopheles gambiae* s.s. in Ghana

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ABSTRACT

The malaria mosquito *Anopheles gambiae* s.s. is rapidly becoming a model for studies on the evolution of reproductive isolation. Debate has centered on the taxonomic status of two forms (denoted M and S) within the nominal taxon identified by point mutations in the X-linked rDNA region. Evidence is accumulating that there are significant barriers to gene flow between these forms, but that the barriers are not complete throughout the entire range of their distribution. We sampled populations from across Ghana and southern Burkina Faso, West Africa, from areas where the molecular forms occurred in both sympatry and allopatry. Neither Bayesian clustering methods nor F_{ST} -based analysis of microsatellite data found differentiation between the M and S molecular forms, but revealed strong differentiation among different ecological zones, irrespective of M/S status and with no detectable effect of geographical distance. Although no M/S hybrids were found in the samples, admixture analysis detected evidence of contemporary interform gene flow, arguably most pronounced in southern Ghana where forms occur sympatrically. Thus, in the sampled area of West Africa, lack of differentiation between M and S forms likely reflects substantial introgression, and ecological barriers appear to be of greater importance in restricting gene flow.

WHEN reproductive isolation between species is incomplete (*i.e.*, when F_1 hybrids are not completely sterile), genes may pass between species (MACHADO *et al.* 2002). Whether such interspecific hybridization is a significant route for the transfer of genetic adaptation remains a major question in evolutionary biology (BARTON 2001). The African malaria vector *Anopheles gambiae* is a unique model for studies of the evolution of reproductive isolation and the importance of hybridization for the transfer of adaptively advantageous genes in incipient species. Within *An. gambiae*, sequence variation in X-linked rDNA of populations from West Africa led to the description of two molecular forms (termed M and S), which differ in both the transcribed and the nontranscribed spacers in the rDNA repeat unit (DELLA TORRE *et al.* 2001; GENTILE *et al.* 2002). Reproductive isolation between the M and S forms has been supported by a lack of hybrids detected by PCR of the rDNA intergenic and also partially by studies of the distribution of the *kdr* gene, a mutation involved in resistance to pyrethroid insecticides (CHANDRE *et al.* 1999). In general, in West African countries, apart from

Benin, the *kdr* resistance locus is at or near fixation in the S-form populations but occurs in the M form at very low levels, even in sympatry (DELLA TORRE *et al.* 2001; YAWSON *et al.* 2004). Importantly, it has been proposed that the *kdr* mutation may have introgressed from the S form into the M form (WEILL *et al.* 2000), raising the possibility that there could be numerous adaptively and epidemiologically important genetic exchanges between forms.

Evidence from *Drosophila* has indicated that isolation does not occur simultaneously for the whole genome (MACHADO *et al.* 2002) and that the least likely parts to exchange are associated with hybrid sterility or mate choice (NOOR *et al.* 2001). This mosaic genome structure was first postulated for *An. gambiae* by DELLA TORRE *et al.* (1997) and more recent evidence was provided by TURNER *et al.* (2005) who used a microarray approach to identify regions of the genome that they term “islands of speciation.” One of the regions that TURNER *et al.* (2005) identified does indeed appear to be intimately involved with the reproductive isolation of these forms (STUMP *et al.* 2005).

Outside the few areas of marked differentiation only slight differences have been observed in allele or haplotype arrays between the M and S forms using a range of genetic markers (GENTILE *et al.* 2002; LEHMANN

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et al. 2003; DONNELLY *et al.* 2004), although microsatellites from regions of the second chromosome associated with putatively selective advantageous chromosomal inversions have been shown to differ between forms (LANZARO *et al.* 1998). The lack of marked differentiation throughout most of the genome has been attributed to the recent isolation of the forms whereby insufficient time has elapsed for even noncoding DNA to accrue differences (GENTILE *et al.* 2001). Premating isolation mechanisms apparently result in positive assortative mating when the forms are in sympatry, with 98.8% within form mating (TRIPET *et al.* 2001). Indeed, evidence from a study in Cameroon suggested that reinforcement between forms might occur in sympatry, with F_{ST} estimates of 0.035–0.052 between allopatric populations compared to 0.060 between sympatric populations (WONDJI *et al.* 2002).

Given that there is strong positive assortative mating and molecular genetic suggestions of reinforcement in sympatry, the question arises, where does interform hybridization occur? Rates of hybridization are unlikely to be equal across the sympatric distributions of the forms and therefore identifying the geographic regions where genetic exchange does occur is central to an understanding of the evolution of these incipient species. Previous evidence has suggested that the area between Cote d'Ivoire and Benin may be an area of transition between populations that show strong positive assortative mating and populations that are more permissive of interform gene flow (BLACK and LANZARO 2001; DELLA TORRE *et al.* 2001). We used microsatellite loci to determine the intra- and interform variation in M and S forms in Ghana and southern Burkina Faso. Our data suggest that interform hybridization occurs at significant levels and that ecological barriers represent far more important barriers to gene flow in this area of West Africa.

MATERIALS AND METHODS

Sample sites and screening: Sample sites are shown in Figure 1, with a detailed description in YAWSON *et al.* (2004). In brief, M and S forms were sympatric in the mangrove swamps along the coast; in northern Ghana and Burkina Faso, samples were overwhelmingly M form (80–86% in northern Ghana and 80% in Burkina Faso) and the samples from the middle rain forest belt were all S forms (Figure 1). Specimens were identified as to form using a combination of the approaches of SCOTT *et al.* (1993) and FANELLO *et al.* (2002). Seven microsatellite loci—two on chromosome X (AgXH7, AgXH99), one on chromosome 2 (Ag2H197), and four on chromosome 3 (Ag3H119, Ag3H812, Ag3H577, 33CI)—were chosen on the basis of physical and linkage maps of *An. gambiae* (ZHENG *et al.* 1996) and, with the exception of Ag2H197 (inversion 2Ra), were not contained within polymorphic inversions. Previous studies had found these markers to be highly variable and to amplify reliably (DONNELLY *et al.* 2001; PINTO *et al.* 2002; WONDJI *et al.* 2002). None of these markers are within the “genomic islands of speciation” identified by other authors

(TURNER *et al.* 2005) but some of the markers show marked differences in allele frequencies between molecular forms (PINTO *et al.* 2002; WONDJI *et al.* 2002). Thirty-one individuals were identified per site unless the forms occurred in sympatry, in which case 31 individuals of each form were identified. Microsatellite genotyping methods followed (DONNELLY *et al.* 1999). In total, genotypes were determined for 462 *An. gambiae* specimens from 11 sites in Ghana and 1 site in Burkina Faso.

Statistical analysis: Tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium among all pairs of loci within populations were done with GENEPOP 3.4 (RAYMOND and ROUSSET 1995). Diversity per locus and per population was assessed using allelic richness (R_s), observed (H_O) and expected (H_E) heterozygosities, and inbreeding coefficients (F_{IS}), and their significance was calculated using FSTAT 2.9.3.2 (GOUDET 2001). To explore the impact of putative null alleles on our analyses, we used the program MICRO-CHECKER (VAN OOSTERHOUT *et al.* 2004) to estimate null allele frequencies (using the “Brookfield 2” estimator; BROOKFIELD 1996) for potentially affected loci that showed a consistent significant excess of homozygotes across sample sites. Using these estimated frequencies, an adjusted data set, with nulls recoded as separate alleles, was produced for comparison with the original data set.

Genetic relationships within and between forms were assessed using three complementary approaches: two Bayesian clustering methods and an F_{ST} -based summary statistic method. A Bayesian clustering algorithm implemented in the program STRUCTURE (PRITCHARD *et al.* 2000) was applied to identify subgroups that have distinctive allele frequencies, without using prior knowledge of sampling sites. We used the admixture model with correlated allele frequencies between populations with a burn-in length of 100,000, and 100,000 Markov chain Monte Carlo replications for each setting of K from 2 to 6 (20 replicate runs of each). To determine the most appropriate number of clusters, we used the approach of EVANNO *et al.* (2005), which is based upon an *ad hoc* quantity (ΔK) that evaluates the second-order rate of change of the likelihood function with respect to K . For the STRUCTURE analysis, we made three predictions: (i) if there was extensive genetic exchange between the forms or if they had only recently become separated, then we would expect M- and S-form samples to form a single cluster; (ii) if sufficient time had elapsed for the forms to diverge, then we would observe clusters corresponding with each of the forms; and (iii) if there were zones of hybridization between the two forms, then we would observe two clusters, one corresponding with each of the forms and one or more additional clusters containing those samples where introgression was occurring.

A second Bayesian clustering analysis, implemented in the software BAPS 4.13, was also applied (CORANDER *et al.* 2003). By contrast to the individual-based algorithm applied in STRUCTURE, we used the group-level option in BAPS 4.13 such that clusters are formed by merging whole samples. Since group-level analysis does not rely on the integrity of individual multilocus genotypes, we were able to use the method to compare solutions from the original and null-allele-corrected data sets. The resulting clusters (from the original data set) were saved, and admixture proportions—estimated for each individual, again using BAPS 4.13 (CORANDER *et al.* 2004), with the posterior probability for the null hypothesis of pure ancestry—were computed by permutation. We used clusters, rather than individual sample sites, as the units for the analysis because admixture results can be unreliable when the power to discriminate populations is low (J. CORANDER, personal communication). Thus, while total contemporary admixture of an individual sample site could be determined, the source of the nonresident admixture proportion(s) could be assigned

TABLE 1
Sampling details

Location	Ecological zone	Latitude	Longitude	% S	% M
Dodowa	Coastal savanna	5° 52.67' N	0° 06.36' W	94	6
Osuogba	Coastal savanna	5° 52.75' N	0° 06.58' W	99	1
Odumasy	Coastal savanna	5° 53.86' N	0° 04.72' W	81	19
Ayikuma	Coastal savanna	5° 55.23' N	0° 03.20' W	97	3
Ayena	Coastal savanna	5° 56.59' N	0° 01.89' W	97	3
Okyereko	Mangrove	5° 24.87' N	0° 36.25' W	35	65
Mampong	Mangrove	5° 24.74' N	0° 36.95' W	69	31
Abia	Mangrove	5° 42.93' N	0° 07.69' W	61	39
Kumasi	Central forest	6° 41.00' N	01° 37.00' W	100	0
Korania	Northern savanna	10° 53.16' N	01° 05.40' W	0	100
Bonia	Northern savanna	10° 52.05' N	01° 07.25' W	0	100
Koubri (Burkina Faso)	Northern savanna	12° 11.56' N	01° 23.46' W	3	97

Locations, habitat type, and the percentage of each molecular form (M and S) are shown.

only to a cluster. Since our aim was to study patterns of admixture, rather than to identify specific potentially migrant individuals, critical α for admixture was set at a posterior probability of 0.05, although we also determined individuals that showed admixture probabilities that remained significant following Bonferroni correction.

F_{ST} estimates and pairwise (permutation-based) tests of genic differentiation, which assume unlinked markers but not Hardy–Weinberg equilibrium, were calculated using Genepop 3.4 (RAYMOND and ROUSSET 1995). Pairwise F_{ST} estimates were linearized following SLATKIN (1995) and the matrix was entered into the MEGA 3.1 program for neighbor-joining tree construction (KUMAR *et al.* 2004). We hypothesize that if introgression is ongoing in sympatry, F_{ST} values between sympatric populations will be lower than those between interform allopatric comparisons, whereas the converse is expected if reinforcement occurs in sympatry. As an *ad hoc* test of these hypotheses, groups were resampled using 1000 bootstrap replicates to estimate 95% confidence intervals around the group mean F_{ST} using SAS (SAS INSTITUTE 1990). Isolation by distance was examined by testing the association of linearized F_{ST} with ln-transformed geographical distance using a Mantel test with 5000 permutations implemented by the POPTOOLS add-in for Microsoft EXCEL (written by Greg Hood, CSIRO; available from <http://www.cse.csiro.au/poptools>). Unless stated otherwise, where multiple tests were used, we adjusted the significance level using a sequential Bonferroni procedure (HOLM 1979).

RESULTS

Hardy–Weinberg equilibrium and genotypic disequilibrium: An excess of homozygotes was very common in the data set (APPENDIX A), with 48 of a total of 105 tests significant at $P < 0.05$, and 26 remaining so following sequential Bonferroni correction. Hardy–Weinberg deviations were not restricted to particular loci nor geographic samples (APPENDIX A). Of 315 tests for linkage disequilibrium, 16 were significant at $P < 0.05$, which is exactly the number expected by chance as type I errors at this critical α -level, and following sequential Bonferroni correction, no tests remained significant in any

sample or at any locus. Since within-population structure would be expected to cause both excess homozygosity and linkage disequilibrium, the widespread prevalence of the former but the absence of the latter in our data suggested the presence of null alleles. Estimates of null allele frequencies per population and per locus were generated, assuming random mating, using MICRO-CHECKER (VAN OOSTERHOUT *et al.* 2004) whenever a significant excess of homozygotes was detected. The mean estimated null allele frequency of 8.7% was similar to the mean frequency of null alleles (5–8%) estimated for *An. gambiae* on the basis of X-linked loci in males (BARNES *et al.* 2005; STUMP *et al.* 2005).

Intra- and interform genetic differentiation: Individual-based Bayesian clustering using STRUCTURE determined the most appropriate number of clusters as two, and successive increases in K did not split the two major groupings into additional clusters (supplemental materials at <http://www.genetics.org/supplemental/>). The ΔK value provided support for the division into two groups with a marked peak at $K = 2$ and a rapid decline at higher values. The clusters did not partition the molecular forms, but rather grouped individuals by ecological zone, although not by geographic proximity (see Figure 1). One cluster comprised M- and S-form individuals from the coastal mangrove strand zone, with the other cluster containing all individuals from the other sites, sampled from the northern Sahel savanna, coastal savanna and deciduous forest (Figure 2a).

Population differentiation was widespread with 101 of 105 pairwise tests significant following sequential Bonferroni correction (APPENDIX B). The major division in an F_{ST} -based neighbor-joining tree was in accord with neither geographic proximity of the sample locations nor with the molecular status of the samples, but represented a primary division between the populations in the mangrove strand zone of southern Ghana (in which M and S are sympatric) and all remaining M and S

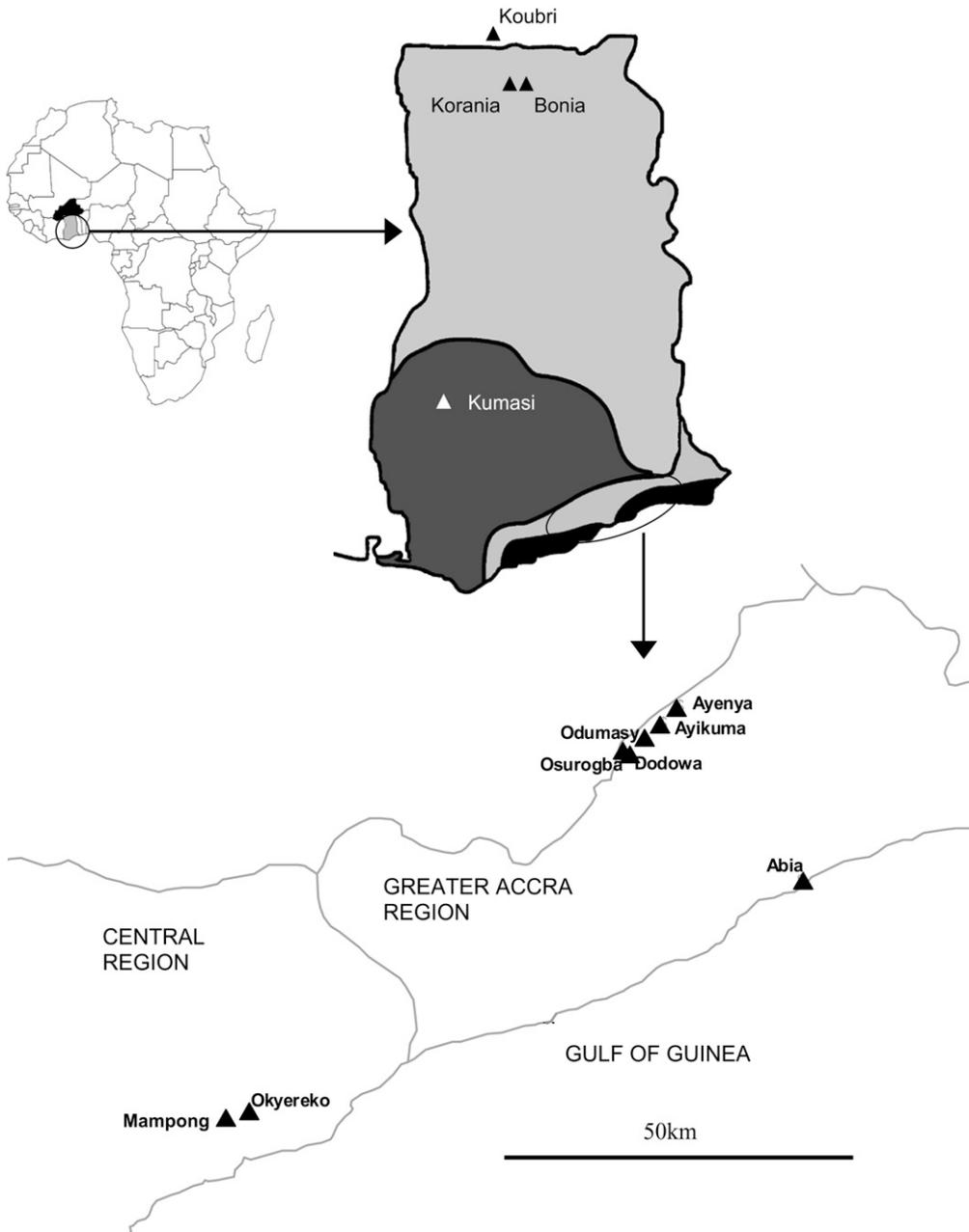


FIGURE 1.—Sampling locations. Ghana and Burkina Faso are marked on the outline map of Africa as shaded and solid, respectively. The more detailed map of Ghana shows the limits of the ecological zones within Ghana. Solid, mangrove strand zone; light shading, coastal savanna; dark shading, deciduous forest; mid-level shading (largest zone), Sahel (northern) savanna. The sample from Koubri (Burkina Faso) is also within the Sahel savanna. Sampling locations are marked by triangles. A large-scale map of sampling locations within 50 km of the coast is given.

populations from the north and the south (Figure 2b). Results from group-level Bayesian clustering analysis performed by BAPS also revealed a major split between the mangrove strand zone and other populations, but in addition partitioned the Kumasi (deciduous forest zone) separately (Figure 2b). This optimal clustering solution was well supported since the next most likely solution—of two clusters, in which Kumasi grouped with all other non-mangrove zone samples—could be confidently rejected ($P < 0.0001$). Nevertheless, the Kumasi site was clearly far more distinct from the mangrove zone samples (mean pairwise $F_{ST} = 0.10$) than from the northern and coastal savanna samples ($F_{ST} = 0.04$).

There was limited evidence to suggest a contribution of molecular form to differentiation. For comparisons

made either within or between ecological zones, interform mean F_{ST} values were only marginally higher than intraform values and none of these differences were significant (Figure 3). However, since the difference between inter- and intraform comparisons was, if anything, greater in the non-mangrove cluster of allopatric M and S populations (categories B and E in Figure 3) than in the mangrove cluster of sympatric M and S occurrence (categories C and F in Figure 3), our *a priori* hypothesis of reinforcement in sympatry (see MATERIALS AND METHODS) seems unlikely. Evidence for interform hybridization in sympatry was more equivocal since both inter- and intraform differentiation within the mangrove strand cluster were lower than elsewhere. Such generally greater genetic homogeneity in the mangrove

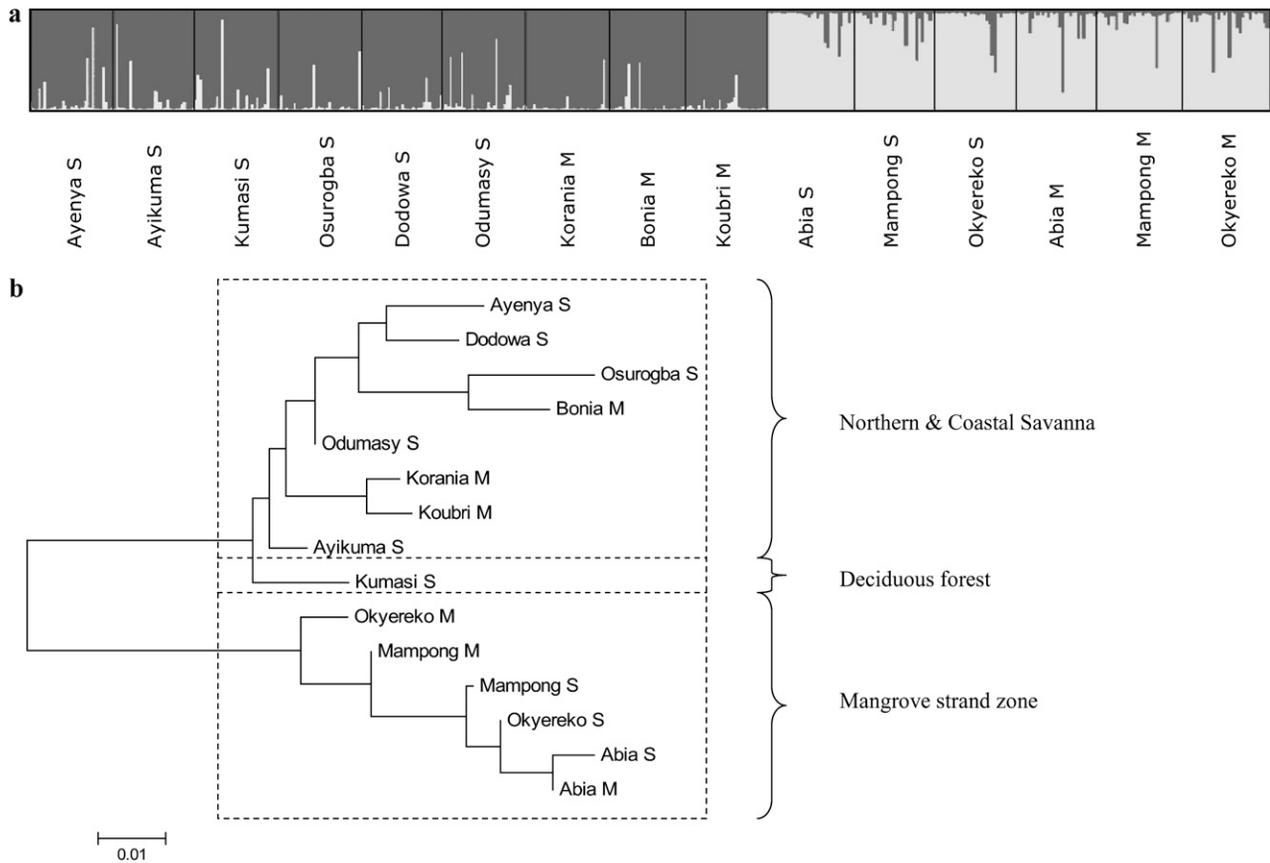


FIGURE 2.—(a) Results from an individual-level Bayesian assignment/cluster analysis in which the optimal number of clusters was two. Vertical bars indicate the proportionate assignment of an individual to each cluster. (b) Neighbor-joining tree based on linearized F_{ST} . Partitions among clusters of sample sites detected by a group-level Bayesian analysis (BAPS 4.13) are delineated by dashed boxes, with ecological zones from which samples originated indicated by parentheses. In both a and b, the molecular form of the samples (M or S) is given after the location name.

zone might be attributable to lower average pairwise separation (mangrove mean = 61 km; non-mangrove mean = 336 km). Yet isolation by distance (analyzed via linearized F_{ST} vs. ln geographical distance) was not significant, either over all samples (Mantel's test $P = 0.14$) or within samples from outside of the mangrove zone ($P = 0.092$); too few sites were available to permit separate analysis within the mangrove zone. Moreover, one of the sampling sites from the mangrove strand zone was actually closer geographically to those within the coastal savanna than to the other two sites within its own ecological zone (see Figure 1), further suggesting that distance is not an important determinant of differentiation.

The concordant sample groupings observed in both the STRUCTURE analysis (Figure 2a) and the F_{ST} neighbor-joining tree (Figure 2b) were not unduly influenced by a single locus. The number of loci with F_{ST} values significantly different from zero was markedly different in comparisons from within the major clusters (mean = 3.1 loci) compared to comparisons between clusters (mean = 6.2 loci) (APPENDIX B). Similarly, null alleles exerted little influence on our results. A data set

in which null alleles were recoded according to their estimated frequencies showed a lower global F_{ST} (0.065, compared to 0.076 for the original data set), but the neighbor-joining tree and group-level clustering analysis results were almost indistinguishable between data sets (supplemental materials at <http://www.genetics.org/supplemental/>).

Genetic diversity and admixture: The seven microsatellites analyzed were highly polymorphic in all populations (APPENDIX C). Variation in genetic diversity among populations was quite moderate, whether measured as allelic richness (R_S , range 7.69–10.01) or expected heterozygosity (H_E , range 0.71–0.82). Both R_S and H_E differed significantly between the two major clusters identified above, with each higher in the mangrove strand zone than in the cluster of other populations (FSTAT 2.9.3.2, 1000 permutations, both $P < 0.05$).

Since we have no evidence that the populations in this study are close to migration-drift equilibrium, we applied a nonequilibrium admixture analysis. Admixture analysis allows us to determine whether individuals have recent ancestors originating from more than one

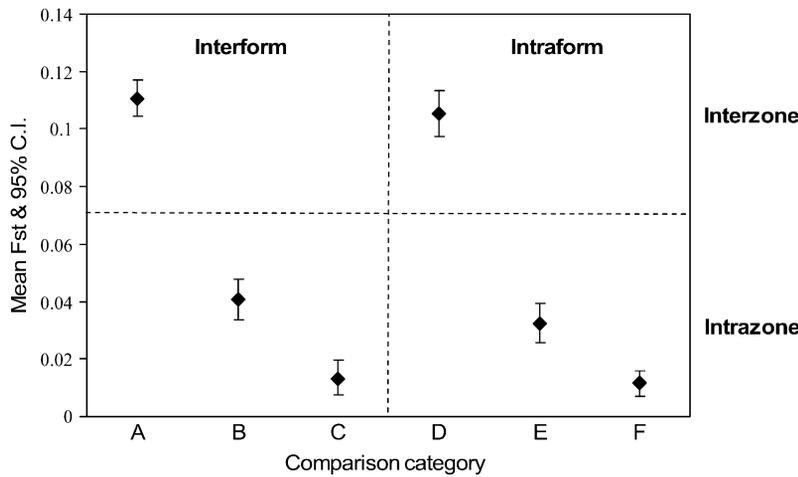


FIGURE 3.—Bootstrapped mean F_{ST} values 95% confidence intervals for comparisons made within and among molecular forms (M and S) and ecological zones. Comparison categories are: A, mangrove zone *vs.* non-mangrove zones between form comparisons; B, non-mangrove zones all between form comparisons; C, mangrove zone all between form comparisons; D, mangrove zone *vs.* non-mangrove zones within form comparisons; E, non-mangrove zones all within form comparisons; F, mangrove zone all within form comparisons.

sample population. We used this analysis to study patterns of very recent migration, using the three clusters identified by the BAPS group-level method (namely, Kumasi, mangrove strand zone, and savanna clusters) as potential source populations (see MATERIALS AND METHODS). Of a total of 411 individuals, 33 were identified as having multilocus genotypes exhibiting significant evidence of population mixing (APPENDIX D), representing a total multilocus admixture level of 4.3%. Individual admixture proportions rarely approached 1.0; that is, no individual could be unequivocally assigned to a population other than the one in which it was sampled (APPENDIX D), suggesting that our samples contained few first-generation (*i.e.*, F_0) immigrants. Four of the eight populations composing the savanna cluster contained no individuals showing significant evidence of mixed cluster ancestry, whereas all from the mangrove cluster showed some degree of admixture (Table 2). Indeed, mean admixture proportions were significantly higher in the mangrove than in the savanna cluster (Mann–Whitney U -test, $U_{6,8} = 3.5$, $P < 0.01$). Interestingly, Kumasi, a single-population cluster from deciduous forest, contained no admixed individuals but was identified as the source for more admixture than either of the other clusters (Table 2). Moreover, since the Kumasi sample was composed entirely of the S molecular form, apparent introgression into M-form populations could be identified. Admixture proportions from Kumasi (S) did not differ between M or S recipient populations ($U_{6,8} = 30.0$, $P = 0.49$).

DISCUSSION

In this study, we used microsatellites to screen *An. gambiae* from Ghana and southern Burkina Faso, with samples from two savanna zones and a deciduous forest zone, in which the M and S molecular forms are largely allopatric, and a coastal mangrove strand zone,

in which M and S are sympatric. Significant genetic differentiation between sample sites was near ubiquitous, but the major genetic division, supported by both individual-level Bayesian cluster analysis and F_{ST} -based analysis, was found between samples from the mangrove strand zone *vs.* the other ecological zones. A group-level Bayesian analysis supported an additional

TABLE 2

Results from Bayesian admixture analysis assessing very recent migration among population clusters based on individuals assessed to show significant evidence of admixture

Into (site)	Form	From savanna cluster: M and S	From Kumasi cluster: S	From mangrove cluster: M and S
Savanna cluster				
Ayenia S	S	<u>0.97</u>	0.051	0.045
Ayikuma S	S	<u>0.94</u>	0.057	0.018
Osuregba S	S	<u>1.00</u>	0	0
Dodowa S	S	<u>1.00</u>	0	0
Odumasy S	S	<u>0.97</u>	0.045	0.024
Korania M	M	<u>0.98</u>	<i>0.042</i>	0.001
Bonia M	M	<u>1.00</u>	0	0
Koubri M	M	<u>1.00</u>	0	0
Kumasi (deciduous forest) cluster				
Kumasi S	S	0	<u>1.00</u>	0
Mangrove cluster				
Abia S	S	0.034	0.018	<u>0.93</u>
Mampong S	S	0.029	0.014	<u>0.94</u>
Okyereko S	S	0.025	0.029	<u>0.95</u>
Abia M	M	0.021	<i>0.039</i>	<u>0.93</u>
Mampong M	M	0.017	<i>0.012</i>	<u>0.97</u>
Okyereko M	M	0.039	<i>0.019</i>	<u>0.93</u>

Average proportions of resident (*i.e.*, not admixed) multilocus DNA are underlined. Italic values show admixture proportions between molecular forms.

partition between the single sample from the deciduous forest zone and the savanna zones, although this was far less distinct from the savanna cluster than from the mangrove zone. Differentiation attributable to molecular form was limited and not significant. These patterns were found consistently across loci and were not the result of the major effect of a single locus, nor could they be attributed to null alleles, although these appeared to be common in the data set.

Determinants of genetic structure: Such high differentiation ($F_{ST} > 0.1$ between the mangrove zone and other areas) is unusual in *An. gambiae*. To date, estimates of genetic differentiation have been low even between populations separated by many thousands of kilometers (LEHMANN *et al.* 2003; DONNELLY *et al.* 2004), unless there are major hydrographic or geographic barriers to migration (*e.g.*, PINTO *et al.* 2002; LEHMANN *et al.* 2003). Yet in the south of Ghana (all samples from south of Kumasi) there are no obvious extant or former geographic barriers to gene flow, with all samples from this region taken from a continuous coastal plain that does not rise >100 m above sea level. Moreover, the effect of geographical distance on the patterns of differentiation in our study appears to be negligible.

High levels of differentiation are sometimes detected among the chromosomal forms of *An. gambiae*. For example, using AFLP markers, SLOTMAN *et al.* (2006) obtained estimates of Φ_{ST} (an F_{ST} analog) between ~ 0.05 and 0.15 among Bamako, Savanna, and Mopti forms. Although estimates of differentiation from microsatellites and AFLPs may not be directly comparable because of the downward bias to the former caused by size homoplasy, the estimates of SLOTMAN *et al.* (2006) seem compatible with those found among ecological zones in our study. Moreover, different chromosomal forms are often associated with different habitats and are thought to play an important role in environmental adaptation (DELLA TORRE *et al.* 2001). However, studies of the presence and distribution of chromosomal forms in the area that we sampled suggest that it is unlikely that high divergence results from the presence of different chromosomal forms in different ecological zones. APPAWU *et al.* (1994) reported that although the FOREST chromosomal form is dominant in the deciduous forest zone, the SAVANNA and MOPTI chromosomal forms were sympatric in both of the savanna zones. Furthermore, MBOLE *et al.* (2004) found all three of these chromosomal forms within the mangrove strand zone. Since we do not have karyotypic information for the samples screened in this study, and the relationship between molecular and chromosomal forms is often ambiguous (DELLA TORRE *et al.* 2001), we cannot rule out the possibility that the different habitat types are dominated by different and genetically divergent chromosomal forms. Nevertheless, unless there are major chance, spatial, or temporal disconti-

nities between the chromosomal forms represented in our samples and those obtained by APPAWU *et al.* (1994) and MBOLE *et al.* (2004), atypical chromosomal forms within a cluster should have been readily detectable via our clustering/assignment analyses and would resemble first-generation immigrants. By contrast, very few individuals in sample sites within each of the two clusters (STRUCTURE) or three clusters (BAPS) identified could be interpreted as immigrants from a different cluster (see Figure 2a).

Therefore, the genetic structure that we have identified appears to be unrelated to physical barriers to gene flow, geographic distance, or molecular form and is unlikely to be attributable to habitat specificity of chromosomal forms. A further possibility is that the very high differentiation is temporally unstable and results from a relatively recent colonization (or recolonization) of, and spread within, the mangrove strand zone from a highly divergent source. While considerable restriction of gene flow from the coastal savanna zone would presumably be required for this scenario to persist for more than a few generations, the significantly lower interpopulation differentiation in the mangrove zone is consistent with this colonization–expansion hypothesis. Yet, the significantly higher genetic diversity and admixture within the mangrove zone are the opposite of what would be expected under a colonization–expansion scenario unless multiple differentiated source populations colonized the area, and this is at odds with the relatively low genetic structure therein. Consequently, we think that recent colonization can also be ruled out. Rather, the most likely explanation would seem that habitat-type-specific selection against interzone immigrants represents a barrier to gene flow.

Gene flow between the molecular forms: Studies of gene flow between the molecular forms of *An. gambiae* have found differentiation between the two forms at four genomic regions (but see WONDJI *et al.* 2002; TURNER *et al.* 2005). One of these is on chromosome 2L, associated with insecticide resistance (GENTILE *et al.* 2004 and references therein). The other loci (including the rDNA) are at the proximal (centromeric) end of the X chromosome (WANG *et al.* 2001; LEHMANN *et al.* 2003). However, little differentiation has been found at loci elsewhere in the genome (GENTILE *et al.* 2001; MUKABAYIRE *et al.* 2001; WANG *et al.* 2001; TURNER *et al.* 2005). Debate is ongoing as to whether such generally limited differentiation primarily reflects insufficient time for differentiation to have occurred or ongoing introgression among forms. Neither the F_{ST} -based analysis nor the Bayesian clustering methods provided conclusive evidence to support or reject our main hypothesis that interform hybridization/introgression would be more common when forms are sympatric than (primarily) allopatric. The alternative hypothesis—that reinforcement might occur between the forms in

sympatry—seems very unlikely, given that the difference between the mean level of intra- and interform comparisons was lower for sympatric than for allopatric populations.

Nevertheless, results from our admixture analysis suggest that genetic exchange between forms might be more frequent than previous molecular genetic and assortative mating studies would imply (TRIPET *et al.* 2001; WONDJI *et al.* 2002). The Kumasi population, which is entirely composed of the S form (YAWSON *et al.* 2004), was sufficiently differentiated by the group-level cluster analysis to be identified as a source of admixture. Recipient M and S populations received low but very similar proportionate admixture from Kumasi, suggesting that intra- and interform gene flow is low but equally common from the Kumasi population. In high-differentiation systems, pairwise admixture proportions in the BAPS analysis that we applied appear to be insensitive to unsampled “ghost” populations (see HANFLING and WEETMAN 2006). By contrast, since the M and S forms are virtually indistinguishable, it is possible that admixture proportions allocated to M recipient populations from our Kumasi S sample might have originated from an unsampled ghost M-form population closely resembling Kumasi. While it is important to recognize this kind of limitation when applying any admixture or assignment analysis, there is no evidence for any M forms occurring within the deciduous forest zone.

A further line of evidence that higher interform exchange occurs in sympatry comes from typing of the pyrethroid insecticide knockdown resistance (*kdr*) genotype. This segregated according to form: very low frequencies in the M form and near fixation in the S form; but the highest frequencies of the *kdr* genotype were all found in M forms from the sympatric M/S sample sites (YAWSON *et al.* 2004). The absence of any M/S hybrids in the 935 specimens from which the data presented here are drawn (YAWSON *et al.* 2004) suggests that at least during our collection period direct introgression between M and S forms is rare (TOURÉ *et al.* 1998; TAYLOR *et al.* 2001; TRIPET *et al.* 2001, 2003). Of course, our data represent a short sampling period and there could be a seasonal component to introgression that we have not detected. Collections were made in the rainy season when mosquito numbers were high and it is possible that patterns of hybridization differ during the dry season when mosquito numbers are low and either one of the two forms may predominate.

Conclusion: Although there is evidence to suggest that the M and S forms of *An. gambiae* may be separate entities that have been evolving under divergent selection pressures (STUMP *et al.* 2005), data from our study area suggest that interform gene flow is occurring at appreciable levels, perhaps most frequently in the southern mangrove strand zone of M/S sympatry. Our most striking and unexpected finding was of extremely

strong genetic structure that correlated with ecological zones, with little, if any, contribution of distance, molecular form, and probably also chromosomal form. Results such as these suggest that climatic modeling and Geographic Information Systems-based approaches will be fruitful in the prediction of gene flow in *An. gambiae*, the understanding of which is critical for malaria control in Africa.

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APPENDIX A
Tests for deviation from HWE

Location	Molecular form	Abbreviation	Locus											Mean
			Ag3H119	Ag2H197	33CI	Ag3H812	Ag3H577	AgXH99	AgXH7					
Ayanya	S	Ae	<u>0.313</u>	0.053	<u>0.289</u>	0.167	<u>0.405</u>	0.144	<u>0.193</u>	<u>0.224</u>				
Ayikuma	S	Ai	0.096	0.069	<u>0.563</u>	<u>0.348</u>	<u>0.312</u>	<u>0.328</u>	<u>0.271</u>	<u>0.297</u>				
Kumasi	S	Km	0.104	0.126	<u>0.335</u>	<u>0.235</u>	<u>0.528</u>	<u>0.349</u>	<u>0.46</u>	<u>0.349</u>				
Osurugba	S	Os	0.035	0.112	<u>0.321</u>	0.095	0.116	<u>0.116</u>	0.07	<u>0.116</u>				
Dodowa	S	Do	0.129	0.086	<u>0.244</u>	<u>0.538</u>	0.055	<u>0.202</u>	<u>0.513</u>	<u>0.202</u>				
Odumasy	S	Od	<u>0.429</u>	<u>0.178</u>	<u>0.212</u>	<u>0.189</u>	<u>0.407</u>	<u>0.236</u>	<u>0.176</u>	<u>0.264</u>				
Korania	M	Kr	<u>0.155</u>	<u>0.247</u>	<u>0.339</u>	<u>0.393</u>	<u>0.195</u>	<u>0.338</u>	<u>0.224</u>	<u>0.267</u>				
Bonia	M	Bo	<u>0.188</u>	<u>0.303</u>	<u>0.2</u>	<u>0.211</u>	<u>0.185</u>	<u>0.032</u>	<u>0.047</u>	<u>0.182</u>				
Koubri	M	Ku	0.12	0.1	<u>0.355</u>	<u>0.344</u>	<u>0.328</u>	<u>0.125</u>	<u>0.189</u>	<u>0.218</u>				
Abia	S	Abx	0.065	<u>0.195</u>	0.1	0.146	<u>0.329</u>	0.109	<u>0.335</u>	<u>0.177</u>				
Mampong	S	Mnx	0.131	<u>0.412</u>	<u>0.403</u>	<u>0.27</u>	<u>0.223</u>	<u>0.259</u>	<u>0.259</u>	<u>0.24</u>				
Okyereko	S	Okx	<u>0.146</u>	<u>0.152</u>	<u>0.224</u>	-0.078	<u>0.302</u>	<u>0.306</u>	<u>0.256</u>	<u>0.185</u>				
Abia	M	Abz	<u>0.414</u>	<u>0.252</u>	0.01	<u>0.24</u>	<u>0.269</u>	0.07	<u>0.435</u>	<u>0.244</u>				
Mampong	M	Mnz	<u>0.247</u>	<u>0.56</u>	<u>0.182</u>	<u>0.24</u>	<u>0.101</u>	<u>0.166</u>	<u>0.566</u>	<u>0.298</u>				
Okyereko	M	Okz	<u>0.277</u>	<u>0.295</u>	<u>0.182</u>	<u>0.243</u>	<u>0.015</u>	<u>0.152</u>	<u>0.683</u>	<u>0.264</u>				
		N locations ($P < 0.05$):	8	9	13	13	11	9	6					

F_{IS} values in italics are significantly different from zero ($P < 0.05$) and, where underlined, remain significant following sequential Bonferroni correction.

APPENDIX B
Population differentiation

Location ^a	Ae	Ai	Km	Os	Do	Od	Kr	Bo	Ku	Abx	Mnx	Okx	Abz	Mnz	Okz
Ae															
Ai	0.031														
Km	0.047	0.023													
Os	0.047	0.048	0.065												
Do	0.025	0.041	0.041	0.042											
Od	0.030	0.011	0.013	0.034	0.011										
Kr	0.055	0.032	0.031	0.052	0.046	0.013									
Bo	0.052	0.048	0.060	0.030	0.043	0.025	0.028								
Ku	0.060	0.014	0.039	0.055	0.059	0.022	0.011	0.036							
Abx	0.122	0.115	0.124	0.150	0.118	0.112	0.134	0.158	0.121						
Mnx	0.106	0.097	0.102	0.138	0.106	0.095	0.110	0.135	0.100	0.007					
Okx	0.105	0.095	0.103	0.149	0.109	0.094	0.114	0.144	0.103	0.008	0.008				
Abz	0.101	0.107	0.108	0.146	0.103	0.097	0.123	0.148	0.119	-0.001	0.012	0.003			
Mnz	0.084	0.082	0.090	0.118	0.088	0.076	0.101	0.125	0.092	0.021	0.016	0.012	0.013		
Okz	0.085	0.080	0.085	0.123	0.091	0.079	0.103	0.124	0.091	0.030	0.034	0.019	0.013	0.021	

Pairwise F_{ST} (below diagonal) and the number of loci $P < 0.05$ in genic tests of population differentiation. F_{ST} values in italics correspond to genic tests combined across loci that are *not* significant following sequential Bonferroni correction.

^a See APPENDIX A for definitions of location abbreviations.

APPENDIX C
Genetic diversity

Location ^a	Ag2H11		Ag2H19		33C1		Ag3H81		Ag3H57		AgXH99		AgXH7		Mean	
	<i>H_E</i>	<i>R_S</i>														
Ae	0.85	9.95	0.85	11.19	0.82	9.60	0.62	8.45	0.76	5.00	0.72	4.87	0.87	11.94	0.78	8.71
Ai	0.83	7.94	0.89	11.50	0.69	9.41	0.72	6.61	0.73	7.29	0.71	5.90	0.87	9.69	0.77	8.33
Km	0.85	10.16	0.84	9.74	0.73	7.97	0.72	5.72	0.82	6.93	0.63	4.53	0.84	9.41	0.77	7.78
Os	0.86	10.51	0.86	11.32	0.83	10.18	0.68	8.84	0.73	5.68	0.23	2.94	0.75	6.65	0.71	8.01
Do	0.81	9.51	0.91	13.94	0.79	9.41	0.51	7.38	0.78	5.77	0.66	4.72	0.82	7.89	0.75	8.37
Od	0.85	9.73	0.90	13.76	0.74	11.43	0.72	8.71	0.82	7.65	0.72	8.04	0.78	9.23	0.79	9.79
Kr	0.84	9.40	0.85	8.76	0.68	11.57	0.82	7.75	0.72	4.00	0.63	3.94	0.77	8.38	0.76	7.69
Bo	0.79	9.56	0.87	8.76	0.85	14.82	0.72	8.60	0.75	4.82	0.55	5.64	0.49	5.61	0.72	8.26
Ku	0.88	11.18	0.86	8.48	0.65	9.82	0.79	10.03	0.77	7.41	0.66	4.93	0.84	10.28	0.78	8.87
Abx	0.89	11.46	0.86	9.74	0.70	7.07	0.84	8.84	0.70	9.91	0.70	4.91	0.66	4.90	0.76	8.12
Mnx	0.90	10.00	0.85	12.62	0.78	9.14	0.82	9.81	0.69	10.21	0.79	7.30	0.65	5.75	0.78	9.26
Okx	0.89	11.33	0.87	11.40	0.73	9.50	0.77	9.78	0.76	11.16	0.77	8.42	0.81	8.48	0.80	10.01
Abz	0.91	13.09	0.88	12.04	0.77	8.82	0.75	8.81	0.68	10.13	0.75	6.48	0.71	6.69	0.78	9.44
Mnz	0.84	12.23	0.88	9.42	0.76	9.92	0.70	4.99	0.83	12.21	0.79	7.60	0.79	7.36	0.80	9.11
Okz	0.86	10.78	0.89	10.47	0.80	9.77	0.78	10.88	0.79	10.53	0.81	7.15	0.79	7.36	0.82	9.56
Mean	0.86	10.46	0.87	10.87	0.75	9.89	0.73	8.35	0.75	7.91	0.67	5.82	0.76	7.97		

Gene diversity (*H_E*) and allelic richness (*R_S*) are shown for each population and locus combination.
^aSee APPENDIX A for definitions of location abbreviations.

APPENDIX D

Bayesian admixture analysis (using BAPS 4.13) assessing very recent migration among population clusters

Into (site)	Form	From savanna cluster: M and S	From Kumasi cluster: S	From mangrove cluster: M and S
Savanna cluster				
Ayenyaa	S	0.36	0.62	<i>0.02</i>
Ayenyaa	S	0.46	<i>0.01</i>	0.53
Ayenyaa*	S	<i>0.02</i>	0.44	0.54
Ayenyaa*	S	0.18	0.51	0.31
Ayikuma	S	0.46	<i>0.02</i>	0.52
Ayikuma	S	0.47	0.52	<i>0.01</i>
Ayikuma	S	0.45	0.55	<i>0</i>
Ayikuma	S	0.39	0.61	<i>0</i>
Odumasy	S	0.39	0.17	0.44
Odumasy	S	0.35	0.35	0.30
Odumasy*	S	0.11	0.89	<i>0</i>
Korania	M	0.39	<u>0.59</u>	<i>0.02</i>
Korania*	M	0.30	<u>0.70</u>	<i>0</i>
Mangrove cluster				
Abia	S	<i>0.05</i>	0.36	0.59
Abia	S	0.43	<i>0.01</i>	0.56
Abia	S	0.43	<i>0.01</i>	0.56
Abia	S	0.18	0.18	0.64
Mampong	S	<i>0</i>	0.40	0.60
Mampong	S	0.35	<i>0.03</i>	0.62
Mampong	S	0.51	<i>0</i>	0.49
Okyereko	S	0.36	<i>0.05</i>	0.59
Okyereko*	S	0.38	0.23	0.39

APPENDIX D

(Continued)

Into (site)	Form	From savanna cluster: M and S	From Kumasi cluster: S	From mangrove cluster: M and S
Okyereko*	S	<i>0</i>	0.60	0.40
Abia	M	0.12	<u>0.21</u>	0.67
Abia*	M	0.28	<u>0.41</u>	0.31
Abia	M	<i>0</i>	<u>0.47</u>	0.53
Abia	M	0.24	<u>0.09</u>	0.67
Mampong	M	<i>0</i>	<u>0.34</u>	0.66
Mampong*	M	0.55	<i>0.05</i>	0.40
Okyereko*	M	0.46	<u>0.15</u>	0.39
Okyereko	M	0.42	<u>0.08</u>	0.50
Okyereko	M	<i>0.02</i>	<u>0.38</u>	0.60
Okyereko	M	0.34	<i>0</i>	0.66

Each line shows an individual multilocus genotype that is significantly (*P* < 0.05) admixed, with the proportion assigned to each cluster: resident (*i.e.*, not admixed) proportions are in boldface. Underlined values show admixture proportions between molecular forms. To aid interpretation, very low proportions (<0.1) are in italics. *Significant after Bonferroni correction.

(continued)