HIGH AMOUNTS OF GENETIC DIFFERENTIATION BETWEEN POPULATIONS OF THE MALARIA VECTOR ANOPHELES ARABIENSIS FROM WEST AFRICA AND EASTERN OUTER ISLANDS

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Abstract. Polymorphism at nine microsatellite loci was examined to assess the level of genetic differentiation between four Anopheles arabiensis populations from Senegal, the high plateau of Madagascar, and Reunion and Mauritius islands. Eight of nine loci showed great polymorphism (2–16 alleles/locus) and significant genetic differentiation was revealed between all four populations by F- and R-statistics, with Fst estimates ranging from 0.080 to 0.215 and equivalent Rst values ranging between 0.022 and 0.300. These high amounts of genetic differentiation are discussed in relation to geographic distance including large bodies of water, and history of mosquito settlement, and insecticide use on the islands. The results suggest that historical events of drift rather than mutation are probably the forces generating genetic divergence between these populations, with homogenization of the gene pool by migration being drastically restricted across the ocean.

Members of the Anopheles gambiae complex are the principal human malaria vectors occurring in sub-Saharan Africa and surrounding islands. Among the seven sibling species belonging to the complex, An. gambiae Giles and An. arabiensis Patton are the most efficient malaria vectors and widely coexist throughout the continent. The latter species, however, is more adapted to drier environments and remains active significantly longer than An. gambiae after the end of the Sahelian rainy season. While both species are highly anthropophilic, An. arabiensis shows a greater tendency to feed and rest outdoors, particularly in east Africa, and is accordingly less susceptible than An. gambiae to the effect of house spraying, the most commonly used method to control adult mosquito populations.

Alternative strategies for efficient malaria control are desperately needed and current efforts tend to focus on a long-term goal of using transgenic mosquitoes with reduced malaria competence. Provided reliable techniques for arthropods transformation and efficient, stable, and safe transgenes are available, a comprehensive knowledge of the genetic structure of the target mosquito population appears absolutely necessary to properly evaluate the outcome of field releases of transgenic vectors. The area of insecticide management will also benefit from the study of gene flow that allows prediction of the spread of resistance genes. Conflicting results have been obtained for An. gambiae populations in west and east Africa using different genetic markers. These include chromosomal inversions, allozymes, random amplified polymorphic DNA, mitochondrial DNA sequences, and microsatellite loci analysis. Some of these markers were found to correlate with environmental parameters. For example, spatial and temporal variability of chromosomal inversion frequencies suggest important constraints acting on these markers that could lead to erroneous observations in term of gene flow.

Microsatellites are relatively short tracts of tandemly repeated DNA sequences of 2–6 basepairs. These loci have been described in recent studies as being powerful markers for population genetics because of their abundance throughout eukaryote genomes, high polymorphism, codominance, and relative ease of scoring. While they are considered selectively neutral, evidence for constraints acting on some of these loci include biased mutation rate or selection on allele size and distribution. However, the forces that shape allele composition at microsatellite loci remain poorly understood and the intensity of constraints they experience may differ from one locus to another. Nevertheless, because of high mutation rates estimated to range between 10^(-2) and 10^(-3) mutations per generation due mostly to replication slippage (e.g., for a review, see Levinson and Gutman), those fast-evolving loci have been shown to be appropriate for fine-scale population genetics studies and should provide accurate descriptions of the actual levels of gene flow accessible by indirect studies (see Bossart and others and references therein).

While An. gambiae has been extensively studied, very little work has been conducted on An. arabiensis population genetics despite its importance as a malaria vector. Furthermore, this species should be a more suitable candidate than its sibling An. gambiae in testing the efficiency of transgenic mosquito control strategies in the fields to reduce malaria burden. Indeed, malaria control by antivectorial means should prove more efficient in low or unstable malaria areas where transmission could be reduced in a sufficient amount to allow subsequent epidemiologic impact. Such areas, located on the edges of sub-Saharan Africa (dry savannas on the southern border of the Sahara desert, northern South Africa, or African and surrounding island highlands), are generally colonized only by An. arabiensis because the environmental conditions are highly restrictive to the establish-
The village of Barkedji (15°17′N, 14°53′W) is located in the Sahelian region. The rainy season is short, extending from July to October, with little annual variation in the amount of rainfall (300–400 mm). An extensive study of malaria vectors ecology and population dynamics in this location showed that the vectors are An. arabiensis and An. gambiae.2 Because no mosquito breeding sites are available during the dry season, malaria transmission is highly seasonal.

Madagascar. Fenoarivo (19°47′S, 46°34′E) is a village on the high plateau of Madagascar, the largest island in the Indian Ocean (587,000 km²) located 350 km from the east coast of the African continent. At an elevation of 1,235 m, Fenoarivo is surrounded by flooded rice fields. The hot rainy season lasts from November to April with an annual rainfall of approximately 1,400 mm. A malaria eradication program based on DDT spraying and chemoprophylaxis was conducted from 1949 until elimination of the disease in the early 1960s, with some local, irregular DDT spraying being continued until 1975 (for a review, see Mouchet and others30). However, malaria was still occurring in certain particular areas in the island, and due to the lack of control measures, the situation worsened until the occurrence of an epidemic of malaria in 1986–1988. Yearly DDT spraying was then reintroduced in 1993 and is still implemented at the present time.

Malaria transmission in Madagascar is carried out by the An. gambiae complex and by An. funestus,27 but An. arabiensis and An. funestus are the only two malaria vectors present on the high plateau.28

Mascarene Islands. Reunion (2,500 km²) and Mauritius (2,000 km²), two islands 240 km apart, belong to the Mascarene archipelago. They are located in the Indian Ocean (20°–25°S, 55°E) 800 km and 1000 km, respectively, from the east coast of Madagascar. Mosquitoes were collected in a single location on the northwestern part of Reunion (Etang de Saint Paul), while mosquitoes from Mauritius were collected all over the island (17 locations) and pooled. The annual amount of rainfall ranges from 5,000 mm in upwind locations to 500 mm in downwind ones. Cyclones often occur during the December to April rainy season. Following strong malaria vector control measures and intensive quinine prophylaxis campaigns in the early 1950s, malaria was declared eradicated from Mauritius in 1973 and from Reunion in 1979 by the World Health Organization.29 Presently, these islands are in an anopheline without malaria state with An. arabiensis as the only potential malaria vector.30,31 To prevent re-emergence of the disease, the malaria situation relies on careful epidemiologic and entomologic surveillance. Anopheles merus Dönitz, another member of the An. gambiae com-

<table>
<thead>
<tr>
<th>Locus</th>
<th>Cytologic location</th>
<th>Repeat</th>
<th>Primers</th>
<th>Number of alleles in this study</th>
<th>Allele size range (basepairs)</th>
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</thead>
<tbody>
<tr>
<td>24D†</td>
<td>2 La:24D (marker 11/Sur1)</td>
<td>CT</td>
<td>5′GCCGACGGCTTTATCAGT 3′</td>
<td>3</td>
<td>103–121</td>
</tr>
<tr>
<td>141*</td>
<td>2 Rd</td>
<td>GT</td>
<td>5′CGGACCAACTGAGGCTG 3′</td>
<td>7</td>
<td>102–114</td>
</tr>
<tr>
<td>26*</td>
<td>2 Rb:12</td>
<td>GT</td>
<td>5′GGTTCTTAGTTCTTCGCTG 3′</td>
<td>16</td>
<td>82–112</td>
</tr>
<tr>
<td>7§</td>
<td>X:1C</td>
<td>GT</td>
<td>5′CAGCAGGTTTTGCGGTTG 3′</td>
<td>11</td>
<td>82–102</td>
</tr>
<tr>
<td>49§</td>
<td>X:1D</td>
<td>GT</td>
<td>5′CAGCAGCTCCATATAAGGACG 3′</td>
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<td>87</td>
</tr>
<tr>
<td>803*</td>
<td>2 R:7B</td>
<td>TG</td>
<td>5′CTCGATATAACGGCGCTTGG 3′</td>
<td>7</td>
<td>120–132</td>
</tr>
<tr>
<td>45C</td>
<td>3 L:45C (ATPase unit2-like gene)</td>
<td>GA</td>
<td>5′AAAAGTGGTGACGGATGAC 3′</td>
<td>7</td>
<td>146–158</td>
</tr>
<tr>
<td>29C</td>
<td>3 R:29C</td>
<td>TGA</td>
<td>5′ATGGTACAGAGCGGACCAT 3′</td>
<td>2</td>
<td>145–148</td>
</tr>
<tr>
<td>147*</td>
<td>2 R:19</td>
<td>GT</td>
<td>5′CTGCCTTGCCTGCAAAGT 3′</td>
<td>8</td>
<td>167–181</td>
</tr>
</tbody>
</table>

* Data from Zheng and others.34
† Data from Zheng and others.35
‡ Data from Besansky and others.36

** Table 1 **
Anopheles arabiensis microsatellite loci: cytologic location, repeat motif, and primer sequences

** MATERIALS AND METHODS **

** Study sites. ** Senegal. The village of Barkedji (15°17′N, 14°53′W) is located in the Sahelian region. The rainy season is short, extending from July to October, with little annual variation in the amount of rainfall (300–400 mm). An extensive study of malaria vectors ecology and population dynamics in this location showed that the vectors are An. arabiensis and An. gambiae.2 Because no mosquito breeding sites are available during the dry season, malaria transmission is highly seasonal.

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plex, is also present in Mauritius, where salt-water breeding sites are available.

**Mosquito collection.** Female specimens of the *An. gambiae* s. l. complex from the four study sites were collected either by landing on human volunteers or after indoor pyrethrum spraying early in the morning. The study protocol was carefully explained to the assembled village population. Informed consent was obtained individually from all participants or their parents. The study was approved by the Conseil de Perfectionnement of the Pasteur Institute of Dakar and the Senegalese Ministry of Health. Mosquito collections were carried out in August 1994 in Senegal, in February 1995 in Reunion, in November–December 1996 in Madagascar, and in March–April 1997 in Mauritius. All mosquitoes were stored individually in 1.5-ml tubes containing desiccant and kept at −20°C until further analyzed.

**Extraction of DNA and microsatellite polymerase chain reaction (PCR) processing.** The DNA was extracted from a leg or wing of each individual mosquito as described by Collins and others and resuspended in 100 μl of sterile distilled water. Only *An. arabiensis* were included in the analysis after species identification using the PCR technique described by Scott and others. A set of approximately 50 specimens per location was investigated, excepted for the Mauritius population, where only 44 specimens were available.

Microsatellites loci were selected from published *An. gambiae* sequence data and nine were chosen for further analysis based on high polymorphism exhibited in *An. gambiae*, with allele sizes ranging from 100 to 200 basepairs (to optimize detection by electrophoresis using 10% polyacrylamide gels), and successful preliminary amplification of *An. arabiensis* DNA (Table 1).

A standard PCR was run in a Perkin-Elmer (Norwalk, CT) 9600 thermal cycler in a 25-μl reaction volume containing 2.5 μl of 10× PCR buffer (Pharmacia Biotech, Uppsala, Sweden) with (final concentrations) 1.5 mM MgCl₂, 0.2 mM of each dNTP, 100 ng of each primer (approximately 15 pmol), one unit of Taq polymerase (Pharmacia Biotech), and 5 μl of template DNA. The PCR conditions were denaturation at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 25 sec, 55°C for 28 sec, and 72°C for 30 sec. The last elongation step was at 72°C for 10 min. The PCR products were then mixed with 6× loading buffer containing 30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol. A 10-μl aliquot of this mixture was loaded onto a
10% non-denaturing polyacrylamide gel. The allelic bands were visualized after electrophoresis and rapid silver staining.\(^7\) A few samples that provided the whole spectrum of alleles present at a particular locus were amplified and mixed to form an allelic ladder for this locus. All samples were scored against such allelic ladders and sequencing of one of those alleles allowed exact determination of the molecular length of each allele at each locus.

**Data analysis.** Genetic variability parameters (allelic frequencies, observed and expected heterozygosity under Hardy-Weinberg equilibrium, and mean number of alleles per locus) were assessed for each locus in each population using BIOSYS-1 software.\(^8\) Genotype frequencies were tested against Hardy-Weinberg expectations for each locus in the pooled population and for each location. Exact goodness-of-fit tests were performed using GENEPOP 3.1.\(^9\) Possible nonrandom allelic associations between pairs of polymorphic loci were assessed within each population using Fisher’s exact test on contingency tables. The same procedure was then performed across all populations (Fisher’s method for a global test).\(^9\) The significance level for each test was adjusted to take into account the other tests using the sequential Bonferroni procedure.\(^40,41\)

Differentiation between populations was examined by F-statistics (Wright,\(^42\) calculated according to Weir and Cockerham\(^43\)) and their microsatellite’s equivalent, R-statistics.\(^44\) The latter was assessed using a program written in the SAS language\(^45\) by one of the authors (TL).\(^12\) Significance of Fis and Fst was assessed using the exact probability test and the exact test of a contingency table testing homogeneity of genotypic frequencies among populations, respectively (available in GENEPOP\(^46\)). Significance of Ris and Rst was evaluated by an F-test in a nested analysis of variance (ANOVA) on the allele length in a model including the individual and the population as factors.\(^44\) The average Ris across loci and/or populations was estimated by calculating the simple means of individual Ris estimates, which was possible due to the sufficient sample sizes (between 44 and 52 individuals per population) and the relative homogeneity of the results. Average Rst was calculated following the procedure of Slatkin\(^44\) from the averages of the within-population and total variance components across loci.

The number of effective migrants (Nm) was estimated by three different methods: the Slatkin private alleles method\(^47\) (namely, alleles found in only one population as referred to by Neel\(^48\)), and by F- and R-statistics of each locus according
RESULTS

Genetic variability and Hardy-Weinberg equilibrium. Microsatellite loci were highly polymorphic with a number of distinct alleles per locus ranging from two for locus 29C up to 16 for locus 26 (Table 1, mean number of alleles per locus on pooled populations = 7.6). Locus 49 was found to be fixed in most An. arabiensis populations with a single allele of 87 basepairs. In the Senegal population, two other alleles were found only once (frequency < 0.01 for each one). This locus was therefore considered polymorphic and was not taken into account in further analyses. Loci 7 and 29C were also found to be monomorphic in the Reunion population.

The most common allele was the same for all four populations for five of eight loci. Locus 26 showed an interesting pattern of allele distribution with very restricted spectrums for Mauritius and Reunion in comparison with Senegal and Madagascar, with the former populations possessing only the longer alleles. Furthermore, the most common allele shared by the Senegalese and Madagascan populations was different than that of the Mascarene island populations (Figure 1).

Hardy-Weinberg predictions were significantly rejected \( (P < 0.05) \) for each locus when considering the pooled samples as belonging to a single population with significant deficit of heterozygotes (i.e., positive values of \( F_{IS} \) that are equal to \( F_{IT} \)) in all cases (Table 3), indicating a strong Wahlund effect (pooling of separate gene pools). At the population level, slight deviations from Hardy-Weinberg expectations occurred with unpooled data. They remained significant only in two cases after the sequential Bonferroni procedure was applied: for locus 24D in the Senegal population and for locus 147 in the Madagascan population. The occurrence of null alleles due to mutation within the DNA sequence complementary to the oligoprimers, which prevented their binding and inhibited DNA amplification by \( Taq \) polymerase, could explain such a pattern of excess homozygosity. It is an unlikely explanation in this particular case because no homozygote for this null allele has been recorded. Nevertheless, comparable values of heterozygote deficiency at locus 147 have also been observed when studying Kenyan An. gambiae populations (Kamau L, Lehmann T, unpublished data). These two instances suggesting heterozygote deficiency were not strengthened by the rest of the dataset and do not suggest obvious violation of Hardy-Weinberg equilibrium. Moreover, all loci conformed when differences were assessed with a chi-square test on pooled alleles. No subdivision of the gene pool was then seen at the population level.

Linkage disequilibrium. Exact tests for linkage disequilibrium within each population resulted in five significant values in 112 comparisons (5.6 significant values are expected at the 5% level) with \( P \) values between 0.0046 and 0.0388. No pair of loci appeared in linkage disequilibrium in more than one population, suggesting the absence of physical linkage between loci. On the other hand, when the Fisher tests were done on the pooled populations, all but four loci pairs (of 28 possible combinations) showed highly significant \( P \) values, ranging from \( <10^{-6} \) to 0.045.

Alleles of different loci are in linkage disequilibrium if they occur together in the same individual with a probability higher or lower than would be expected by chance alone. It is an indicator of nonrandom mating or of selection for certain genotypes, thus being reliable for separation of the gene pool in a single population. The Wahlund effect detected in the above section when the different populations are fused has generated the pattern observed here. These results corroborate our findings of homogeneity of the gene pool within each of the four populations that can be considered as distinct gene pools.

Genetic differentiation. Pairwise \( F_{ST} \) and \( R_{ST} \) and global values over all loci and over all populations were assessed using GENEPOP version 3.1 and SAS programs, respectively. Estimations are shown in Table 4, together with their corresponding \( N_m \) values.

### Table 1: Genetic variability at eight microsatellite loci for each population*

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean sample size per locus</th>
<th>Mean no. of alleles per locus</th>
<th>Percentage of loci polymorphic</th>
<th>Mean heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senegal</td>
<td>53.8 (0.2)</td>
<td>7.3 (1.3)</td>
<td>100.0</td>
<td>0.558 (0.068)</td>
</tr>
<tr>
<td>Madagascar</td>
<td>52.9 (0.1)</td>
<td>5.9 (0.1)</td>
<td>100.0</td>
<td>0.549 (0.084)</td>
</tr>
<tr>
<td>Reunion</td>
<td>51.9 (0.1)</td>
<td>2.6 (0.5)</td>
<td>75.0</td>
<td>0.383 (0.087)</td>
</tr>
<tr>
<td>Mauritius</td>
<td>43.8 (0.2)</td>
<td>3.4 (0.5)</td>
<td>100.0</td>
<td>0.406 (0.074)</td>
</tr>
</tbody>
</table>

* Values in parentheses are standard errors.
† A locus is considered polymorphic if more than one allele was detected.
‡ Unbiased estimates.
### Table 3

Hardy-Weinberg expectations*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Pooled population?</th>
<th>Senegal</th>
<th>Madagascar</th>
<th>Reunion</th>
<th>Mauritius</th>
<th>All populations?</th>
</tr>
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<tbody>
<tr>
<td>24D</td>
<td></td>
<td>0.0056</td>
<td>1.00</td>
<td>0.0377</td>
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<td>&lt;0.005</td>
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<tr>
<td></td>
<td></td>
<td>+0.320</td>
<td>+0.259</td>
<td>-0.09</td>
<td>+0.149</td>
<td>-0.036</td>
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<tr>
<td></td>
<td></td>
<td>+0.275</td>
<td>+0.079</td>
<td>-0.031</td>
<td>+0.296</td>
<td>-0.036</td>
</tr>
<tr>
<td>141</td>
<td></td>
<td>0.0156</td>
<td>0.299</td>
<td>0.3301</td>
<td>0.1652</td>
<td>0.3524</td>
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<tr>
<td></td>
<td></td>
<td>+0.004</td>
<td>+0.128</td>
<td>+0.055</td>
<td>-0.205</td>
<td>-0.156</td>
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<tr>
<td></td>
<td></td>
<td>+0.291</td>
<td>+0.043</td>
<td>+0.008</td>
<td>-0.089</td>
<td>-0.027</td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>&lt;10^-4</td>
<td>0.261</td>
<td>0.0507</td>
<td>0.6893</td>
<td>0.3542</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.147</td>
<td>+0.090</td>
<td>-0.013</td>
<td>-0.085</td>
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<td></td>
<td></td>
<td>+0.291</td>
<td>+0.043</td>
<td>+0.008</td>
<td>-0.089</td>
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<tr>
<td>7</td>
<td></td>
<td>&lt;10^-4</td>
<td>0.0225</td>
<td>0.3614</td>
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<td>0.5854</td>
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<tr>
<td></td>
<td></td>
<td>+0.236</td>
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<tr>
<td></td>
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<td>+0.128</td>
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<td>803</td>
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<tr>
<td></td>
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<td>+0.224</td>
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<td></td>
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<td>-0.082</td>
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<tr>
<td>147</td>
<td></td>
<td>&lt;10^-4</td>
<td>0.2764</td>
<td>0.0025</td>
<td>1.00</td>
<td>0.5561</td>
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<td></td>
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<td>+0.138</td>
<td>+0.107</td>
<td>+0.025</td>
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<td>+0.346</td>
<td>+0.069</td>
<td>+0.368</td>
<td>+0.041</td>
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<tr>
<td>All $</td>
<td>$</td>
<td></td>
<td>&lt;10^-4</td>
<td>0.0107</td>
<td>0.0015</td>
<td>0.0848</td>
</tr>
<tr>
<td>loci</td>
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<td>+0.214</td>
<td>+0.122</td>
<td>+0.039</td>
<td>-0.028</td>
<td>+0.044</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.233</td>
<td>+0.045</td>
<td>+0.124</td>
<td>+0.015</td>
<td>+0.027</td>
</tr>
</tbody>
</table>

* PHW = exact goodness-of-fit test probability (see Materials and Methods); Bold numbers indicate values that remain significant after the sequential Bonferroni procedure was applied to take into account the other tests, underlined numbers: $P < 0.05$, $- =$ irrelevant data because of fixed allele.

† Fis = Fit and Ris = Rit.

‡ Refers to a one-sided test probability of obtaining by chance a value as large or larger than the observed one (permutation of alleles within samples).

§ All sections: Ris is the simple mean of individual Ris (see Materials and Methods).

¶ PHW refers to Fisher’s combined probability test across loci.

### Table 4

Genetic differentiation between populations

<table>
<thead>
<tr>
<th>Locus</th>
<th>Senegal</th>
<th>Senegal</th>
<th>Senegal</th>
<th>Madagascar</th>
<th>Madagascar</th>
<th>Reunion</th>
<th>Mauritius</th>
<th>Reunion</th>
<th>Mauritius</th>
<th>All Populations</th>
</tr>
</thead>
</table>
| 24D   | Fst (Nm) | 0.202† | 0.176† | 0.262† | 0.297† | 0.012† | 21.12 | 0.379† | 0.243† | 0.78%
|       | Rst (Nm) | 0.162† | 0.144† | 0.184† | 0.000† | 20.01 | 0.162† | 0.218† | 0.90%
| 141   | Fst (Nm) | 0.031† | 0.013† | 0.011† | 0.227 | 0.001† | 227.03 | 0.0000† | 0.038† | 6.23%
|       | Rst (Nm) | 0.010† | 0.059† | 0.042† | 0.012† | 35.60 | 0.0000† | 35.60 | 0.031† | 7.90%
| 26    | Fst (Nm) | 0.022† | 0.241† | 0.128† | 0.249† | 0.110† | 2.02 | 0.122† | 1.80 | 1.50†          |
|       | Rst (Nm) | 0.060† | 0.517† | 0.137† | 0.295† | 0.166† | 7.59 | 0.157† | 0.67 | 2.75†          |
| 7     | Fst (Nm) | 0.148† | 0.357† | 0.111† | 0.142† | 0.058† | 5.40 | 0.280† | 0.86 | 1.83†          |
|       | Rst (Nm) | 0.010† | 0.065† | 0.025† | 0.000† | 0.208 | 0.165 | 0.0165 | 15.20 |
| 803   | Fst (Nm) | 0.036† | 0.155† | 0.128† | 0.126† | 0.101† | 2.23 | 0.0000† | 0.959† | 2.37†          |
|       | Rst (Nm) | 0.000I† | 0.141† | 0.086† | 0.064† | 0.032† | 3.72 | 0.0000† | 0.076† | 3.00†          |
| 45C   | Fst (Nm) | 0.300† | 0.172† | 0.441† | 0.158† | 0.115† | 1.92 | 0.223† | 0.87 | 2.46†          |
|       | Rst (Nm) | 0.406† | 0.181† | 0.696† | 0.055† | 0.150† | 0.71 | 0.302† | 0.29 | 0.42†          |
| 29C   | Fst (Nm) | 0.074± | 0.074± | 0.011± | 0.216 | 0.122± | 0.86 | 0.141± | 1.52 | 0.227 | 8.97 | 1.20± |
|       | Rst (Nm) | 0.039± | 0.038± | 0.090± | 0.165 | 0.174± | 0.59 | 0.221± | 5.61 | 0.132 | 0.82 | 0.21± |
| 147   | Fst (Nm) | 0.053± | 0.136± | 0.909± | 0.340± | 0.004± | 28.30 | 0.382± | 0.40 | 0.258± |
|       | Rst (Nm) | 0.109± | 0.206± | 0.188± | 0.214± | 0.080± | 2.87 | 0.215± | 0.91 | 0.169± |
| All   | Fst (Nm) | 0.095± | 0.119± | 0.169± | 0.174± | 0.021± | 5.61 | 0.132± | 0.82 | 0.21± |
| loci  | Rst (Nm) | 0.35±  | 0.56±  | 0.69±  | 0.59±  | 0.87±  | 0.50  | 1.33±  | 0.87 | 1.33± |

* Values in parentheses are Nm estimates. Fst = probability of homogeneity over all loci was estimated by Fisher’s combined probability test; Rst = estimated as described by Slatkin.

† P < 0.005.

‡ Not significant at the 5% level (values in italics).

§ P < 0.05.
High amounts of genetic differentiation were generally observed in this study. The mean Fst estimate based on the whole dataset (eight loci and four populations) was 0.169 and was highly significant ($P < 10^{-3}$, by Fisher's combined probability test). The corresponding Rst value was slightly higher, with an estimate of 0.213.

To compare each population to the others, pairwise estimates of F- and R-statistics were assessed. Mean pairwise Fst over all loci between different locations ranged from 0.080 (between Madagascar and Mauritius) to 0.215 (between Reunion and Mauritius) and were very significant (probability of homogeneity $< 0.005$). Mean pairwise Rst estimates were accordingly lower than their Fst equivalent in all cases, excepted between Senegal and Reunion where it reached 0.302 (corresponding Fst estimate = 0.206). The associated Nm values ranged from 0.91 to 2.88 using the Fst method, from 0.29 to 5.61 using Rst values and from 0.50 to 1.35 using Slatkin's private alleles method. As a result, all four populations were found to be significantly differentiated from one another with subsequent gene flow (Nm $> 1$) arising only between Senegal and Madagascar and between Madagascar and Mauritius.

Each locus was then studied independently to investigate their contribution to the global Fst and Rst values observed. Mean locus-specific Fst estimates over all populations were all very significant (probability of homogeneity $< 0.005$) and varied from 0.038 for locus 141 up to 0.246 for locus 45C. The Rst estimates were in good agreement with their Fst equivalents although most were lower. Only five of 48 locus-specific pairwise Fst estimates were found to be not significant ($P > 0.05$). Three of them occurred between Reunion and Mauritius, populations that share very similar allelic compositions and poor polymorphism at those particular loci (namely loci 141, 803, and 29C). The Rst estimates were also not significant in these particular cases and in 10 additional pairwise comparisons.

**DISCUSSION**

In this study, four *An. arabiensis* populations from different locations were investigated for variation in polymorphism and allele distribution at nine microsatellite DNA loci. The PCR primers described for *An. gambiae* proved very useful in amplifying *An. arabiensis* DNA and showed high polymorphism of the loci in this sibling species. The observed number of alleles per locus ranged from one for locus 49 up to 16 for locus 26. Subdivision of the gene pool was revealed by both a highly significant deviation from Hardy-Weinberg equilibrium with heterozygote deficits and subsequent linkage disequilibrium between pairs of loci when all samples were considered as belonging to one single population in panmixia. At the population level, no outstanding deviation from Hardy-Weinberg equilibrium was observed. High amounts of genetic divergence were measured by Wright’s Fst and Slatkin’s Rst, leading to low estimates of the average migration index (Nm $< 1$ in most of the cases), suggesting the occurrence of a strong genetic structuring of the gene pool.

High levels of genetic variability as observed in the present study are very favorable to detecting separation of the gene pool. Indeed, significant differentiation was detected between all four populations we investigated with both F- and R-statistics. This indicates that *An. arabiensis* populations from those four locations have diverged from one another and that the genetic markers we used are prone to detect geographically differentiated units of the same species.

Faiiloux and others found comparable estimates of Fst when studying *Aedes aegypti* populations from islands of French Polynesia using allozyme markers. In contrast, much lower values were obtained in a study comparing *An. gambiae* populations from Senegal and Kenya using both allozyme and microsatellite markers. The investigators in the later study found a mean Fst of 0.016 and an equivalent Rst of 0.036, suggesting a high level of gene flow in this species across the African continent. Although these populations were 6,000 km apart, they were both carriers of the savanna cytotype and a continuous savanna-type habitat bridges them. Such a difference in amounts of genetic differentiation described in this study and ours underlines the expected major influence of the sea acting as a barrier to gene flow. Our high estimates of interpopulation differentiation correspond to low estimates of the migration index (Nm) between pairs of populations, indicating restricted migration of individuals. The three methods used to estimate Nm gave comparable results and a significant correlation was found between Nm estimates from the Fst and Rst ($r^2 = 0.811, P = 0.014$).

The highest Fst and Rst values were observed between Reunion and each of any other location, while Mauritius and Madagascar showed the lowest degree of differentiation. This suggests no correlation between the level of genetic differentiation and geographic distance. Moreover, distance alone, even across large bodies of water, does not appear to be a significant hindrance to mosquito migrations. A similar pattern of absence of significant correlation between genetic differentiation and geographic distance was observed in a recent study of island *Ae. polynesiensis* populations. Interestingly, the investigators found a significant correlation between gene flow (Nm estimates) and commercial traffic by planes and/or boats between islands: modern intensification of maritime and air travel could then thwart in a non-negligible manner the effect of geographic barriers to insect dispersion.

In light of the history of malaria and human population migrations in the Mascarene islands and Madagascar, vector settlement in these particular sites are thought to be due to active transportation by steamship lines, first from the African continent to Madagascar and then from Madagascar to Mauritius and Reunion. Also, transport of vectors by wind during a cyclone from Mauritius to Reunion cannot be excluded. Indeed, Mauritius, Reunion and the highlands of Madagascar were originally described as free of malaria vectors. Their introduction occurred after environmental reshaping due to human settlement (deforestation, development of agriculture), suggesting a strong founder effect that occurred very recently (Mauritius and Reunion were settled by humans at the beginning of the 17th century). Later, intensive insecticide use on both islands during the 1950s considerably diminished malaria vectors density, thus constraining local populations to experience a severe bottleneck. Then, after eradication of malaria from Mauritius and Reunion, great efforts were continued on autochthonous mosquito popula-
tion control. Today, particular attention is still paid to avoid unintentional importation of foreign mosquitoes by focusing on areas around harbors and airports and by conducting entomologic control in airplanes and boats. These strong control efforts are likely to be one of the major components responsible for such an isolation of the Reunion population and for the overall high amount of genetic differentiation observed in this study by constantly maintaining a reduced population size of the malaria vectors on the islands and by limiting the possibilities of migration and the introduction of foreign alleles. This observation is supported by the high level of genetic differentiation observed between Reunion and Mauritius populations, which despite a comparable entomologic history, revealed significantly different patterns of allelic variation at the microsatellite loci studied here.

The heterogeneity of allelic variation patterns observed between the Mauritius and Reunion populations also has a more fundamental implication as indicating no evidence for overall constraints acting on allele size or for selection for one (or few) particular genotype(s) despite fairly high insecticide selection pressure. Nevertheless, three of eight microsatellite loci showed no significant differentiation between these populations (loci 141, 803, and 29C, Table 4) and could therefore reflect the occurrence of such constraints acting on at least some of those loci.

These findings have shown great influence of both genetic drift and restricted migration opportunities on a large scale genetic structuring of An. arabiensis populations. We will now consider their effect at a local population level.

One of the nine loci we investigated, locus 49, located on the X-chromosome, was found to be fixed for the same allele in all four populations and was therefore not informative in An. arabiensis populations, despite high polymorphism observed in An. gambiae test samples. This locus should therefore have experienced different molecular evolution since speciation occurred between An. arabiensis and An. gambiae. Locus 7, located less than 1 cM away from locus 49, was found to be monomorphic in the Reunion population but showed great polymorphism in the three other samples. If we assume a 1:1 sex ratio in natural diploid species, the X-chromosome is accordingly 25% less represented than the autosomes. Thus, loci of this particular chromosome are subject to lower effective population sizes than their autosomal homologs, and therefore are more susceptible to random genetic drift. Such a high level of genetic drift occurring on those particular loci together with high insecticide selection pressure could have led to the loss of variability observed on locus 7 in the Reunion population, since effective population size seems to be reduced on this island, according to the overall low genetic variability observed (Table 2). Indeed, as shown in Figure 1, of 62 distinct alleles across eight loci, no private allele (e.g., diagnostic allele) was found for the Reunion population, one was found for Mauritius (namely allele 102 at locus 7), three for Madagascar, and 11 for Senegal, with the remaining 47 alleles being shared by two or more populations. This pattern of variability would be expected if alleles are lost by enhanced genetic drift in the former populations and is consistent with lower effective population sizes of the Mascarene populations.

Analysis of genotype frequencies and distribution across loci suggest random mating within each of the four populations investigated since no obvious indication of inbreeding or of genotypic selection was shown by both Hardy-Weinberg and linkage disequilibrium exact probability tests. Furthermore, even when genetic variability was low and putative selection pressure by intensive use of insecticides was high (e.g., in the Reunion and Mauritius populations), no evidence for selection of certain genotypic combinations was observed. Up to now, no resistance to the different insecticides used in those locations (either to Temephos in the larvae or to DDT or other organophosphorous compounds in the adults) has been reported. The outcome of such an intensive effort, rather than selecting one particular insecticide-resistant strain of malaria vector, probably contributes to genetic isolation (e.g., limiting gene flow with other continental or island populations) and to restriction in density (and therefore effective population size) of the local population of vectors.

It should be noted that a possible bias towards the results we obtained may have been generated by the way our sampling was realized. Indeed, mosquitoes were collected at times from August 1994 to April 1997, but also at different geographic scales (e.g., in one location in Reunion and from all over the island in Mauritius). Collection in one location at one time point may severely limit the genetic diversity observed, thus increasing the amount of genetic differentiation detected. However, homogeneity of the gene pool was verified within each of the four samples, and comparable studies on western Kenyan An. gambiae populations failed to detect significant genetic differentiation at the microgeographic level (up to 50 km in diameter). It is also possible that allelic and gametic frequencies changes have occurred during the 1994–1997 time interval. Studies are currently being conducted to assess for temporal variations within An. arabiensis natural populations.

Our survey showed that island populations of the malaria vector An. arabiensis have significantly diverged from each other essentially by founder effect and enhanced genetic drift due to permanent limitation of their effective population size. Homogenization of the gene pool by exchange of migrants between the different demes (basic reproductive units) is strongly restricted by the presence of a geographic barrier limiting passive dispersion, and intensive control of the different human-made active means of transportation. Further studies are needed to confirm the high amounts of genetic differentiation observed and to investigate the temporal behavior of the genetic structure within and among populations. Comparative studies and estimation of the effective population sizes of these four populations have to be investigated to check for the accuracy of the assumptions used to estimate F- and R-statistics: populations have to be at mutation-drift equilibrium and care should be taken not to overlook significant differences in Ne between them that would lead to an overestimation of the level of genetic differentiation. Further studies on linkage disequilibrium and temporal variation in allelic frequencies and heterozygosities should prove very helpful to investigate putative differences in effective population sizes. Furthermore, such studies should help to quantify and appreciate to a greater extent the amplitude of the founder effect that allowed colonization of the Mascarene islands by An. arabiensis populations and to as-
sess more precisely the effect of intensive control measures on locale vector populations.

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