

Genetic Structure of *Plasmodium falciparum* and Elimination of Malaria, Comoros Archipelago

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The efficacy of malaria control and elimination on islands may depend on the intensity of new parasite inflow. On the Comoros archipelago, where falciparum malaria remains a major public health problem because of spread of drug resistance and insufficient malaria control, recent interventions for malaria elimination were planned on Moheli, 1 of 4 islands in the Comoros archipelago. To assess the relevance of such a local strategy, we performed a population genetics analysis by using multilocus microsatellite and resistance genotyping of *Plasmodium falciparum* sampled from each island of the archipelago. We found a contrasted population genetic structure explained by geographic isolation, human migration, malaria transmission, and drug selective pressure. Our findings suggest that malaria elimination interventions should be implemented simultaneously on the entire archipelago rather than restricted to 1 island and demonstrate the necessity for specific chemoresistance surveillance on each of the 4 Comorian islands.

Plasmodium falciparum causes malaria worldwide; 250 million cases and ≈1 million deaths occur annually, mostly in sub-Saharan Africa. However, recently increased international financial commitment has revived hope for malaria elimination from selected areas to which it is endemic, and the feasibility of elimination has become a topic of research (1). The successful elimination of malaria from several Caribbean islands, Cyprus, Reunion, Mau-

ritius, Maldives, Taiwan, and Singapore in the context of the Global Malaria Eradication Program (1955–1968) (2) suggests that islands are prime targets for elimination interventions. Because most parasites among neighboring areas are exchanged through human migrations, the geographic isolation of islands can limit malaria importation and may make control easier (3,4).

Several molecular epidemiologic studies have shown that *P. falciparum* populations are more or less homogeneous within malaria-endemic areas and may exhibit genetic structure patterns shaped by various transmission rates and geographic isolation levels (4–6). Although geographic isolation may be more relevant on islands than within continents, the role of parasite genetic structure in malaria-endemic archipelagos or among malaria-endemic islands and the nearest continent remains unknown. Past failures of malaria elimination in Zanzibar off the coast of mainland Tanzania; in Sri Lanka (1); or in Mayotte, a France-administered island of the Comoros archipelago (7), raise the question of the minimal geographic isolation level and the optimal size of intervention area required for malaria elimination success. Analysis of malaria epidemiology in Comoros archipelago, where a limited malaria elimination program is ongoing, may help to answer this question.

Falciparum malaria remains a major public health problem on the 4 islands of the Comoros archipelago (Grande Comore, Moheli, Anjouan, and Mayotte) (Table 1) in the Indian Ocean between Madagascar and the eastern coast of Africa. Malaria control has been hampered by the emergence of *P. falciparum* resistance to chloroquine and to pyrimethamine/sulfadoxine in the early 1980s (7,10) and of *Anopheles* mosquitoes resistance to DDT. Malaria con-

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Table 1. Epidemiologic and sampling characteristics of 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France

| Characteristic | Grande Comore | Moheli | Anjouan | Mayotte | Marseille |
|---|-----------------------------|-----------------------------|-----------------------------|-------------|---------------|
| Area, km ² | 1,148 | 290 | 424 | 374 | |
| Total population | 330,000 | 40,000 | 280,000 | 190,000 | 50,000–80,000 |
| No. bites by infected mosquitoes/person* | 10–20/y (up to 200/y) | 10/y (up to 1/night) | No data | Low | None |
| Endemicity*† | Mesoendemic to hyperendemic | Mesoendemic to hyperendemic | Mesoendemic to hyperendemic | Hypoendemic | None |
| Total no. reported cases (% confirmed cases), 2006* | 51,148 (34) | 7,866 (27) | 15,408 (19) | 496 (100) | 84 (100) |
| Incidence/1,000 inhabitants, 2006* | 150 | 150 | 50 | 3 | Null |
| % <i>P. falciparum</i> malaria cases,* 2006 | 96 | 96 | 96 | 90 | 97 |
| Period of sampling, 2007 | Apr–May | Apr–May | Apr–May | Entire year | Entire year |
| No. patients sampled, 2007 | 62 | 61 | 63 | 227 | 111 |
| Median age of sampled patients, y (IQR) ‡ | 4 (2–14.8) | 7.5 (2.6–21) | 7 (3.3–18) | 19 (15–25) | 33 (9.8–40) |
| No. sampled patients in site (A) with history of recent arrival from another site (B) | 7 | 17 | 6 | 13 | 111§ |
| Anjouan | 6 | 9 | – | 7 | |
| Grande Comore | – | 8 | 5 | 6 | |
| Mayotte | 1 | 1 | 0 | – | |
| Moheli | 0 | – | 2 | 0 | |
| No. randomly genotyped isolates | 36 | 36 | 36 | 36 | 36 |

*Data from World Malaria Report 2008 (8) and from various Comorian and French official reports and references published in French, all reviewed by the first author in a recent, unpublished thesis (Rebaudet S. Molecular epidemiology and population genetics study of *Plasmodium falciparum* in Comoros archipelago. Impacts on malaria control [thesis] [in French]. Marseille (France): Université de la Méditerranée; 2009). Cases for Marseille represent those in persons with history of recent arrival from one of the 4 islands.

†Malaria endemicity levels based on 2–9 years of available parasite prevalence data, according to the World Health Organization classification: hypoendemic, 0–10%; mesoendemic, 11%–50%; hyperendemic, 51%–75%; and holoendemic, >75% (9).

‡IQR, interquartile range.

§Mainly from Grande Comore (S. Rebaudet, pers. comm.).

tol also has had recurrent political, economic, and structural weaknesses in the Union of the Comoros (the state comprising Grande Comore, Moheli, and Anjouan islands). Under stable political and economic conditions, notable efforts in case management and vector control in Mayotte failed to eliminate falciparum malaria and to prevent recurrent epidemics (Table 1). During the past 6 years Since 2004, health authorities in Grande Comore and France have introduced an artemisinin-based combined therapy (artemether plus lumefantrin) as first-line treatment for uncomplicated falciparum malaria (7,10). Large-scale distribution of insecticide-treated mosquito nets also has been gradually implemented on Grande Comore, Moheli, and Anjouan (8), with the goal of reaching up to 89.1% and 46.3% of the households with at least 1 mosquito net and 1 insecticide-impregnated mosquito net, respectively, among 1,620 households from the 3 islands (Comoran National Malaria Control Program, unpub. data, 2007).

In Mayotte, anti-*Anopheles* spp. mosquito larvae measures have been strengthened. Finally, by late 2007, a controversial malaria elimination project was launched on the sole island of Moheli with assistance from China. Mass treatment of the residing and disembarking population with artemisinin plus piperazine (Artequick; Artepharm Co., Guangzhou, People's Republic of China) and primaquine was initiated without enhancement of vector control. Be-

cause of continual human travel across the archipelago, the long-term success of such a spatially limited elimination attempt is questionable.

In addition, surveillance of *P. falciparum* chemosusceptibility has been chaotic and unequal among the islands of the archipelago, and results of the few available therapeutic efficacy tests and in vitro and molecular resistance studies often have been discordant. A more rational and efficient surveillance system is urgently needed. Because Marseille, France, houses a Comorian community of 50,000–80,000 persons who annually import several hundred malaria cases, the city was proposed as a relevant surveillance site for chemosusceptibility of *P. falciparum* imported from Comoros (11). However, extrapolating these chemoresistance data to the entire archipelago remains difficult.

As already proposed for Borneo (12) and the Philippines (13), our main objective was to analyze the genetic structure of *P. falciparum* on the Comoros islands to 1) forecast the chances of middle-term and long-term success for the current elimination program focalized in Moheli, 2) guide future malaria elimination programs on the archipelago, and 3) adjust its chemoresistance monitoring and treatment policies. Study results also would provide a pertinent model for determining which other malaria-endemic areas might be eligible for malaria elimination. A secondary objective was to assess whether the diversity of the *P.*

falciparum strains imported into Marseille were representative of the *P. falciparum* populations from Comoros so we could evaluate the relevance of distant chemoresistance surveillance from Marseille.

We characterized *P. falciparum* populations from each of the 4 islands and from Marseille (imported from the archipelago) by multilocus microsatellite genotyping. The genetic polymorphism of 3 genes involved in *P. falciparum* resistance to chloroquine, pyrimethamine and cycloguanil, or sulfadoxine was also investigated.

Materials and Methods

P. falciparum Isolates

The study was conducted in 2007 (before the malaria elimination program was launched in Moheli) in each of the 4 islands of the Comoros archipelago and in Marseille. The protocol was approved by the ethics committee of the university hospitals of Marseille and by the Comorian Ministry of Health. Blood samples were obtained after informed consent from patients seeking care for symptomatic *falciparum* malaria at healthcare centers of the archipelago or at emergency departments of hospitals in Marseille.

Blood samples were absorbed onto Whatman FTA Elute absorbent filter paper in Grande Comore, Moheli, and Anjouan islands, on Whatman 903 Protein Saver filter paper (Whatman Inc., Florham Park, NJ, USA) in Mayotte, and collected into Vacutainer tubes (Becton Dickinson, Le Pont-De-Claix, France) in Marseille. All samples were frozen and kept at -20°C . After eliminating samples with missing data or the lowest parasitaemia levels ($<0.01\%$), 36 isolates per site were randomly chosen for genotyping, a sample size considered adequate for the planned population genetics analyses.

Collection of Epidemiologic Data

Patient's age, sex, history of travel across or outside the archipelago (during the past year for Grande Comore, Moheli, and Anjouan; during the past 3 weeks for Mayotte) and history of recent clinical malaria episodes and intake of antimalarial drugs (during the previous month) were collected by oral questioning. Distances between each island were measured by using Google Earth software.

Genotyping Procedures

DNA was extracted from filter papers according to the manufacturer's recommendations (Whatman Inc.) and from whole blood from Vacutainer tubes by using the EZNA Blood DNA Kit (Biofidal, Vaulx-en-Velin, France). Next, the entire genome was amplified by using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK).

Molecular Markers

Length polymorphism was analyzed for 6 complex and putatively neutral microsatellite loci previously described (4): *Pf2689*, *7A11*, *C4M79*, *Pf2802*, *TRAP*, and *C4M69* (online Appendix Table, www.cdc.gov/EID/content/16/11/1686-appT.htm). The studied chemoresistance markers were the K76 point mutation of the *P. falciparum* chloroquine resistance transporter (*Pfcr1*) gene (associated with *P. falciparum* resistance to chloroquine) (14); point mutations of *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene codons 108, 16, 51, 59, and 164 (associated with *P. falciparum* resistance to pyrimethamine and cycloguanil, i.e., proguanil metabolite) (15); and *P. falciparum* dihydropteroate synthase (*Pfdhps*) gene codons 436, 437, 540, 581, and 613 (associated with *P. falciparum* resistance to sulfadoxine) (15) (online Appendix Table).

Genotyping by PCR

Microsatellite loci were amplified by nested PCR with fluorescent end-labeled primers before electrophoresis on polyacrylamide gels with Genescan-500 LIZ labeled size standards on an ABI 3130XL capillary sequencer (Applied Biosystems, Warrington, UK) (online Appendix Table). Their length was then analyzed by using GENESCAN software (Applied Biosystems, Carlsbad, CA, USA), as described (4). The *Pfcr1* gene was amplified by seminested PCR, and the codon 76 mutation was genotyped by using a simple PCR-restriction fragment digest assay and fluorescent detection of products on an ABI 3130XL capillary sequencer, as described (16). The *Pfdhps* and *Pfdhfr* genes were amplified by nested PCR, and their mutations were genotyped by using a primer extension method, as described (17) and electrophoresis on the ABI 3130XL capillary sequencer.

Statistical Analysis

The multiplicity of infection (MOI, i.e., the number of parasites genetically distinguishable by different alleles) with *P. falciparum* was estimated for each isolate from the microsatellite locus that exhibited the highest number of alleles. The mean MOI for each *P. falciparum* population (Grande Comore, Moheli, Anjouan, Mayotte, and Marseille) was then calculated. Each pair of sites was compared for MOI by using the Mann-Whitney U test.

For parasites with multiple infection, i.e., >1 allele at each locus, we conducted separate subsequent analysis considering the following: 1) complete dataset, 2) curtailed dataset with single or main clones after elimination of isolates unsuccessfully genotyped at all 6 microsatellite loci, or 3) reconstructed multilocus genotypes after elimination of samples with impossible reconstruction (>1 allele at >1 locus with equivocal peak intensities) and elimination of unsuccessfully genotyped isolates (Table 1).

Genetic Diversity

Genetic diversity of the 5 *P. falciparum* populations was assessed by the number of alleles per locus and by the Nei unbiased expected heterozygosity index (H_e) calculated from allelic frequencies on the 6 microsatellites for complete datasets by using GENETIX software version 4.05 (18,19). Comparison between H_e of the 5 different populations was performed on FSTAT software version 2.9.4 with a 1,000 permutations bilateral comparison test (20).

Population Genetic Structure

Population genetic structure was investigated by using the Wright F statistic (F_{ST}) (21). The F_{ST} index was computed for the 6 microsatellite markers and 5 populations on FSTAT software version 2.9.4 (20,22) and by using the Slatkin index on ARLEQUIN software (23). A canonical correspondence analysis of the reconstructed multilocus genotypes set was conducted to illustrate measures of population structure (24) by using CANOCO software (25), and its graphic representation was performed by using R software. A Monte Carlo procedure permuting genotypes among the populations was used to test the significance of the canonical axes and estimate the 95% confidence intervals of the centroid of each population (25).

Frequency of Mutations Associated with Chemoresistance

We estimated the frequency of point mutations on the *Pfcr1* (K76T mutation), *Pfdhfr* (108 + 59 + 51 triple mutation), and *Pfdhps* (437 + 540 double mutation) genes. Differences among sites were tested by using the Fisher exact test.

Associations between F_{ST} and Estimations of Parasite Flux

The association between genetic distance (transformed as $F_{ST}/[1 - F_{ST}]$) and the natural log of the geographic distance in kilometers was investigated for each pair of islands according to the isolation-by-distance model (5,12,26). When we considered the number of patients in each sampled island (A) with history of recent arrival from each of the neighboring islands (B) and thus possibly with imported malaria (Table 1), the relationship between F_{ST} and the mean proportion of these travelers among patients, calculated as $([N_{B \rightarrow A}/N_A] + [N_{A \rightarrow B}/N_B])/2$, was investigated for each pair of islands.

Results

P. falciparum was detected by PCR in each of the 36 genotyped blood samples from all 5 sites. Microsatellite genotyping was complete for 149 (83%) of the 180 samples (Table 2).

Mean MOIs

Of the 180 samples, 59 isolates were multi-infected; the proportion of multi-infection among islands differed substantially (Table 2). The mean MOI ranged from 1.22 in Mayotte to 2.11 in Anjouan (Table 3). It was significantly higher in Anjouan than in Grande Comore ($p = 0.0015$), Moheli ($p = 0.0051$), and Mayotte ($p = 0.0001$) and higher in Marseille than in Mayotte ($p = 0.0093$).

Genetic Diversity

Genetic diversity (H_e) of each population, estimated by unbiased expected heterozygosity based on allelic frequencies of the 6 microsatellites and the complete dataset, is shown in Table 4. The highest diversity was observed for Anjouan and Moheli (each $H_e = 0.71$) and the lowest diversity for Mayotte ($H_e = 0.63$). The mean H_e was significantly lower for Mayotte than for Marseille ($p = 0.04$) and lower than for the other sites combined ($p = 0.001$).

Genetic Differentiation among Islands and Population Structure

Figure 1 shows the centroid of each *falciparum* population surrounded by its 95% confidence interval, and both axes were significant ($p = 0.0001$ and $p = 0.0004$ for 1,000 permutations, respectively). Grande Comore, Moheli, and Anjouan nearby centroids suggest closely related populations. The detached Mayotte centroid suggests a marked differentiation from all the other populations.

Figure 2 shows the pairwise differentiation coefficients (F_{ST}) estimated for the 5 parasite populations according to the 6 microsatellite loci and the complete dataset ($n = 281$). The number of *P. falciparum* clones used to calculate F_{ST} between sites was 50, 53, 76, 44, and 58 in Grande Comore, Moheli, Anjouan, Mayotte, and Marseille, respectively. The Moheli parasite population did not differ significantly from the Grande Comore and Anjouan populations. Conversely, the Mayotte population differed significantly from the populations of the 4 other sites. Marseille parasite populations differed significantly from those from all sites except Grande Comore. Similar differentiation index were

Table 2. Genotyping results of the 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France

| Characteristic | Grande Comore | Moheli | Anjouan | Mayotte | Marseille | Total |
|--|---------------|--------|---------|---------|-----------|-------|
| No. randomly genotyped isolates | 36 | 36 | 36 | 36 | 36 | 180 |
| No. detected parasites | 50 | 53 | 76 | 44 | 58 | 281 |
| No. single or main clones successfully genotyped | 28 | 20 | 29 | 36 | 36 | 149 |
| No. reconstructed multilocus genotypes | 30 | 24 | 40 | 37 | 39 | 170 |
| No. multi-infected isolates | 9 | 10 | 22 | 4 | 14 | 59 |

Table 3. MOIs of *Plasmodium falciparum* infections for the 5 sites studied, Comoros archipelago and Marseille, France*

| Locus | Grande Comore | | Moheli | | Anjouan | | Mayotte | | Marseille | |
|----------------------------|---------------|------|--------|------|---------|------|---------|------|-----------|------|
| | No. | MOI | No. | MOI | No. | MOI | No. | MOI | No. | MOI |
| All 6 loci | 36 | 1.39 | 36 | 1.47 | 36 | 2.11 | 36 | 1.22 | 36 | 1.64 |
| <i>Pf2689</i> | 34 | 1.03 | 27 | 1.07 | 36 | 1.19 | 36 | 1.08 | 36 | 1.22 |
| <i>7A11</i> | 34 | 1.12 | 32 | 1.19 | 35 | 1.37 | 36 | 1.14 | 36 | 1.39 |
| <i>C4M79</i> | 34 | 1.21 | 25 | 1.32 | 33 | 1.55 | 36 | 1.11 | 36 | 1.42 |
| <i>Pf2802</i> | 31 | 1.00 | 23 | 1.00 | 32 | 1.00 | 36 | 1.00 | 36 | 1.00 |
| <i>TRAP</i> | 35 | 1.42 | 29 | 1.14 | 36 | 1.67 | 36 | 1.08 | 36 | 1.19 |
| <i>C4M69</i> | 32 | 1.06 | 21 | 1.19 | 34 | 1.12 | 36 | 1.03 | 36 | 1.11 |
| Mean no. alleles per locus | 8.5 | | 8 | | 8.5 | | 4.5 | | 7.8 | |

*Based on no. multi-infected isolates as shown in Table 2. MOI, multiplicity of infection (no. multi-infected isolates/no. randomly genotyped isolates); no., no. randomly genotyped isolates;

obtained by using a curtailed dataset (n = 149) or reconstructed multilocus genotypes (n = 170) and by using the Slatkin index (data not shown).

Relations between F_{ST} and Estimations of Parasite Flux

Association between genetic and geographic distances for each pair of islands is shown in Figure 3. No association was significant. The Anjouan–Mayotte pair exhibited a large F_{ST} despite the close proximity of the 2 islands.

Of the 414 patients sampled in the archipelago, 35 reported recent travel to neighboring islands (Table 1). Figure 3 suggests a negative relationship between the mean percentage of travelers among patients and the corresponding F_{ST} .

Frequency of Point Mutations associated with Chemoresistance

Prevalence of *Pfcr*, *Pfdhfr*, and *Pfdhps* mutations in the 5 *P. falciparum* study populations are presented in Table 5. Prevalence of the *Pfcr* mutation (i.e., isolates with the 76T allele or with the 76 K, and T alleles) was significantly lower in Anjouan than in the other parasite populations ($p \leq 0.002$). Prevalence of the *Pfcr* mutation was significantly higher in Mayotte than in any other population ($p \leq 0.0001$). The prevalences of the *Pfcr* mutation in Grande Comore, Moheli, and Marseille did not differ significantly.

When mutated, the *Pfdhfr* gene frequently exhibited the association of the 3 mutations 108N + 59R + 51I. Prevalence of this triple mutation was significantly lower in the

Anjouan population than in the Grande Comore ($p = 0.04$), Moheli ($p = 0.003$), or Marseille populations ($p = 0.0004$). Its prevalence also was significantly higher in Marseille than in Mayotte ($p = 0.02$). The prevalence of *Pfdhps* gene mutations appeared low in the 5 populations.

Multi-infected isolates with genotype ambiguities and impossible distinction between associated clones were rare. However, prevalence of these mutations varied little, regardless whether these ambiguous multi-infected isolates were considered.

Discussion

The mean MOIs remained low for the Comoros archipelago in comparison with African areas, where malaria is highly endemic (4), most likely because of moderate levels of malaria transmission (4,5). Likewise, the significantly higher MOI in Anjouan may reflect a higher level of malaria transmission in the rainy and swampy sampled areas, where vector control has for a long time been impaired by recurrent island-specific political crises. The genetic diversities appeared lower on the archipelago than on most of the African continent (4,5,27–30), probably because of the geographic isolation of the islands and their lower malaria transmission levels that could limit effective parasite population sizes and outbreeding. However, genetic diversities remained higher than in Asia (5,6,12,13) and South America (5,31).

The genetic differentiation index (F_{ST}) exhibited a contrasted genetic structure between the studied *P. falciparum* populations. Genetic distances were low among parasites

Table 4. Genetic diversity of *Plasmodium falciparum* at the 5 sites studied, Comoros Archipelago and Marseille, France*

| Locus | Grande Comore | | Moheli | | Anjouan | | Mayotte | | Marseille | |
|---------------|---------------|-------|--------|-------|---------|-------|---------|-------|-----------|-------|
| | No. | H_e | No. | H_e | No. | H_e | No. | H_e | No. | H_e |
| All 6 loci | 50 | 0.63 | 53 | 0.71 | 76 | 0.71 | 44 | 0.51 | 58 | 0.63 |
| <i>Pf2689</i> | 35 | 0.47 | 29 | 0.44 | 43 | 0.52 | 39 | 0.15 | 42 | 0.53 |
| <i>7A11</i> | 38 | 0.81 | 38 | 0.85 | 48 | 0.82 | 41 | 0.41 | 50 | 0.87 |
| <i>C4M79</i> | 41 | 0.86 | 33 | 0.85 | 51 | 0.83 | 40 | 0.74 | 51 | 0.87 |
| <i>Pf2802</i> | 31 | 0.00 | 23 | 0.48 | 32 | 0.43 | 36 | 0.48 | 36 | 0.20 |
| <i>TRAP</i> | 39 | 0.79 | 33 | 0.80 | 57 | 0.85 | 39 | 0.60 | 43 | 0.56 |
| <i>C4M69</i> | 34 | 0.87 | 25 | 0.82 | 38 | 0.84 | 37 | 0.68 | 40 | 0.77 |

*No., no. detected parasites. H_e , Nei unbiased expected heterozygosity index.

on Grande Comore, Moheli, and Anjouan islands. However, F_{ST} s among these 3 populations and Mayotte were as significant as between *P. falciparum* populations of Senegal and Djibouti when the same microsatellite loci were used (4) or as between populations of Africa and Southeast Asia when other microsatellite loci were used (5). In addition, the genetic distances between falciparum populations across the archipelago seemed associated with the parasite flows among islands, estimated from the proportion of travelers among sampled patients, in particular for Moheli, Grande Comore, and Anjouan.

Our results strongly suggest that despite the insular geographic isolation of Moheli and the malaria elimination program launched in late 2007 on this island only, the mass treatment without enhanced vector control may soon be impaired by the continuous importation of new parasites through intense human migrations. In addition to flights and ferries regularly traveling across the archipelago, humans in Comoros move mainly by small fishing boats, especially from and toward Moheli (S. Rebaudet, pers. comm.). Their ubiquitous and informal traffic makes human flux estimations unreliable (probably several tens of thousands of persons each way annually [S. Rebaudet, pers. comm.]) and their control difficult. These factors might explain why, despite the substantial resources that France has allocated in Mayotte to malaria control since the mid-1970s, malaria importation to this island could not be stopped and autochthonous falciparum malaria could not be eliminated. The disease persists in Mayotte with a hypoendemic-epidemic setting, genetically characterized by low MOI , low H_e , significant linkage disequilibrium (data not shown), and high F_{ST} s, artificially overestimated by the sampling of multiple repeated genotypes (data not shown).

The persistent efforts for malaria elimination in Moheli can be hypothesized to create a Mayotte-like setting requiring efficient vector control to prevent epidemics in a Mohelian population that is losing its immunity. In the Union of the Comoros, the extension of the elimination program based on artemisinin-based combined therapy mass treatment to Grande Comore and Anjouan is being considered by Comorian health authorities and their Chinese interlocutors (R. Silai, pers. comm.). Its success and the prevention of epidemics will depend on the rapid and large implementation of the preventive, diagnostic, and therapeutic measures planned with the funds granted in 2010 by Round 8 of the Global Fund (<http://portfolio.theglobalfund.org/Grant/Index/COM-810-G03-M?lang=en>).

Isolation of a specific *P. falciparum* population before planning its elimination needs to be appropriately evaluated. Results from the present Comorian epidemiologic study illustrate how it could be evaluated by a population genetics approach. In that type of geographic setting, population genetics studies provide a probably more direct and reliable

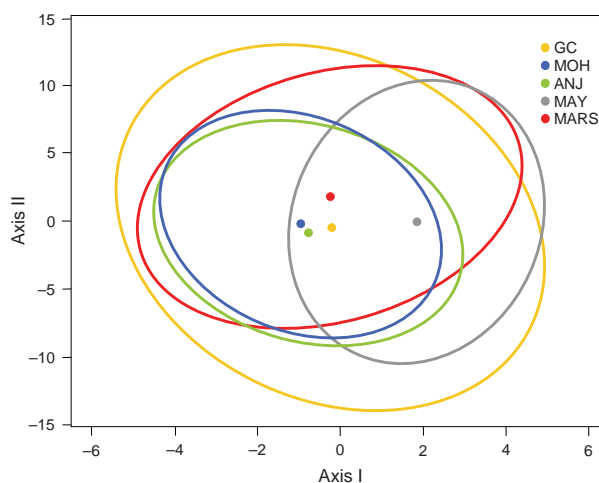


Figure 1. Results of canonical correspondence analysis (CCA) of *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. CCA is used as a 2-dimensional representation of genetic distance between plasmodial populations assessed from 6 microsatellite loci (*Pf2689*, *C4M79*, *Pf2802*, *7A11*, *TRAP*, and *C4M69*). This representation requires the projection of data from 6-dimensional space to 2-dimensional space. Canonical axes I and II of the new 2-dimensional space are calculated to conserve the highest genetic variance between populations after projection of data, and their significance was tested by Monte Carlo permutation that also enabled estimation of the 95% confidence intervals (ellipses) of the centroid (dots) of each population.

estimation of parasite flows and risk for re-introduction than does the evaluation of human population movements by sociodemographic methods. Therefore, the relevance of parasite inflow from Africa (mostly the Tanzania coast, Madagascar, or other malaria-endemic areas) should be evaluated before the elimination project is extended to the rest of the Comoros archipelago. Similar data would also be useful for Sri Lanka, Malaysia, Indonesia, the Philippines, Solomon islands, or Vanuatu, several islands where national or localized malaria elimination projects are being implemented (2).

According to the genetic structure of *P. falciparum* populations in Comoros demonstrated by microsatellite genotyping, resistance levels would be expected to be fairly similar across the archipelago, except for Mayotte. However, the study of *Pfprt* and *Pfdhfr* resistance-associated mutations differed markedly, explainable only by contrasting levels of drug selective pressure among islands. Indeed, the prevalence of the K76T mutation on the *Pfprt* gene was high in both Grande Comore and Moheli as found in previous studies (32,33) but substantively lower in Anjouan and significantly higher in Mayotte where chloroquine use was massive during 1975–2007

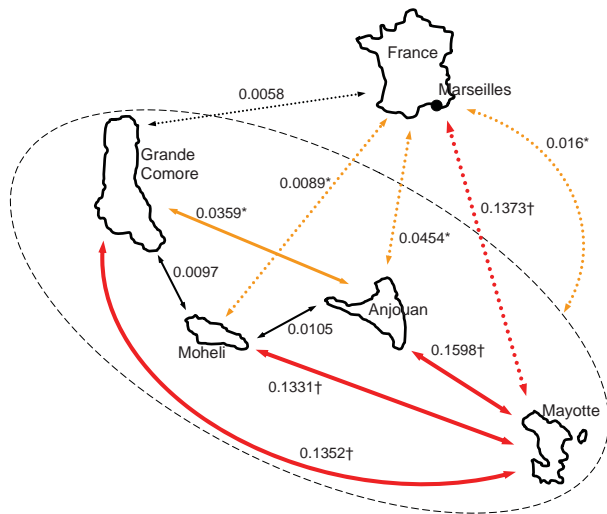


Figure 2. Genetic differentiation (F_{ST}) between *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. Pairwise comparison among sites that used complete dataset ($n = 281$) and 6 microsatellite loci (*PF2689*, *C4M79*, *PF2802*, *7A11*, *TRAP*, and *C4M69*). Departure of F_{ST} from 0 tested after 10,000 bootstrap simulations and by using Bonferroni corrected p values obtained after 200 permutations. Difference is significant if adjusted $p < 0.005$. Black arrows indicate negligible ($F_{ST} < 0.01$) and nonsignificant differentiation. Asterisks (*) and orange arrows indicate moderate ($0.01 \leq F_{ST} < 0.1$) and/or statistically significant differentiation. Daggers (†) and red arrows indicate important ($F_{ST} \geq 0.1$) and significant differentiation. Plain arrows indicate genetic differentiation between the parasite populations of the Comoros islands. Dotted arrows indicate genetic differentiation between the parasite population imported in Marseille (from Comoros) and either the overall parasite population of the entire Comoros archipelago (dotted oval and extreme right arrow) or the parasite populations of each of the 4 islands.

(7,34). Similarly, the prevalence of *Pfdhfr* triple mutants was higher in Moheli than in Anjouan and the prevalence of *Pfdhfr* double or triple mutants higher in Marseille than in Grande Comore.

Although no reliable estimation of past use of antimalarial drugs in Comoros is available, these differences may be explained by a greater use in Moheli of pyrimethamine (in the sulfadoxine/pyrimethamine combination for malaria treatment) and trimethoprim (in cotrimoxazole compound, which is widely prescribed in this island as an antimicrobial drug) and in Marseille of proguanil (in association with chloroquine or atovaquone, used as malaria chemoprophylaxis by travelers to the archipelago) (S. Rebaudet, pers. comm.). Trimethoprim and proguanil are 2 antifolate drugs whose cross-resistance with pyrimethamine has been suspected (35,36) and that may have selected these *Pfd-*

hfr mutations. Because of the contrasting resistance levels among islands, the risk for rapid propagation of resistant *P. falciparum* strains across the archipelago suggested by the low F_{ST} s among Grande Comore, Moheli, and Anjouan (4,5), and the easier selection of multigenic resistance and multiresistance from low MOIs limiting the possibilities of genetic recombinations that could break apart allele combinations (5,37,38), French and Comorian health authorities should organize surveillance of chemoresistance, both regular and separated for each island.

Finally, microsatellite genotypes of the *P. falciparum* population in Marseille substantially differed from those populations on all islands except Grande Comore. Because most of the Comorian inhabitants living in Marseille originated from Grande Comore, malaria is imported mainly from this particular island (S. Rebaudet, pers. comm.). Therefore, if we consider that the *P. falciparum* population in Marseille may be representative only of the Grande Comore population and the distinct levels of drug pressure between Marseille and the other populations, the relevance of distant chemosusceptibility surveillance from Marseille is likely to be limited.

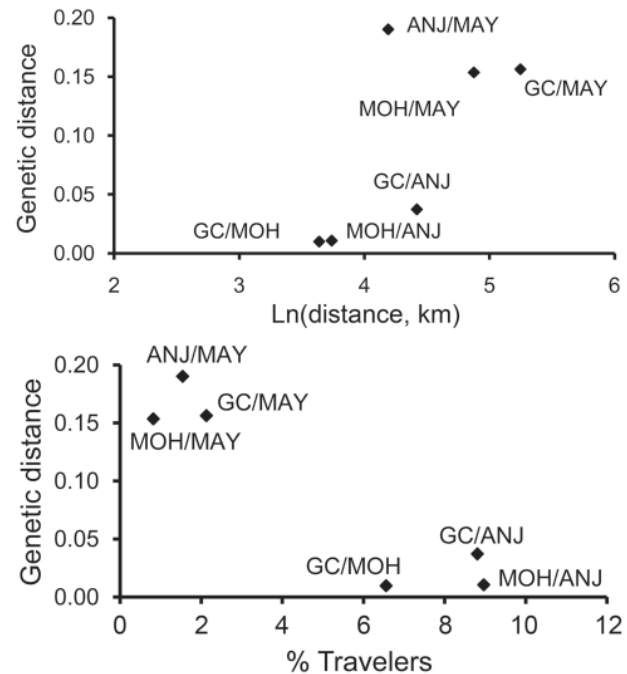


Figure 3. Relationship between geographic and genetic distances for each pair of Comoros islands (top) and between mean percentage of travelers among sampled patients and genetic distance for each pair of Comoros islands (bottom). Genetic distances were calculated as $(F_{ST}/1 - F_{ST})$, where F_{ST} is the Wright F statistic. Mean percentage of travelers was calculated from the total number of sampled patients in one site (N_A) with history of recent arrival from another site (N_B) by using the equation $([N_{B \rightarrow A}/N_A] + [N_{A \rightarrow B}/N_B])/2$; data in Table 1. GC, Grande Comore; MOH, Moheli; ANJ, Anjouan; MAY, Mayotte; Ln, logarithmically transformed.

Table 5. Frequency of chemoresistance-associated point mutations of 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France*

| Locus | No. isolates (% mutations) | | | | |
|----------------------|----------------------------|-----------|------------|------------|------------|
| | Grande Comore | Moheli | Anjouan | Mayotte | Marseille |
| <i>Pfcr</i> | | | | | |
| 76T | 33 (45.5) | 33 (45.5) | 36 (13.9) | 36 (91.7) | 36 (52.8) |
| 76 T and K | 33 (9.1) | 33 (6.1) | 36 (5.6) | 36 (2.8) | 36 (0) |
| K76 (Wt) | 33 (45.5) | 33 (48.5) | 36 (80.6) | 36 (5.6) | 36 (47.2) |
| <i>Pfdhfr</i> | | | | | |
| 108N | 26 (50.0) | 19 (84.2) | 34 (38.2) | 36 (50.0) | 36 (80.6) |
| 108 N and S | 26 (15.4) | 19 (5.3) | 34 (11.8) | 36 (0) | 36 (2.8) |
| S108 (Wt) | 26 (34.6) | 19 (10.5) | 34 (50.0) | 36 (50.0) | 36 (16.7) |
| 59R | 26 (50.0) | 19 (78.9) | 34 (26.5) | 36 (44.4) | 36 (77.8) |
| 59 R and C | 26 (7.7) | 19 (10.5) | 34 (11.8) | 36 (0) | 36 (2.8) |
| C59 (Wt) | 26 (42.3) | 19 (10.5) | 34 (61.8) | 36 (55.6) | 36 (19.4) |
| 51I | 26 (38.5) | 19 (63.2) | 34 (23.5) | 36 (44.4) | 35 (65.7) |
| 51 I and N | 26 (11.5) | 19 (5.3) | 34 (2.9) | 36 (0) | 35 (2.9) |
| N51 (Wt) | 26 (50.0) | 19 (31.6) | 34 (73.5) | 36 (55.6) | 35 (31.4) |
| 108N and 59R | 26 (57.7) | 19 (89.5) | 34 (38.2) | 36 (44.4) | 36 (80.6) |
| 108N and 59R and 51I | 26 (50.0) | 19 (68.4) | 34 (26.5) | 36 (44.4) | 35 (68.6) |
| <i>Pfdhps</i> | | | | | |
| 437G | 25 (4.0) | 24 (20.8) | 31 (0) | 36 (0) | 36 (8.3) |
| 437 G and A | 25 (0) | 24 (4.2) | 31 (0) | 36 (0) | 36 (5.6) |
| A437 (Wt) | 25 (96.0) | 24 (75.0) | 31 (100.0) | 36 (100.0) | 36 (86.1) |
| 540E | 25 (0) | 24 (4.2) | 31 (0) | 36 (0) | 36 (0) |
| 540 E and K | 25 (4.0) | 24 (0) | 31 (0) | 36 (0) | 36 (0) |
| K540 (Wt) | 25 (96.0) | 24 (95.8) | 31 (100.0) | 36 (100.0) | 36 (100.0) |
| 437G and 540E | 25 (0) | 24 (0) | 31 (0) | 36 (0) | 36 (0) |

**Pfcr*, *P. falciparum* chloroquine resistance transporter; *Pfdhfr*, *P. falciparum* dihydrofolate reductase; *Pfdhps*, *P. falciparum* dihydropteroate synthase.

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Appendix Table. Primer sequences and amplification conditions of the 6 microsatellite loci and *Pfcr*, *Pfdhfr*, and *Pfdhps* genes of *Plasmodium falciparum**

| Loci | GenBank accession no. | Size, bp | Hr | Primer ID | Primer sequences for first and second rounds of nested PCR (5' → 3') | Ta, °C |
|---------------|-----------------------|----------|----|--|--|------------|
| <i>Pf2689</i> | G37854 | 86 | 2 | Pf2689 PCR1 | TTA ACC TTA TAG CTT CAG AG TCT TCT TCA CTT ACA TTA AAG | 56 |
| | | | | Pf2689 PCR2 | TAT GCA CAC ACG TTT CTA 6-FAM—CTC CAA GGC ATT CAC GTA | 54 |
| 7A11 | G38831 | 92 | 7 | 7A11 PCR1 | ACA TAT TAT TTC TTC GTA A TTA TCT CTT CTC TGA GTA A | 53 |
| | | | | 7A11 PCR2 | ATG TGT AAG GAG ATA GTA TA 6-FAM—CAA CTT TCT CTT TTT AAA TAT TAC | 54 |
| <i>C4M79</i> | G42726 | 220 | 3 | C4M69 PCR1 | TTT TGT AGG AAC ATG TAA GGA GAC TAG CTC TAC AAT A | 53 |
| | | | | C4M69 PCR2 | TTT ATA TCA AGA ATG ACA ACC NED—TAG CAA CAA TAA ACA ATA TGG | 57 |
| <i>Pf2802</i> | G37818 | 136 | 5 | Pf2802 PCR1 | GAT GCT TAG TTT AAT CTT ATA ACA AAT A GAC TTA CTT TCT TAC ATA AAA TCA TTA AC | 60 |
| | | | | Pf2802 PCR2 | GTA TAA AAG GAA ATA CCT A NED—CAG ACT ATC TTA AGG GAA | 52 |
| <i>TRAP</i> | G37858 | 134 | 3 | TRAP PCR1 | ATA AAA CAA ATT ACC GAG TA ACA ATT CAG ATT ACC TGA A | 56 |
| | | | | TRAP PCR2 | CAT AAT AGT AGC AAG AGA PET—GAT TAT ATA TAG CGA TTT AC | 49 |
| <i>C4M69</i> | G37956 | 362 | 4 | C4M69 PCR1 | AGA AAT GGA GAT AAA CTA TTA CAA CTA AGC GCA CGA GAA CAA TC | 60 |
| | | | | C4M69 PCR2 | GAA ATG GAG ATA AAC TAT TAC VIC—AAT TAC ACA ACA GAT GTG AA | 61 |
| <i>Pfcr</i> | | | 7 | Pfcr PCR1 | GTT CTT GTC TTG GTA AAT GT CCA ATT TTG TTT AAA GTT CT | 50 then 45 |
| | | | | Pfcr PCR2 | GTT CTT GTC TTG GTA AAT GT 6-FAM—TAA ATG TGC TCA TGT GTTTA | 50 then 45 |
| <i>Pfdhfr</i> | | | 4 | <i>Pfdhfr</i> primers (template) | | |
| | | | | dhfr PCR1 | TTC TCC TTT TTA TGA TGG AAC AAG T ATA TTT GAA AAT CAT TTG GAT GTA TAG | 56 |
| | | | | dhfr PCR2 | ACG TTT TCG ATA TTT ATG C TCA CAT TCA TAT GTA CTA TTT ATT C | 47 |
| | | | | dhfr51-f dhfr16-r dhfr108-f dhfr164-r | <i>Pfdhfr</i> primers (SNaPshot) AGG AGT ATT ACC ATG GAA ATG TA gactgactCTC ATT TTT GCT TTC A AC CTT ACA ACA T gactgactACA AAA TGT TGT AGT TAT GGG AAG AAC AA ctgactgactgactgactAAT TCT TGA TAA ACA ACG GAA CCT CCT A | |
| | | | | dhfr59-r | ctgactgactgactgactgactTGA TTC ATT CAC ATA TGT TGT AAC TGC AC | |
| <i>Pfdhps</i> | | | 8 | <i>Pfdhps</i> primers (template) | | |
| | | | | dhps PCR1 | GATTCTTTTTTCAGATGGAGG TTCCTCATGTAATTCATCTGA | 52 |
| | | | | dhps PCR2 | GTT GAA CCT AAA CGT GCT GT TTC ATC ATG TAA TTT TTG TTG TG | 49 |
| | | | | dhps613-r dhps540-f dhps581-r dhps436(1)-r dhps436(2)-r dhps437-r | <i>Pfdhps</i> primers (SNaPshot) TTG ATC ATT CAT GCA ATG GG gactGAG GAA ATC CAC ATA CAA TGG AT TAA GAG TTT AAT AGA TTG ATC ATG TTT CTT C gactgactAGT GTT ATA GAT ATA GGT GGA GAA TCC gactgactgactgactTGG ATT AGG TAT AAC AAA AAG GAI CA gactgactgactgactTTTT TTG GAT TAG GTA TAA CAA AAG GA | |

*Size in basepairs for 3D7 clone. *Pfcr*, *P. falciparum* chloroquine resistance transporter; *Pfdhfr*, *P. falciparum* dihydrofolate reductase; *Pfdhps*, *P. falciparum* dihydropteroate synthase; Chr, chromosome. Primer sequences are given for reactions no. 1 (first round) and no. 2 (second round) of the nested PCRs and are 5' → 3' with fluorescent label (VIC, NED, 6-FAM or PET) and annealing temperature (Ta, °C). Thermocycling performed in a Biometra (Goettingen, Germany) 96-well T3 thermocycler.