

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 \approx 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

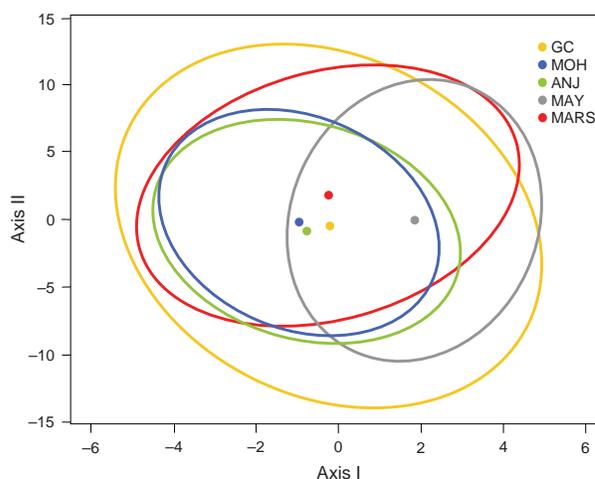


Figure 1. Results of canonical correspondence analysis (CCA) of *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. CCA is used as a 2-dimensional representation 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

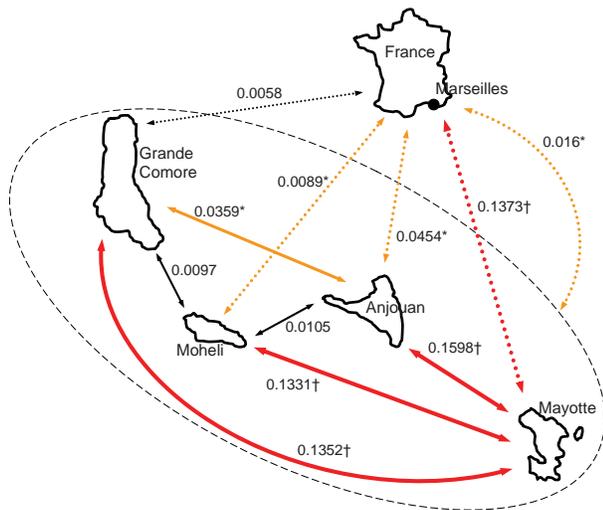


Figure 2. Genetic differentiation (F_{ST}) between *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. Pairwise comparison among sites that used complete dataset ($n = 281$) 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.

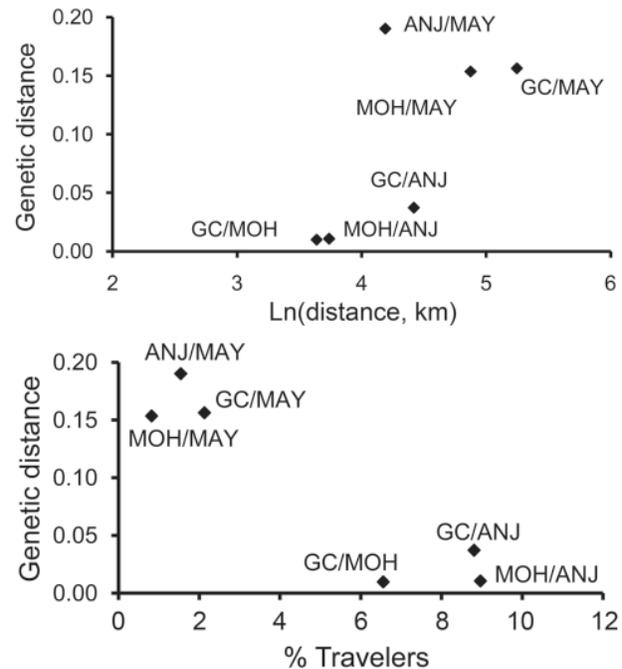


Figure 3. Relationship between geographic and genetic distances for each pair of Comoros islands (top) and between mean percentage of travelers among sampled patients and genetic distance for each pair of Comoros islands (bottom). Genetic distances were calculated as $(F_{ST}/1 - F_{ST})$, where F_{ST} 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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References

1. Roll Back Malaria Partnership. The global malaria action plan, for a malaria-free world. Geneva: World Health Organization; 2008 [cited 2010 Sep 5]. <http://www.rollbackmalaria.org/gmap/index.html>
2. World Health Organization. World malaria report 2009. Geneva: The Organization; 2009 [cited 2010 Sep 5]. <http://www.who.int/malaria/publications/atoz/9789241563901/en/index.html>
3. Rogier C, Pradines B, Bogreau H, Koeck JL, Kamil MA, Mercereau-Puijalon O. Malaria epidemic and drug resistance, Djibouti. *Emerg Infect Dis*. 2005;11:317–21.
4. Bogreau H, Renaud F, Bouchiba H, Durand P, Assi SB, Henry MC, et al. Genetic diversity and structure of African *Plasmodium falciparum* populations in urban and rural areas. *Am J Trop Med Hyg*. 2006;74:953–9.
5. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol*. 2000;17:1467–82.
6. Pumpaibool T, Arnathau C, Durand P, Kanchanakhan N, Siripoon N, Suegorn A, et al. Genetic diversity and population structure of *Plasmodium falciparum* in Thailand, a low transmission country. *Malar J*. 2009;8:155. DOI: 10.1186/1475-2875-8-155
7. Quatresous I, Pettinelli F, Le Bras J, Solet JL, Lepère JF, Giry C, et al. What do we know about malaria resistance in Mayotte, France, in 2007? [in French]. *Bulletin Epidémiologique Hebdomadaire*. 2007;48–49:409–12.
8. World Health Organization. World malaria report 2008. Geneva: The Organization; 2008 [cited 2010 Sep 5]. <http://malaria.who.int/wmr2008/malaria2008.pdf>

9. World Health Organization. Malaria control and elimination: report of a technical review. Geneva: The Organization; 2008 [cited 2010 Sep 5]. <http://www.who.int/malaria/publications/atoz/9789241596756/en/index.html>
10. Silai R, Moussa M, Abdalli Mari M, Astafieva-Djaza M, Hafidhou M, Oumadi A, et al. Surveillance of falciparum malaria susceptibility to antimalarial drugs and policy change in the Comoros [in French]. *Bull Soc Pathol Exot.* 2007;100:6–9. DOI: 10.3185/pathexo2898
11. Parola P, Pradines B, Simon F, Carlotti MP, Minodier P, Ranjeva MP, et al. Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates imported from Comoros to Marseille, France, 2004–2006. *Am J Trop Med Hyg.* 2007;77:431–7.
12. Anthony TG, Conway DJ, Cox-Singh J, Matusop A, Ratnam S, Shamsul S, et al. Fragmented population structure of *Plasmodium falciparum* in a region of declining endemicity. *J Infect Dis.* 2005;191:1558–64. DOI: 10.1086/429338
13. Iwagami M, Rivera PT, Villacorte EA, Escueta AD, Hatabu T, Kawazu S, et al. Genetic diversity and population structure of *Plasmodium falciparum* in the Philippines. *Malar J.* 2009;8:96. DOI: 10.1186/1475-2875-8-96
14. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, et al. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med.* 2001;344:257–63. DOI: 10.1056/NEJM200101253440403
15. Kublin JG, Dzinjalama FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis.* 2002;185:380–8. DOI: 10.1086/338566
16. Anderson TJ, Nair S, Jacobzone C, Zavai A, Balkan S. Molecular assessment of drug resistance in *Plasmodium falciparum* from Bahr El Gazal province, Sudan. *Trop Med Int Health.* 2003;8:1068–73. DOI: 10.1046/j.1360-2276.2003.01144.x
17. Nair S, Brockman A, Paiphun L, Nosten F, Anderson TJ. Rapid genotyping of loci involved in antifolate drug resistance in *Plasmodium falciparum* by primer extension. *Int J Parasitol.* 2002;32:852–8. DOI: 10.1016/S0020-7519(02)00033-4
18. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics.* 1978;89:583–90.
19. Belkhir K, Borsa P, Chikhi L, Raufaste L, Bonhomme F. GENETIX 4.05, Windows software for population genetics (author's translation) [in French]. Montpellier, France: Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II; 1996–2004 [cited 2010 Sep 5]. <http://www.genetix.univ-montp2.fr/genetix/genetix.htm>
20. Goudet J. FSTAT (version 2.9.3.2), a program to estimate and test population genetics parameters; 2002 [cited 2010 Sep 5]. <http://www2.unil.ch/popgen/softwares/fstat.htm>
21. Wright S. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution.* 1965;19:395–420. DOI: 10.2307/2406450
22. Weir B, Cockerham C. Estimating F-statistics for the analysis of population structure. *Evolution.* 1984;38:1358–70. DOI: 10.2307/2408641
23. Excoffier L, Laval G, Schneider S. ARLEQUIN (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online.* 2005;1:47–50.
24. Ter Braack C. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology.* 1986;67:1167–79. DOI: 10.2307/1938672
25. Ter Braack C, Šmilauer P. CANOCO. Fortran program for canonical community ordination [cited 2010 Sep 5]. <http://www.pri.wur.nl/UK/products/Canoco/>
26. Rousset F. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics.* 1997;145:1219–28.
27. Durand P, Michalakis Y, Cestier S, Oury B, Leclerc MC, Tibayrenc M, et al. Significant linkage disequilibrium and high genetic diversity in a population of *Plasmodium falciparum* from an area (Republic of the Congo) highly endemic for malaria. *Am J Trop Med Hyg.* 2003;68:345–9.
28. Razakandrainibe FG, Durand P, Koella JC, De Meeus T, Rousset F, Ayala FJ, et al. “Clonal” population structure of the malaria agent *Plasmodium falciparum* in high-infection regions. *Proc Natl Acad Sci U S A.* 2005;102:17388–93. DOI: 10.1073/pnas.0508871102
29. Annan Z, Durand P, Ayala FJ, Arnathau C, Awono-Ambene P, Sismard F, et al. Population genetic structure of *Plasmodium falciparum* in the two main African vectors, *Anopheles gambiae* and *Anopheles funestus*. *Proc Natl Acad Sci U S A.* 2007;104:7987–92. DOI: 10.1073/pnas.0702715104
30. Bonizzoni M, Afrane Y, Baliraine FN, Amenya DA, Githeko AK, Yan G. Genetic structure of *Plasmodium falciparum* populations between lowland and highland sites and antimalarial drug resistance in western Kenya. *Infect Genet Evol.* 2009;9:806–12. DOI: 10.1016/j.meegid.2009.04.015
31. Ferreira MU, Karunaweera ND, da Silva-Nunes M, da Silva NS, Wirth DF, Hartl DL. Population structure and transmission dynamics of *Plasmodium vivax* in rural Amazonia. *J Infect Dis.* 2007;195:1218–26. DOI: 10.1086/512685
32. Arieu F, Randrianarivojosia M, Duchemin JB, Rakotondramarina D, Ouledi A, Robert V, et al. Mapping of a *Plasmodium falciparum* Pfcrt K76T mutation: a useful strategy for controlling chloroquine resistance in Madagascar. *J Infect Dis.* 2002;185:710–2. DOI: 10.1086/339000
33. Randrianarivojosia M, Raherinjafy RH, Migliani R, Mercereau-Puijalon O, Arieu F, Bedja SA. *Plasmodium falciparum* resistant to chloroquine and to pyrimethamine in Comoros. *Parasite.* 2004;11:419–23.
34. Quatresous I, Jeannel D, Sissoko D. Epidémiologie du paludisme à Mayotte. Etat des lieux 2003–2004 et propositions. Paris, France: Institut de Veille Sanitaire; 2005.
35. Iyer JK, Milhous WK, Cortese JF, Kublin JG, Plowe CV. *Plasmodium falciparum* cross-resistance between trimethoprim and pyrimethamine. *Lancet.* 2001;358:1066–7. DOI: 10.1016/S0140-6736(01)06201-8
36. World Health Organization. Susceptibility of *Plasmodium falciparum* to antimalarial drugs: report on global monitoring: 1996–2004. Geneva: The Organization; 2005 [cited 2010 Sep 5]. http://www.malaria.org/ABOUT%20MALARIA/SusceptibilityPlasmodium_report%20WHO%202004.pdf
37. Talisuna AO, Langi P, Mutabingwa TK, Van Marck E, Speybroeck N, Egwang TG, et al. Intensity of transmission and spread of gene mutations linked to chloroquine and sulphadoxine-pyrimethamine resistance in falciparum malaria. *Int J Parasitol.* 2003;33:1051–8. DOI: 10.1016/S0020-7519(03)00156-5
38. Hastings IM, Watkins WM. Intensity of malaria transmission and the evolution of drug resistance. *Acta Trop.* 2005;94:218–29.

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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	<i>C4M69</i> PCR1	TTT TGT AGG AAC ATG TAA GGA GAC TAG CTC TAC AAT A	53
				<i>C4M69</i> PCR2	TTT ATA TCA AGA ATG ACA ACC NED—TAG CAA CAA TAA ACA ATA TGG	57
<i>Pf2802</i>	G37818	136	5	<i>Pf2802</i> PCR1	GAT GCT TAG TTT AAT CTT ATA ACA AAT A GAC TTA CTT TCT TAC ATA AAA TCA TTA AC	60
				<i>Pf2802</i> PCR2	GTA TAA AAG GAA ATA CCT A NED—CAG ACT ATC TTA AGG GAA	52
TRAP	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	<i>C4M69</i> PCR1	AGA AAT GGA GAT AAA CTA TTA CAA CTA AGC GCA CGA GAA CAA TC	60
				<i>C4M69</i> PCR2	GAA ATG GAG ATA AAC TAT TAC VIC—AAT TAC ACA ACA GAT GTG AA	61
<i>Pfcr</i>			7	<i>Pfcr</i> PCR1	GTT CTT GTC TTG GTA AAT GT CCA ATT TTG TTT AAA GTT CT	50 then 45
				<i>Pfcr</i> PCR2	GTT CTT GTC TTG GTA AAT GT 6-FAM—TAA ATG TGC TCA TGT GTTTA	50 then 45
<i>Pfdhfr</i>			4	dhfr PCR1	<i>Pfdhfr</i> primers (template) 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 <i>Pfdhfr</i> primers (SNaPshot) AGG AGT ATT ACC ATG GAA ATG TA	47
				dhfr51-f	gactgactCTC ATT TTT GCT TTC A AC CTT ACA ACA T	
				dhfr16-r	gactgactACA AAA TGT TGT AGT TAT GGG AAG AAC AA	
				dhfr108-f	ctgactgactgactgactAAT TCT TGA TAA ACA ACG GAA CCT CCT A	
dhfr164-r	ctgactgactgactgactgactTGA TTC ATT CAC ATA TGT TGT AAC TGC AC					
<i>Pfdhps</i>			8	dhps PCR1	<i>Pfdhps</i> primers (template) GATTCTTTTTTCAGATGGAGG TTCCTCATGTAATTCATCTGA	52
				dhps PCR2	GTT GAA CCT AAA CGT GCT GT TTC ATC ATG TAA TTT TTG TTG TG <i>Pfdhps</i> primers (SNaPshot) TTG ATC ATT CAT GCA ATG GG	49
				dhps613-r	gactGAG GAA ATC CAC ATA CAA TGG AT	
				dhps540-f	TAA GAG TTT AAT AGA TTG ATC ATG TTT CTT C	
				dhps581-r	gactgactAGT GTT ATA GAT ATA GGT GGA GAA TCC	
dhps436(1)-r	gactgactgactgactTGG ATT AGG TAT AAC AAA AAG GAI CA					
dhps436(2)-r	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.