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Plasmodium falciparum Genetic Diversity in Continental Equatorial Guinea before and after Introduction of Artemisinin-Based Combination Therapy

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ABSTRACT Efforts to control malaria may affect malaria parasite genetic variability and drug resistance, the latter of which is associated with genetic events that promote mechanisms to escape drug action. The worldwide spread of drug resistance has been a major obstacle to controlling *Plasmodium falciparum* malaria, and thus the study of the origin and spread of associated mutations may provide some insights into the prevention of its emergence. This study reports an analysis of *P. falciparum* genetic diversity, focusing on antimalarial resistance-associated molecular markers in two socioeconomically different villages in mainland Equatorial Guinea. The present study took place 8 years after a previous one, allowing the analysis of results before and after the introduction of an artemisinin-based combination therapy (ACT), i.e., artesunate plus amodiaquine. Genetic diversity was assessed by analysis of the *Pfmsp2* gene and neutral microsatellite loci. *Pfdhps* and *Pfdhfr* alleles associated with sulfadoxine-pyrimethamine (SP) resistance and flanking microsatellite loci were investigated, and the prevalences of drug resistance-associated point mutations of the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* genes were estimated. Further, to monitor the use of ACT, we provide the baseline prevalences of K13 propeller mutations and *Pfmdr1* copy numbers. After 8 years, noticeable differences occurred in the distribution of genotypes conferring resistance to chloroquine and SP, and the spread of mutated genotypes differed according to the setting. Regarding artemisinin resistance, although mutations reported as being linked to artemisinin resistance were not present at the time, several single nucleotide polymorphisms (SNPs) were observed in the K13 gene, suggesting that closer monitoring should be maintained to prevent the possible spread of artemisinin resistance in Africa.

KEYWORDS ACT introduction, drug resistance molecular markers, Equatorial Guinea, genetic diversity, malaria, neutral and flanking microsatellites, *Plasmodium falciparum*, *Pfcrt*, *Pfdhps*, *Pfdhfr*, *Pfmdr1*, Kelch propeller protein K13 polymorphisms

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Malaria control has highly progressed globally, but this parasitic disease persists as one of the deadliest in the world, with 584,000 deaths recorded in 2013, particularly in Africa, where 90% of fatal cases occur (1). In Equatorial Guinea, *Plasmodium* infections are among the leading causes of disease, with an incidence rate of 24,767 cases per 100,000 people, and they are the cause of 15% of mortality among children under 5 years of age (2).

In a previous study, the circulating populations of *Plasmodium* spp. and *Plasmodium falciparum* in blood isolates collected in 2005 at two villages, located 80 km from each

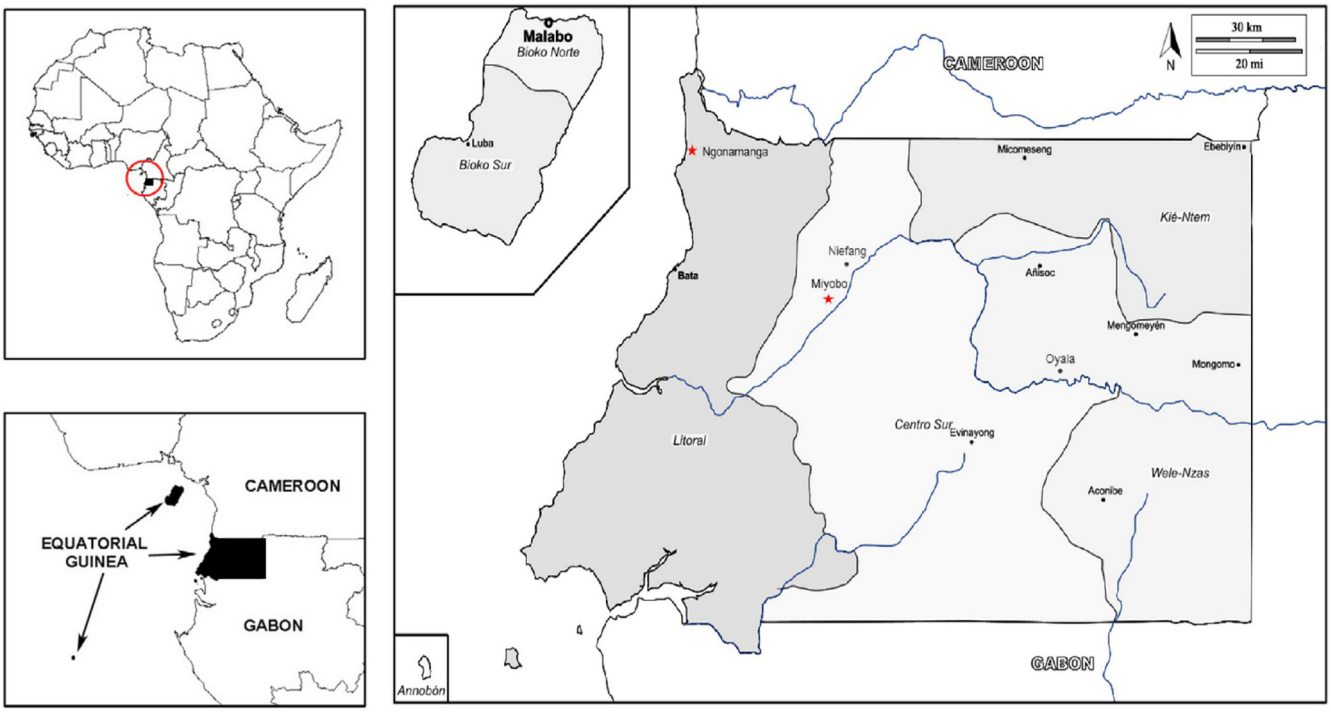

FIG 1 Map of Equatorial Guinea with study areas. The country's capital is Malabo, located on Bioko Island. Sampling took place in two villages of mainland Equatorial Guinea: Ngonamanga and Miyobo (red stars). Ngonamanga (Litoral Province; 02°09′34.5″N, 009°47′54.4″E) is a coastland village, isolated from the main trade routes and with an older population, whereas Miyobo (Centro Sur Province; 01°44′56.40″N; 10°10′40.05″E) is a village in the interior, but closer to the developing city Niefang, near a main road, and with a younger population. (Adapted from http://d-maps.com/.)

other (Fig. 1) and both situated in mainland Equatorial Guinea, were characterized (3). *Plasmodium falciparum* diversity was described through the analysis of *Pfmsp2*, a set of neutral microsatellite loci, also referred to as short tandem repeats (STRs), and four antimalarial resistance-associated genes (*Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps*); the dissemination of *P. falciparum* drug resistance-associated alleles was also investigated.

In 2005, in mainland Equatorial Guinea, chloroquine (CQ) was, and continued to be until 2009, the first-line treatment for uncomplicated malaria (4). The combination sulfadoxine (SFX)-pyrimethamine (PYR) (SP) was introduced in 2004 and is presently used as intermittent preventive therapy during pregnancy (IPTp). After 2009, artemisinin-based combination therapy (ACT) with artesunate plus amodiaquine (AS+AQ) was adopted as a first-line treatment (5), which is still recommended for Equatorial Guinea by the WHO (1) (Fig. 2). However, Romay-Barja et al. (6) reported that artemether monotherapy is the antimalarial treatment most often prescribed for

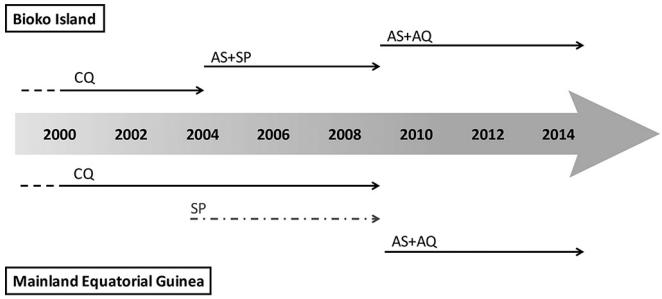

FIG 2 Chronology of antimalarial drug policies adopted on Bioko Island and mainland Equatorial Guinea. On the mainland, SP was presumably adopted at the same time as that on Bioko Island (gray dashed arrow). CQ, chloroquine; SP, sulfadoxine-pyrimethamine; AS+SP, artesunate plus sulfadoxine-pyrimethamine; AS+AQ, artesunate plus amodiaquine. The figure is based on data from the work of Rehman et al. (4), Charle et al. (5), and the WHO (1).

children of up to 5 years of age in Bata District (urban and rural areas), followed by paracetamol and SP.

Furthermore, in 2007, the Equatorial Guinea Malaria Control Initiative (EGMCI) was implemented on the mainland, following the success obtained on Bioko Island, where it was introduced in 2004 (7). This program included vector control interventions (large-scale distribution of long-lasting insecticide-treated nets [LLIN] in Centro Sur and Wele-Nzas Provinces and indoor residual spraying in Litoral and Kie-Ntem Provinces), effective case management, and extensive education and communication (4, 5). Despite the initial success, the EGMCI was discontinued in 2011 due to funding limitations, so the malaria prevalence remained high (4, 5).

Over the years, the expansion of *P. falciparum* strains able to survive under adverse conditions, such as drug pressure, has compromised the efficacy of conventional treatments. Drug resistance is associated with genetic events that promote mechanisms to escape drug action, such as mutations or changes in the gene copy number that can be related to the drug target or can affect pumps regulating the within-parasite drug concentration (8). Studying the origin and spread of mutations associated with drug resistance may provide important insights into preventing the emergence of resistance, especially in the context of combination therapies (9).

In 2005, before the change of the malaria therapeutic regimen to ACT, the prevalences in mainland Equatorial Guinea of the main point mutations associated with CQ resistance, i.e., N75E and K76T mutations of the *Pfcrt* gene and N86Y and D1246Y mutations of the *Pfmdr1* gene, were 56%, 72%, 84%, and 1%, respectively. Regarding SP, the prevalence of a *Pfdhfr* triple mutant (N51I/C59R/S108N [mutant amino acids are underlined]) (IRN mutant) was nearly 80%, and a quintuple mutant (*Pfdhfr* triple mutant plus *Pfdhps* A437G/K540E double mutant [GE mutant]) was absent (3). The analysis of *Pfdhfr*- and *Pfdhps*-flanking STRs revealed a decrease in genetic diversity, and this finding, along with multiple independent introductions of *Pfdhps* mutant haplotypes, suggested a soft selective sweep. Increased differentiation at *Pfdhfr*-flanking STRs hinted at a model of positive directional selection for this gene.

The present study intended to analyze the evolution of *P. falciparum* genetic diversity, with a special focus on antimalarial resistance-associated alleles at the same sites, 8 years after the first analysis and after ACT implementation as the first-line treatment. Genetic diversity was estimated by analysis of the *Pfmsp2* gene and neutral STRs. Again, SP resistance-associated *Pfdhfr* and *Pfdhps* alleles and STRs flanking loci were investigated. Also, the prevalences of drug resistance-associated point mutations of the *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps* genes were estimated.

Furthermore, considering the introduction and use of ACT, we also analyzed the PF3D7_1343700 gene, which encodes the Kelch propeller protein K13, recently identified as a molecular marker associated with *P. falciparum* artemisinin resistance (the single nucleotide polymorphisms [SNPs] Y493H, R539T, I543T, and C580Y were linked to delayed parasite clearance both *in vitro* and *in vivo*) (10, 11). In 2005, the WHO recommended that ACT be used as the first-line treatment for *P. falciparum* malaria, replacing SP in nearly all countries where malaria is endemic. However, in 2008, Noedl et al. (12) confirmed the first cases of *P. falciparum* resistance to artemisinin derivatives in Southeast Asia (where it was introduced for the first time in the mid-1990s), but so far it has not been detected in Africa (13). Since artemisinin resistance constitutes a worrying threat to the global effort, the WHO has taken measures to monitor its possible spread from Asia to Africa for early detection of its possible emergence there. In particular, the WHO established containment projects in hot spot regions (14) and recommends also monitoring African isolates through genotyping of the K13 locus (15). Talundzic et al. (16) recently reported an analysis of the K13 propeller artemisinin resistance mutations and flanking microsatellite loci in parasites collected in Thailand shortly before the implementation of the artemisinin resistance containment project. In this study, we provide a baseline prevalence of K13 polymorphisms as well as an analysis of the *Pfmdr1* copy numbers in Equatorial Guinea before and after the introduction of ACT.

This analysis allows a better understanding of how changes in drug pressure, intensity of malaria transmission, premunition, and even socioeconomic conditions influence the evolution of molecular markers for antimalarial drug resistance.

RESULTS

Detection and identification of *Plasmodium* species. A total of 232 individuals were analyzed (63 in Ngonamanga and 169 in Miyobo), with ages ranging from 6 months to 99 years. The age distributions are not similar for the two locations ($P = 0.001$; chi-square test): older ages are overrepresented in Ngonamanga, whereas in Miyobo, different age groups have a homogeneous distribution. Similar distributions of males and females occur in both locations ($P = 0.072$; Fisher's exact test) (see Table S1 in the supplemental material).

The prevalence of *Plasmodium* sp. infection determined by nested PCR was 71.4% for Ngonamanga and 68.0% for Miyobo, i.e., higher than those determined by optical microscopy (OM), which were 45.6% and 55.6%, respectively, and those determined by a rapid diagnostic test (RDT), which were 49.1% and 55.6%, respectively. Forty-eight samples were found to be positive by PCR and negative by OM, and on subsequent analyses, these were assigned to the parasitemia group with <800 parasites/μl. Overall, we identified three *Plasmodium* species: *P. falciparum*, *Plasmodium malariae*, and *Plasmodium ovale*. *Plasmodium falciparum* was the predominant species, occurring in 98% of the isolates from Ngonamanga and 96% of those from Miyobo, with 21% of infections being mixed (Table S1).

All *P. falciparum*-positive samples, from single or mixed infections, were selected for further genotyping and included 44 samples from Ngonamanga and 110 from Miyobo.

Point mutations of *Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps*. The prevalences of *Pfmdr1*, *Pfcrt*, *Pfdhps*, and *Pfdhfr* mutations in isolates from Ngonamanga and Miyobo are shown in Fig. 3.

The prevalences of the 75E and 76T mutant alleles of the *Pfcrt* gene, in either single or mixed infections, were higher in Miyobo than in Ngonamanga ($P = 0.008$ and $P = 0.002$, respectively). The *Pfcrt* 76T mutant was significantly more prevalent in individuals of younger ages (≤15 years) in both villages ($P = 0.023$ for Miyobo and $P = 0.001$ for Ngonamanga). Regarding *Pfmdr1*, the prevalence of the mutant allele 86Y was also higher in Miyobo (87%) than in Ngonamanga (52%) ($P < 0.001$), and the opposite occurred with the mutant allele 1246Y, which was more prevalent in Ngonamanga (17%) than in Miyobo (4%) ($P = 0.013$). The prevalence of the *Pfmdr1* 86Y and *Pfcrt* 76T haplotype was 17% for Ngonamanga and 58% for Miyobo ($P = 0.003$). The prevalence of this haplotype was also determined in 2005, before ACT introduction, and it was 69% for Ngonamanga and 60% for Miyobo ($P = 0.108$). The mutant alleles linked to PYR resistance, i.e., the *Pfdhfr* 51I, 59R, and 108N alleles, showed prevalences above 90% in both villages. The most prevalent mutation was S108N, which reached fixation in both parasite populations (100%). No mutations were found in codon 164. The *Pfdhfr* triple mutant (IRN mutant), associated with PYR clinical failure, was present in both villages, with prevalences of 90% and 97% in Ngonamanga and Miyobo, respectively (Table 1).

Concerning the *Pfdhps* gene, which is associated with SFX resistance, the 436A mutant allele was found only in mixed infections and was more prevalent in Miyobo (32%) than in Ngonamanga (16%) ($P = 0.033$). The 437G mutant allele was present at high prevalences in both parasite populations, and the 540E and 581G mutations showed prevalences below 10%. The *Pfdhps* double mutant (GE mutant) associated with SFX clinical failure was found in 5% and 10% of the isolates in Ngonamanga and Miyobo, respectively (Table 1).

The *Pfdhfr Pfdhps* quintuple mutant (IRN/GE mutant), associated with SP treatment failure in Africa, occurred in 7% of the total number of isolates, 5% of those from Ngonamanga, and 8% of those from Miyobo (Table 1).

A comparison of the present data and the data collected in 2005 is presented in Fig. S1.

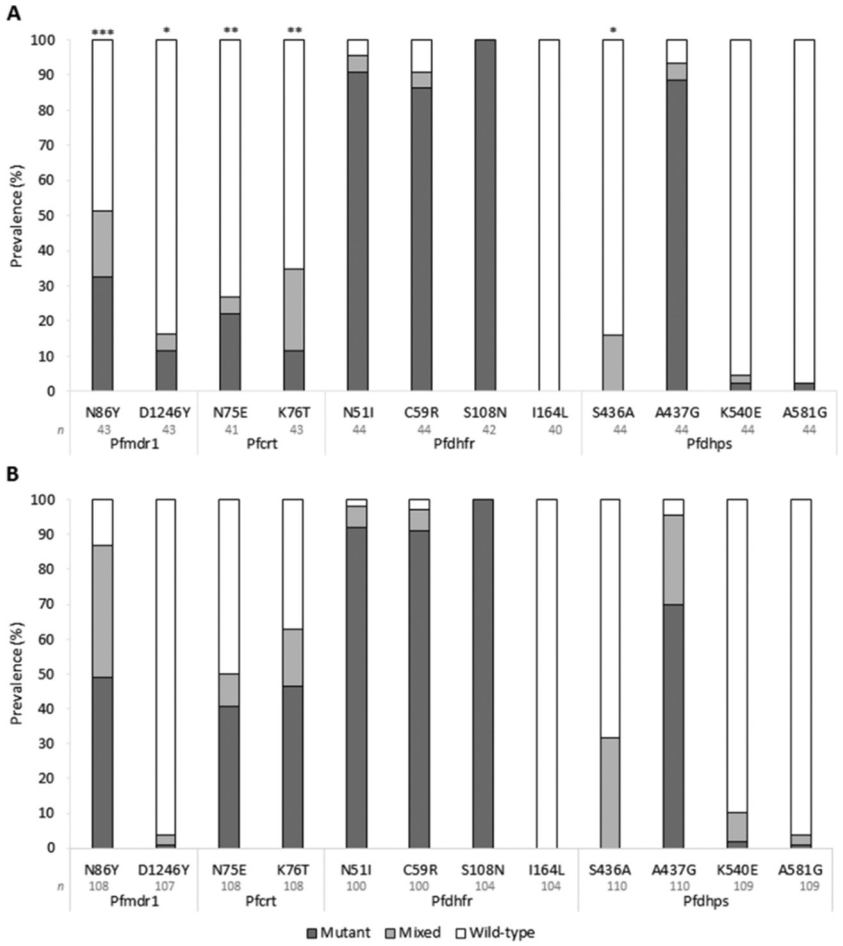

FIG 3 Total prevalences of mutations in the *Pfmdr1*, *Pfcrt*, *Pfdhfr*, and *Pfdhps* genes in Ngonamanga (A) and Miyobo (B). The *Z* test for comparison of two proportions was used to compare data between villages. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Kelch propeller protein K13 locus. We sequenced the gene encoding the K13 propeller domain in 144 *P. falciparum*-infected samples from both single and mixed infections, including 58 samples collected in 2005 (29 from Ngonamanga and 29 from Miyobo, before the implementation of ACT) and 86 samples collected in 2013 (27 from Ngonamanga and 59 from Miyobo, after the implementation of ACT).

Table 2 shows the distributions of K13 propeller polymorphisms in both collections for each village.

TABLE 1 Allele and genotype frequencies of the *Pfdhps* and *Pfdhfr* genes in Ngonamanga and Miyobo[a]

Population	Frequency of genotype							
	Pfdhfr				*Pfdhps*			*Pfdhfr/Pfdhps*
	WT (NCS)	Single mutant (NCN)	Double mutant (ICN)	Triple mutant (IRN)	WT (AK)	Single mutant (GK)	Double mutant (GE)	Quintuple mutant (IRN/GE)
Ngonamanga	$n = 42$				$n = 44$			$n = 42$
	0.00	0.05	0.05	0.90	0.07	0.89	0.05	0.05
Miyobo	$n = 98$				$n = 109$			$n = 98$
	0.00	0.02	0.01	0.97	0.05	0.85	0.10	0.08

[a]*n* values show the total numbers of alleles or genotypes. Point mutations in the *Pfdhfr* genotype were N51I, C59R, and S108N, and those in the *Pfdhps* genotype were A437G and K540E. Mutant amino acids are underlined in the genotypes.

TABLE 2 *Plasmodium falciparum* K13 propeller polymorphisms observed in Ngonamanga and Miyobo in 2005 and 2013[d]

Yr	Village (*n*)	Codon	Type	Wild-type aa (nt)	Mutant aa (nt)	No. of samples carrying mutant allele	PROVEAN score (type of effect)[a]
2005	Ngonamanga (29)	**588**	**NS**	Y (tat)	C (tGt)	1	−6.594 (D)
		609	Syn	B (aat)	B (Gat)	1	
		611	Nonsense	W (tgg)	STOP (tgA)	1	
	Miyobo (29)	**556**[c]	**NS**	E (gaa)	K (Aaa)	1	−3.904 (D)
2013	Ngonamanga (27)	**578**[b]	**NS**	A (gct)	S (Tct)	1	−1.962 (N)
		637[c]	**NS**	V (gtt)	I (Att)	1	−0.111 (N)
	Miyobo (59)	471[b]	Syn	R (cgt)	R (cgC)	1	
		578[b]	**NS**	A (gct)	S (Tct)	1	−1.962 (N)
		621	Syn	A (gct)	A (gcA)	1	
		641	**NS**	D (gat)	G (gGt)	1	−5.498 (D)

[a]Neutral (N) and deleterious (D) effects on protein function were predicted with a cutoff score of −2.5.
[b]The A578S (11, 56, 57) and R471R (54) SNPs have been identified previously.
[c]Different SNPs in the same codon have been described previously, i.e., E556 (16) and V637 (54, 56).
[d]NS, nonsynonymous mutation (shown in bold); Syn, synonymous mutation; aa, amino acid; nt, nucleotides.

Compared to *P. falciparum* clone 3D7, for samples collected in 2005, a total of four different mutations (E556K, Y588C, B609B, and W611STOP) were detected in 3 of 58 samples (5.2%). The synonymous polymorphism B609B and the W611STOP polymorphism, containing a premature stop codon (nonsense), were present in the same isolate from Ngonamanga.

In 2013, a total of five different mutations (R471R, A578S, A621A, V637I, and D641G) were found in 6 of 86 isolates (7%).

Functional assessment of the nonsynonymous polymorphisms detected was performed by using the PROVEAN software tool, with a cutoff score of −2.5 (Table 2). The A578S and V637I SNPs were associated with neutral effects on protein biological function, but deleterious effects were predicted for the E556K (score = −3.904), Y588C, and D641G polymorphisms.

***Pfmdr1* copy number.** The *Pfmdr1* copy number was evaluated in 25 samples collected in Ngonamanga in 2005 and 64 samples collected in 2013 (22 from Ngonamanga and 42 from Miyobo). In 2005, 12 isolates (48%) showed a single copy and 13 isolates had two copies of the *Pfmdr1* gene. In 2013, 49 isolates showed one copy (76%; 17 samples from Ngonamanga and 32 samples from Miyobo) and 15 isolates had two copies (24%; 5 samples from Ngonamanga and 10 samples from Miyobo).

Concerning the presence of the mutant allele 86Y (in single or mixed infections) together with *Pfmdr1* amplification, we found prevalences of 23% and 21% for Ngonamanga and Miyobo, respectively.

Multiplicity of *P. falciparum* infection. Amplification of the *Pfmsp2* repeat region was successful for 79% (121/154 samples) of the *P. falciparum*-positive samples. The IC allelic family was more frequent than the FC27 family, with prevalences of 80% versus 66%, respectively, in Ngonamanga and 69% versus 50%, respectively, in Miyobo. Fourteen and 12 distinct alleles were observed for the IC and FC27 families, respectively. In Ngonamanga, 8 allelic variants associated with the IC type and 7 associated with the FC type were detected, while in Miyobo, 14 and 9 variant alleles corresponding to the IC and FC types were observed. Eight of the IC variants and four of the FC27 variants occurred in both villages.

Fifty-six percent and 73% of the isolates from Ngonamanga and Miyobo, respectively, harbored mixed *Pfmsp2* genotypes, consistent with the results obtained by the neutral STR analysis, i.e., 51% and 66% of isolates from the same villages.

The multiplicity of infection (MOI) varied with age, with higher values associated with younger ages (6 to 15 years), significantly in Miyobo for both markers and in Ngonamanga only for comparing the values obtained by STR analysis. In both analyses, there were no statistically significant differences in MOI values among the 3 levels of parasitemia (Table 3). Although there is a clear difference in age groups between both villages (Table S1), MOI values between villages were not statistically significant in the

TABLE 3 Distribution of MOI values determined by *Pfmsp2* gene and neutral STR analyses, according to study area, age, and parasitemia level

Variable	*Pfmsp2* analysis		Neutral STR analysis	
	Mean MOI (median,[a] range)	Significance	Mean MOI (median,[a] range)	Significance
Village				
Ngonamanga	2.08 (2, 1–4)	0.747[b]	2.59 (2, 1–5)	0.048[b]
Miyobo	2.20 (2, 1–5)		2.33 (2, 1–6)	
Variables for Ngonamanga				
Age (yr)				
0–5	2.33 (2, 2–3)	0.296[c]	3.33 (3, 3–4)	0.003[c]
6–15	2.50 (2.5, 1–4)		3.67 (4, 2–5)	
>15	1.96 (2, 1–4)		2.34 (2, 1–4)	
Parasitemia				
Low	2.03 (2, 1–4)		2.51 (2, 1–4)	
Moderate	2.33 (2, 2–3)	0.470[c]	2.67 (3, 2–3)	0.185[c]
High	2.5 (2.5, 2–3)		4 (4, 3–5)	
Variables for Miyobo				
Age (yr)				
0–5	2.10 (2, 1–4)	0.001[c]	2.21 (2, 1–6)	<0.001[c]
6–15	2.73 (2.5, 1–5)		2.97 (3, 1–6)	
>15	1.79 (2, 1–5)		1.94 (2, 1–4)	
Parasitemia				
Low	2.15 (2, 1–5)	0.217[c]	2.28 (2, 1–6)	0.555[c]
Moderate	3.25 (3, 2–5)		2.75 (2.5, 2–4)	
High	2.50 (2.5, 2–3)		2.00 (2, 2)	
Variables for total population				
Age (yr)				
0–5	2.13 (2, 1–4)		2.33 (2, 1–6)	
6–15	2.69 (2.5, 1–5)	<0.001[c]	3.07 (3, 1–6)	<0.001[c]
>15	1.87 (2, 1–5)		2.10 (2, 1–4)	
Parasitemia				
Low	2.11 (2, 1–5)		2.37 (2, 1–6)	
Moderate	2.86 (2, 2–5)	0.133[c]	2.71 (3, 2–4)	0.287[c]
High	2.50 (2.5, 2–3)		3.00 (2.5, 2–5)	

[a]Median used in Mann-Whitney test.
[b]Mann-Whitney test.
[c]Kruskal-Wallis test.

Pfmsp2 analysis ($P = 0.747$) and were barely significant in the neutral STR analysis ($P = 0.048$). The lack of significance of these results is due mostly to the small numbers of samples from children in Ngonamanga (3 from children of 0 to 5 years and 6 from those of 6 to 15 years) and/or the higher molecular resolution of STR analysis, which is more efficient for determining the MOI.

Analysis of *Pfdhfr*- and *Pfdhps*-flanking STRs and haplotype characterization. Haplotypes were assembled for 57 of 140 isolates genotyped for *Pfdhfr*-flanking STRs (Table S2) and 37 of 153 isolates genotyped for *Pfdhps*-flanking STRs (Table S3).

For the loci flanking the *Pfdhfr* gene, 15 different haplotypes were detected. The H1 haplotype was the most prevalent in both populations (34 of 57 samples), and it was associated with the *Pfdhfr* triple mutant (IRN mutant), consisting of 113/183/210 bp at the loci at 0.3/4.4/5.3 kb. This haplotype matched the microsatellite composition of the *P. falciparum* K1 laboratory strain used as a control.

Only the parasite populations with the H2 and H5 haplotypes carried the double mutant alleles (ICN). The remaining haplotypes carried triple *Pfdhfr* mutations (IRN). The H2 and H4 haplotypes, found in Ngonamanga, had the same microsatellite composition, i.e., 113/185/210 bp at the loci at 0.3/4.4/5.3 kb, but carried double (ICN) and triple (IRN) *Pfdhfr* mutations, respectively. Similarly, in Miyobo, the H5 and H11 haplotypes presented double and triple *Pfdhfr* mutations, respectively, in association with the STR combination of 107/183/200 bp at the loci at 0.3/4.4/5.3 kb. We did not find any

TABLE 4 Allele richness (Rs), expected heterozygosity (He), and F-statistics (F_{ST}) estimates at flanking and neutral microsatellites for wild-type and mutant (single and double) genotypes at the *Pfdhps* locus in *P. falciparum* populations[a]

	Value for indicated sample and population											
	Ngonamanga		Miyobo									
	Single mutant (GK) (n = 39)		Wild type (AK) (n = 5)		Single mutant (GK) (n = 93)		Double mutant (GE) (n = 11)		All samples (n = 109)			
Microsatellite	Rs	He	Rs	He	Rs	He	Rs	He	Rs	He	F_{ST}	P
Pfdhps gene-flanking loci												
0.8 kb	6	0.69	3	0.73	3	0.69	3	0.68	3	0.70	0.018	0.001
4.3 kb	6	0.69	3	0.67	3	0.68	2	0.59	3	0.65	0.071	<0.001
7.7 kb	8	0.71	2	0.63	3	0.70	2	0.62	3	0.65	0.082	<0.001
All loci	7	0.70	3	0.67	3	0.69	2	0.63	3	0.66	0.057	<0.001
Pfdhfr gene-flanking loci												
0.3 kb	5	0.61	3	0.75	2	0.66	3	0.69	2	0.70	0.001	NS
4.4 kb	4	0.57	3	0.71	2	0.57	2	0.56	2	0.61	0.036	0.038–0.037
5.3kb	4	0.63	3	0.75	2	0.59	2	0.59	2	0.64	0.011	NS
All loci	4	0.60	3	0.74	2	0.60	2	0.61	2	0.65	0.015	NS
Neutral loci												
Pfg377	6	0.67	2	0.50	2	0.65	3	0.68	2	0.61	−0.004	NS
PfPK2	10	0.73	2	0.50	3	0.72	3	0.72	3	0.65	0.008	NS
TAA109	8	0.71	3	0.67	3	0.70	3	0.70	3	0.69	0.008	NS
TAA87	9	0.72	3	0.67	3	0.71	3	0.69	3	0.69	0.007	NS
TAA81	9	0.72	3	0.67	3	0.71	3	0.71	3	0.70	−0.002	NS
TAA42	6	0.65	3	0.67	2	0.59	2	0.59	2	0.62	0.019	NS
ARA2	7	0.68	3	0.73	3	0.70	2	0.65	3	0.69	0.017	NS
TA102	9	0.72	3	0.71	3	0.72	3	0.68	3	0.70	0.001	NS
TA1	9	0.72	2	0.50	3	0.71	3	0.71	3	0.64	0.012	NS
All loci	8	0.70	2	0.62	3	0.69	3	0.68	3	0.66	0.007	NS

[a]The *Pfdhps* genotype was A437G/K540E (mutant amino acids are underlined). *n*, number of isolates genotyped. "All loci" refers to all-locus Rs and He values and the global F_{ST} over all loci as calculated by FSTAT. *P* values were determined by permutation tests to assess the significance of F_{ST} values. NS, nonsignificant ($P > 0.05$).

parasite carrying the wild-type allele at the positions of the three point mutations of the *Pfdhfr* gene.

Regarding the *Pfdhps* gene, 31 distinct haplotypes were found, with the H1, H2, and H5 to H8 haplotypes occurring in both locations. As mentioned before, both the SAK haplotype and alleles that contain the S436A mutation alone ($\underline{A}_{436}A_{437}K_{540}A_{581}$) (the mutant amino acid is underlined) are considered to be the wild type. Three haplotypes, H11, H22, and H23, were associated with the wild-type allele (AAKA). The majority of the haplotypes found (18 different haplotypes) were associated with the single mutant allele S<u>G</u>KA (n = 29). The haplotypes that carried the K540E mutation (H7 and H31) shared the microsatellite profile of 131/103/108 bp at the loci at 0.8/4.3/7.7 kb, matching that of the K1 strain.

Genetic diversity and linkage disequilibrium (LD). Microsatellite estimates for parasites carrying wild-type or mutant alleles at the *Pfdhps* loci are shown in Table 4. Mutant parasites, especially double mutants, presented lower levels of genetic diversity at flanking loci than the wild-type ones. Genetic differentiation was higher at loci flanking the *Pfdhps* gene (F_{ST} = 0.057; $P < 0.001$) than at neutral loci (F_{ST} = 0.007; P = 0.205) or *Pfdhfr*-linked loci (F_{ST} = 0.015; P = 0.228). The levels of genetic diversity were similar for neutral and *Pfdhfr*-flanking loci. For Ngonamanga, only single mutants were tested, and the heterozygosity (He) results were similar to those for the single mutants from Miyobo.

All the analyzed parasites harbored at least one *Pfdhfr* mutant allele, with the triple mutant predominating in both locations (Table 5). Lower values for genetic diversity were found for loci flanking the *Pfdhfr* gene (He = 0.59 for Ngonamanga and 0.60 for Miyobo) than for neutral or *Pfdhps*-flanking loci (He = 0.70 for Ngonamanga and 0.69 for Miyobo).

TABLE 5 Allele richness (Rs) and expected heterozygosity (He) estimates at flanking and neutral STRs for triple mutant genotypes at the *Pfdhfr* locus in *P. falciparum* populations[a]

	Value for triple mutant (IRN) in sampled population			
	Ngonamanga (n = 38)		Miyobo (n = 95)	
Microsatellite	Rs	He	Rs	He
Pfdhfr gene-flanking loci				
0.3 kb	4	0.60	5	0.65
4.4 kb	4	0.55	4	0.57
5.3kb	4	0.62	4	0.59
All loci	4	0.59	5	0.60
Pfdhps gene-flanking loci				
0.8 kb	7	0.69	6	0.69
4.3 kb	6	0.69	5	0.68
7.7 kb	8	0.71	8	0.71
All loci	7	0.70	6	0.69
Neutral loci				
Pfg377	6	0.67	5	0.64
PfPK2	9	0.73	9	0.72
TAA109	7	0.71	7	0.70
TAA87	8	0.71	7	0.71
TAA81	9	0.72	7	0.70
TAA42	6	0.65	4	0.59
ARA2	6	0.67	7	0.70
TA102	8	0.72	8	0.71
TA1	8	0.72	8	0.71
All loci	8	0.70	7	0.69

[a]The *Pfdhfr* genotype was N51I/C59R/S108N. *n*, number of isolates genotyped. "All loci" refers to all-locus Rs and He values calculated by FSTAT.

Exact tests of linkage disequilibrium were performed for all pairs of flanking and neutral loci on each mutant group linked to the *Pfdhps* gene and the triple mutant associated with the *Pfdhfr* gene (Table S4). In Miyobo, for the *Pfdhps* gene, three significant associations between pairs of STR loci were detected among 240 tests for the single mutant group, and only one association occurred between two flanking loci (0.8 kb and 4.3 kb). Among the isolates carrying *Pfdhfr* resistance alleles, three significant associations between STRs were found among 105 tests; also, only a single significant association involving flanking microsatellites (loci at 0.3 kb and 5.3 kb) was detected. In Ngonamanga, only one significant association was found among 105 tests. This was detected in the group of the *Pfdhfr* triple mutant and involved the STRs flanking the *Pfdhps* gene (loci at 7.7 kb and 0.8 kb).

DISCUSSION

The mainland Equatorial Guinea population is exposed to one of the highest levels of malaria infection in the world (17). The present study demonstrated that malaria prevalence continues to be substantially high, with a 69% prevalence of *Plasmodium* sp. infection in mainland Equatorial Guinea, mainly due to *P. falciparum* (96% of infections), which has developed resistance to all antimalarial drugs developed over time. The use of artemether monotherapy for *Plasmodium* infection is usual in this country (6), and it fosters the spread of resistance to artemisinin that can compromise the long-term usefulness of ACTs. Nevertheless, little attention has been given to the monitoring of evolution and changes in genetic diversity of *P. falciparum* populations regarding drug resistance markers. The last publication on the subject, to our knowledge, was in 2005, before the introduction of ACT (3).

Although recently declared a high-income country (and no longer eligible for funding from the Global Fund), Equatorial Guinea does not have a national malaria control program, which poses a serious public health challenge for the mainland region

and is an obstacle to malaria elimination on Bioko Island due to the increasing number of travelers from the mainland (18).

Aiming to repeat the success achieved on Bioko Island by the malaria control program implemented in 2004 (7), the same control measures were introduced on the mainland in 2007, under the EGMCI. Some moderate positive impact was attained, especially for the group of children aged 2 to 14 years, reducing the incidence of *P. falciparum* infection from 72% to 61% between 2007 and 2011, but the program was discontinued (4). Considering only this age group, the present study reports a much higher prevalence of *Plasmodium* infection (87% [55/63 samples]) that is comparable to the overall prevalence of 87% in 2005, before the introduction of ACT in this region (3).

The withdrawal of CQ as a first-line treatment led to some regression of CQ resistance in certain areas in Africa, such as Malawi and Kenya (19, 20), and also in Far East regions, such as China and Vietnam (21–23). Nevertheless, in Cameroon, high prevalences of the 76T (83%) and 86Y (93%) mutations were observed after the replacement of CQ by AQ monotherapy in 2002 and by ACT (AS+AQ) in 2004 (24). In fact, AQ and its active metabolite desethylamodiaquine (DEAQ) are correlated with CQ and promote the maintenance of CQ-resistant isolates through active drug pressure (25). Although resistance to CQ, AQ, and DEAQ has been associated with the 76T mutant genotype of *Pfcrt* and the 86Y mutant genotype of *Pfmdr1* (26), AQ/DEAQ efficacy has remained despite the similarities and putative cross-resistance to CQ. Froberg et al. (27) reported that the *Pfmdr1* 1246Y mutation was also associated with low susceptibility to AQ and DEAQ but had a significant fitness cost to the parasite.

In Equatorial Guinea, the same trend seems to occur especially in Ngonamanga, with a decrease of 43% in the prevalence of the 76T mutant allele of *Pfcrt* ($P < 0.001$) from 2005 to 2013. The prevalence of the 86Y allele of *Pfmdr1* also decreased significantly in Ngonamanga, from 85% in 2005 to 51% in 2013, but it remained high in Miyobo (84% in 2005 versus 87% in 2013). Conversely, the mutant allele 1246Y, which was absent in Ngonamanga in 2005, was later present in 16% of the isolates, whereas in Miyobo the prevalence remained similar (2% versus 4%).

The different accessibilities to antimalarial treatment between villages might explain these differences. The AS+AQ combination has been in use in Equatorial Guinea since 2009, leading to a high-drug-pressure environment favorable for the selection and spread of drug-resistant parasites. Miyobo's population is younger, with a larger proportion of individuals belonging to age groups at risk, and therefore more symptomatic individuals are treated with AS+AQ. This does not seem to happen in Ngonamanga, where the majority of individuals are older, semi-immune, and asymptomatic and thus not treated with antimalarial drugs. Such an environment with reduced drug pressure may be the cause of the lower prevalence of mutated parasites and the limited spread of drug resistance.

The interplay of the *Pfmdr1* 86Y and *Pfcrt* 76T alleles is associated with CQ resistance (28) and AQ monotherapy failure and selected by the AS+AQ association (29, 30). Analyzing differences in mutation prevalence and association with age, only those for the *Pfcrt* 76T allele were significant in both villages ($P = 0.023$ for Miyobo and $P = 0.001$ for Ngonamanga). In 2005, before ACT introduction, CQ was used for uncomplicated malaria, and it likely promoted the selection of the 86Y-76T haplotype in both populations, independently of age. After ACT introduction, the prevalence of this haplotype decreased only in Ngonamanga, as it is significantly more prevalent in children of $\leq$15 years of age ($P = 0.016$). If AQ exerts selective pressure on *Pfcrt*, then younger groups have a major role in the selection of mutant variants. In Miyobo, the prevalence did not decrease and there were no significant differences between age groups, but the results seem to present the same trend. The high prevalence still present in Miyobo may have been due to the pressure exerted by AQ, the half-life partner drug of the ACT used.

The genetic diversity of *P. falciparum* populations in the same villages, Ngonamanga and Miyobo, was again characterized by using the *Pfmsp2* and neutral STR markers, the same markers used in the 2005 study. The high genetic diversity of *P. falciparum*

determined by *Pfmsp2* and STR analyses is in accordance with previous studies performed both on the mainland (3) and on Bioko Island (31) before the beginning of ACT.

The prevalences of isolates that harbored mixed genotypes in both villages are within the values described for regions with hyperendemicity (32). Due to lower preimmunity (33), the MOI was found to be the highest for younger age groups (≤15 years) as determined by either *Pfmsp2* or STR analysis. This result is significant for Miyobo, with a younger population, but is observed for Ngonamanga only when STR markers are used. Surprisingly, when MOIs are compared between villages independently of age, there are no remarkable differences between the two villages in the *Pfmsp2* analysis, with barely significant differences when STR markers are considered. This lack of significant differences is probably due to the small number of children present in Ngonamanga and/or the higher molecular resolution of STR analysis, which is more efficient for determining the MOI, as *Pfmsp2* genetic variance is likely fixed in Ngonamanga.

In addition, there was an increase of allelic variation from 2005 to 2013, probably as a consequence of a higher prevalence of mixed-genotype infections, which enhances cross-fertilization and the generation of novel alleles. The parasite population size and transmission intensity remained high enough to allow effective genetic recombination of the parasites (34).

The study carried out in 2005 reported a high prevalence of mutations in the *Pfdhfr* and *Pfdhps* genes associated with resistance to the SP combination, and although the *Pfdhps* double mutant (GE mutant) was absent, 80% of the parasites were *Pfdhfr* triple mutants (IRN mutants) (3).

Our recent results support the fixation of mutations linked to PYR resistance at the *Pfdhfr* gene. In both Ngonamanga and Miyobo, 100% of isolates carried the 108N mutation, which is essential for *in vitro* PYR resistance, increasing the 50% inhibitory concentration (IC_{50}) of PYR 7- to 50-fold above that for the wild type (35). The same result was observed in eight other African countries between 2004 and 2006: Angola, Ethiopia, Kenya, Malawi, Rwanda, São Tomé and Príncipe, Tanzania, and Uganda (36). The prevalence of the *Pfdhfr* triple mutant genotype increased to 90% and 97% in Ngonamanga and Miyobo, respectively, but the "superresistant" 164R allele was not detected in either of the villages, which may have been due to a significant fitness cost for the parasite (37).

Concerning the *Pfdhps* gene, associated with SFX resistance, the prevalence of the 436A mutation remained low and the 581G mutation was absent in this study, despite its increase in prevalence in East and West African countries where the genotype "superresistant" to SP is emerging (36). The 437G mutation was the most common in both villages (93% in Ngonamanga and 95% in Miyobo), and overall, it was more prevalent in 2013 than in 2005 (70%). Consequently, about 85% of the parasites harbored a genotype denominated "partially resistant" to SP. The *P. falciparum* isolates that carried the 540E mutation were practically nonexistent in 2005, in contrast to an overall 8% prevalence identified in this survey (5% in Ngonamanga and 10% in Miyobo).

The combination of the *Pfdhps* double mutant (GE mutant) and the *Pfdhfr* triple mutant (IRN mutant) in a quintuple mutant or "fully resistant" genotype is associated with clinical SP failure (38). In most East African countries, the quintuple mutant genotype has been reported to have a prevalence exceeding 50% (36), and the prevalence of the 540E mutation is used by the WHO as a criterion to decide whether to implement intermittent preventive treatment during infancy (IPTi) (39). In this study, the overall prevalence of the "fully resistant" genotype was 7%, which may be an important predictor of SP therapeutic failure in Equatorial Guinea and a sign of concern. Not only SP exerts drug pressure on the *Pfdhfr* and *Pfdhps* genes, but the sulfa drugs, such as trimethoprim-sulfamethoxazole (co-trimoxazole), commonly given to patients with HIV/AIDS as prophylaxis against opportunistic infections, also do so (40). In 2013, Equatorial Guinea recorded a prevalence of 19% HIV-infected people (41), and HIV-malaria coinfections will thus be frequent. Although recent studies reported the

antimalarial properties of co-trimoxazole prophylaxis in reducing the incidence of malaria among HIV-infected patients even in areas where malaria parasites have antifolate resistance (40), there is some evidence that the efficacy of co-trimoxazole is lower in areas where the prevalence of antifolate resistance mutations of *Plasmodium* is high (42).

The effect of *Pfmdr1* gene amplification on antimalarial drug resistance is still controversial (42–45). In Southeast Asia (SEA), an increase of the *Pfmdr1* gene copy number has been associated with decreased susceptibility to several antimalarial drugs, including artesunate, mefloquine, artesunate-mefloquine, and artemether-lumefantrine (43, 46, 47), but Ariey et al. (10) did not observe an increase of *Pfmdr1* copy number in an artemisinin-resistant parasite line. However, Sidhu et al. (48) observed *in vitro* that an increase of *Pfmdr1* expression might modulate parasite sensitivity to artemisinin.

In Africa, an increase of gene expression has also been reported (89), but the copy number of the *Pfmdr1* gene is usually low compared to those reported for SEA parasites (43, 49). Nevertheless, in Gabon, Uhlemann et al. (46) reported amplification of *Pfmdr1* in more than 5% of patient samples collected in 1995 from patients with mefloquine treatment failure, but 7 years later, none of the samples collected showed *Pfmdr1* amplification. On the other hand, Duah et al. (50), in Ghana, observed that 27% of isolates showed an increased *Pfmdr1* gene copy number after the implementation of AS+AQ therapy in 2005 and 2006, possibly due to drug pressure induced by artesunate.

In Equatorial Guinea in 2005, despite CQ being the official antimalarial drug in use, a high prevalence of isolates with *Pfmdr1* amplification was obtained, but none of the isolates showed more than two copies of the gene. In 2013, the prevalence of *Pfmdr1* amplification was halved, and the majority of the isolates seemed to present a single *Pfmdr1* copy. Thus, it seems that if the number of *Pfmdr1* copies is in fact involved in resistance to artemisinin, it might be not relevant in this setting.

Furthermore, for SEA, it was suggested that *Pfmdr1* amplification was inversely related to the 86Y mutant allele (51), but in Africa, parasites harboring multiple copies of the *Pfmdr1* gene together with the 86Y mutant allele have been reported in Sudan, Gabon, and Ethiopia (46, 52, 53). In the present study, this association was observed at a low prevalence. This may be due to AS+AQ drug pressure, as Holmgren et al. (43) suggested that parasites exhibit the gene variant that may be optimal for survival depending on the drug pressure. CQ has been in use in Africa for a long time, which could explain why the *Pfmdr1* 86Y mutant allele but not the *Pfmdr1* amplification was selected, which would be reassuring with the massive introduction of ACT.

Following WHO recommendations concerning artemisinin resistance surveillance (15), we analyzed K13 propeller polymorphisms in samples collected in 2005 and 2013, before and after the ACT implementation. Four of the five nonsynonymous mutations detected have not been described before, and one SNP, A578S, previously found in sub-Saharan African parasites, may be of interest. Nevertheless, none of the mutations match the SNPs associated with artemisinin resistance in SEA (10), reinforcing recent studies which did not detect the mutations strongly linked to artemisinin resistance in SEA in sub-Saharan African isolates (54–56).

In isolates from 2013, the A578S mutation was the only one found in both villages (Ngonamanga and Miyobo). This mutation has already been detected in six other African countries (Kenya, Democratic Republic of Congo, Gabon, Ghana, Mali, and Uganda) (55, 57) and may have an important role, since it is adjacent to the C580Y mutation, the major mutation associated with delayed parasite clearance in SEA (10). Ashley et al. (11) described that mutations after position 440 in the K13 propeller domain were associated with an increased parasite clearance half-life (116%), probably due to structural changes of the K13 protein (57). Although the A578S mutation was considered neutral in a functional analysis that predicted an effect on protein function (PROVEAN score = −1.962), this mutation leads to the replacement of a neutral nonpolar amino acid (A) by a neutral polar amino acid (S) (55), and based on the prediction of computational modeling for mutational sensitivity (PHYRE2 Investigator),

this may change the K13 protein's biological function by changing the surface charges that disrupt interactions with other proteins (57). According to the PROVEAN analysis, the nondescribed nonsynonymous mutations V637I and D641G are associated with neutral and deleterious effects on protein function, respectively. Although these mutations have not been described before, other SNPs in codon 637 have been reported previously, namely, V637D (56) and V637A (54).

Mutant alleles of the K13 propeller gene were also detected in isolates collected in 2005, before ACT implementation. Two new nonsynonymous mutations, E556K and Y588C, were identified, with both associated with deleterious effects on protein function. Talundzic et al. (16) reported a different SNP in codon 556, i.e., E556D. None of these mutations were associated with delayed parasite clearance in SEA parasites (10). The rise of these mutations in the absence of selective pressure by artemisinin may have resulted from a high transmission level that promoted high parasite genetic diversity. Alternatively, the fact that ACT was first introduced in the neighboring countries earlier than in Equatorial Guinea, in 2004 in Cameroon and in 2003 in Gabon (1), may have led to the nonofficial circulation of artemisinin-derived drugs in the country. Additional biochemical studies are necessary to investigate whether the K13 mutations found in Equatorial Guinea may have a real effect on artemisinin sensitivity/resistance.

In 2009, Equatorial Guinea antimalarial treatment with SP was replaced by ACT due to widespread SP resistance. However, SP remains the only option for IPTp and an important drug for IPTi. In this context, priority remains for continuing surveillance and monitoring of genetic changes of SP resistance in the population. Under drug pressure, drug resistance-associated alleles conferring a selective advantage are selected. As a consequence, genetic variation of sequences flanking them will be reduced substantially compared to that for the wild-type alleles (58, 59).

STR analysis of the *Pfdhfr* gene showed that the majority of haplotypes belong to the triple mutant (96%). These data are predictive of a possible selective sweep of the triple mutant haplotype. The continuous PYR drug pressure is evident in both villages and is corroborated by the reduction of heterozygosity and the increased genetic differentiation of the *Pfdhfr*-flanking loci compared to that of neutral loci. Apparently, the effect of PYR selection is even stronger in Miyobo, where a significant LD of triple mutants between the loci at 0.3 and 5.3 kb was identified. This fact is a well-described signature of genetic hitchhiking due to strong selection (60).

Sixty-two percent (34/55 isolates) of the triple mutant isolates contained the K1-flanking STR profile 113/183/210 bp. This result suggests a common origin for the *Pfdhfr* triple mutant in both villages. In fact, the triple mutant has a common ancestry between African and SEA lineages (61). This haplotype derived from Asia and has been recorded in countries across the African continent: in Senegal, as the second most common haplotype, and in Tanzania, South Africa, Southeast Africa (62), and Kenya (63). This triple mutant haplotype was already observed in samples from Equatorial Guinea collected in 2005 (3). Except for two haplotypes (H8 and H12), all the other non-H1 *Pfdhfr* triple mutant haplotypes share an allele length similar to that for haplotype H1 at one or two STR loci, indicating that the variation observed is probably generated by replication errors (mutations) (64) or genetic recombination between the SEA and African lineages (61, 62, 65).

Mita et al. (66) reported two African lineages of the *Pfdhfr* triple mutant with the same microsatellite haplotypes as those of *Pfdhfr* double mutants, suggesting an indigenous evolution.

The results of this study showed two distinct haplotypes of the *Pfdhfr* double mutant (ICN): H2 and H5. The H5 haplotype was previously reported in São Tomé and Príncipe (67) as a possible African lineage of the *Pfdhfr* triple mutant; in Equatorial Guinea, it is associated with the single 108N mutation (3). The present data support the hypothesis that the triple mutant H11 haplotype is derived from H5. In the same way, the triple mutant H4 haplotype may be derived from the double mutant H2 haplotype through an additional mutation at codon 59 of the *Pfdhfr* gene. However, the fact that the H2

and H4 haplotypes share two STR alleles with SEA lineage parasites may be explained by a *de novo* origin and maintenance of distinct *Pfdhfr* lineages in Equatorial Guinea and/or by gene flow of particular *Pfdhfr* lineages into the Equatorial Guinea population. The triple mutant haplotypes H8 and H12, which do not share any allele with H1, are probably a consequence of a local independent lineage that carried the 181/208-bp profile at the loci at 4.4/5.3 kb. Altogether, the origin of the *Pfdhfr* triple mutant in Equatorial Guinea corresponds to a pattern of "soft selective sweep," the presence of multiple ancestral haplotypes at one selected locus in the population (68).

SFX resistance alleles appeared more recently than PYR resistance alleles and after the spread of the *Pfdhfr* triple mutant (69). STRs flanking *Pfdhps* mutant alleles showed greater genetic variability than those flanking *Pfdhfr*, which indicates that resistance to SFX is still developing in this setting.

Pfdhps mutant resistance alleles emerged independently in multiples sites of Africa, with three major AGK/SGK lineages in West and Central Africa and two major SGE lineages in East Africa predominating (58). In 2005, the majority of STR haplotypes for the *Pfdhps* gene showed the AGK/SGK 1 lineage (117/105 bp at the loci at 0.8/4.3 kb); the double mutant SGE lineage was not detected (3). In the present analysis, at least four independent introductions of mutant alleles in *P. falciparum* populations in both villages were identified. The majority of the 437G single mutant haplotypes match the AGK/SGK 1 lineage, which is found with a higher prevalence in Southwest Africa; the AGK/SGK 2 (121/107 bp at the loci at 0.8/4.3 kb) and AGK/SGK 3 (123/107 bp at the loci at 0.8/4.3 kb) lineages, predominant in West and Central Africa, respectively (58), were less prevalent. The *Pfdhps* double mutant (GE mutant) haplotype H6 identified in both villages has an STR profile coincident with the SGE 1 lineage, the predominant lineage in Southeast Africa. In Central and Southwest Africa (including neighboring countries of Equatorial Guinea), the SGE 1 haplotype was detected at a low prevalence (0 to 9%) (58). Its presence in Equatorial Guinea suggests that given the use of SP not only for IPTi but also frequently for use without medical surveillance, one resistant lineage can eventually displace AGK/SGK (similarly to what occurs with *Pfdhfr*). Two other *Pfdhps* haplotypes were identified, including the AGKG (H14) and AGEA (H31) triple mutants; the latter has been described predominantly in SEA (70).

Although only the STR background for one isolate with the AGE mutant alleles could be constructed, it shared the STR pattern of the SGE 1 lineage (131/103 bp at the loci at 0.8/4.3 kb), suggesting that the triple mutant arose from it. Mita et al. (71) analyzed the ordered accumulation of mutations in *Pfdhfr* and *Pfdhps* and suggested that the AGE lineage appeared after the SGE lineage, and this triple mutant is associated with a higher level of SFX resistance (9.8-fold) than that of the wild-type *Pfdhps* alleles (72).

When samples were grouped into wild-type and *Pfdhps* mutant groups, a reduction in heterozygosity was observed; also, the higher mean F_{ST} at loci flanking the *Pfdhps* gene in double mutants indicated that the *Pfdhps* double mutants had undergone positive directional selection. However, the SFX selection signature was also evident in single mutants, through a significant LD value between flanking loci at 0.8 and 4.3 kb. This result was reported in 2005 by Mendes et al. (3) and was considered suggestive of soft selective sweep. The *Pfdhps* gene analysis showed the presence of wild-type populations due to widely diverse haplotypes in the mutant groups and selection signatures in a single mutant group. These data showed a recent and continuous selection of the SFX resistance alleles, which have not yet reached fixation as the PYR-associated resistance alleles have. Nevertheless, our data support a positive selection of the SFX double mutants. According to related literature, in a few years the *Pfdhps* double mutant alleles combined with *Pfdhfr* triple mutants may reach fixation if SP continues to be used in Equatorial Guinea (73). These results are a matter of concern, since a recent study (6) reported that SP is the third most prescribed antimalarial in Bata (urban and rural areas) for children of up to 5 years of age.

Based on data from 2005 (3), the developing parasite resistance to SP could have been established first in Ngonamanga, a coastal area closer to the Equatorial Guinea-Cameroon border with more accessibility to antimalarial drugs. Miyobo, located in the

interior of the country, seemed to have developed resistance to SP later on, with a lower prevalence of the triple mutant (IRN mutant).

Since the discovery of oil and gas deposits in the mid-1990s, Equatorial Guinea has undergone rapid economic growth. The oil and gas income allowed the rapid development of basic infrastructures, such as roads, ports, and airports, over the last years; however, the population's living conditions did not improve much, and the country continues to appear at the bottom of the social indicator scale (74). The improved road from Bata to Niefang, connecting the political and economic center of Bata (the main city of mainland Equatorial Guinea) to the three inland provincial capitals, Ebebiyin, Mongomo, and Evinayong, and the future capital, Oyala, allowed the local social and economic development of Niefang, which is 15 km from Miyobo. This development has made better access to resources possible for the villages around Niefang, such as Miyobo. In fact, Miyobo now has a younger population and a higher population density. In contrast, the rural-urban migration of people from Ngonamanga looking for the better living conditions offered by the oil industry and construction became increasingly evident. The huge development driven by the oil industry led to declines in agriculture, fisheries, and forestry, making the economy highly dependent on the oil and gas sector. This policy measure led to migration to urban areas from the coastal region, such as Ngonamanga, where fishing was the main economic activity.

The genetic diversity of the *Plasmodium* population and the selection of resistant strains in Equatorial Guinea have accompanied human migration. In 2005, we observed a higher genetic diversity and a larger number of strains resistant to CQ and SP in coastal areas (Ngonamanga). On the other hand, in 2013, we detected higher parasite diversity in Miyobo and the migration of resistant *Plasmodium* strains to the interior of Equatorial Guinea, where antimalarial drugs may be more accessible, therefore increasing the probability of drug resistance development.

After the adoption of ACT, noticeable differences appeared in the distribution of genotypes conferring resistance to CQ and SP. The spread of mutated genotypes differed according to the setting. In both villages, the high prevalence of resistance genotypes may compromise the efficacy of SP for IPTp/IPTi. Regarding artemisinin resistance, despite our data showing that mutations strongly linked to artemisinin resistance are not present at the time, several SNPs in the K13 gene were observed, meaning that close monitoring should be maintained in order for timely detection of a possible advancement of artemisinin resistance in Africa. These data are of utmost interest for a global adequate drug policy as well as for the monitoring of malaria drug resistance in Equatorial Guinea.

MATERIALS AND METHODS

Study area. Equatorial Guinea is a sub-Saharan country located in Central Africa. It consists of two regions: the mainland, called Rio Muni, lying between Cameroon and Gabon, and the insular region (Bioko Island, where the capital city of Malabo, Annobón, and Corisco Bay are located). The mainland area of 26,000 km^2 is divided into four provinces (Litoral, Centro Sur, Kié-Ntem, and Wele-Nzas), with an estimated population of 736,000 in 2012 (2). Sampling took place in two mainland villages: Ngonamanga and Miyobo (Fig. 1).

Equatorial Guinea has a tropical climate, with two dry seasons in continental regions: one from December to mid-February and the other from July to September (75). Malaria is a major cause of morbidity and the fourth cause of death (5.7%), following HIV/AIDS (14.8%), lower respiratory infections (10%), and diarrheal diseases (8%) (76). In the continental region, malaria is hyperendemic, with stable transmission, and the main mosquito vector is *Anopheles gambiae sensu lato* (4, 77).

Ethics statement. Each person (or parent/guardian) who participated in this study was informed of its nature and main objectives and was told that participation was voluntary and that he or she could withdraw from the study at any time. Blood samples were collected from all donors after written informed consent (parents or guardians responded on behalf of children). A code number was assigned to each participant who answered a questionnaire, and confidentiality is maintained regarding all information obtained. The study was approved by the local health authorities from these villages, the Ethical Committee of Equatorial Guinea's Ministry of Health and Social Welfare, and the National Malaria Control Program. Ethical clearance was also given by the Ethical Committees of the Instituto de Higiene e Medicina Tropical (IHMT) and the Instituto de Salud Carlos III (ISCIII), according to European Union norms.

Sampling. Sampling took place in 2013, from February to April. Collections were made in all inhabited households of each village, i.e., a total of 29 in Ngonamanga and 40 in Miyobo, corresponding

to a total of 232 individuals (63 in Ngonamanga and 169 in Miyobo). According to the Centro de Referencia para el Control de Endemias, the estimated population density in October 2012 was 343 inhabitants in Miyobo and 69 in Ngonamanga. The individuals were fully identified and blood samples were collected by finger prick. For each person, a rapid diagnostic test (RDT) (Nadal malaria test 4 species) was performed, thick and thin blood films were made, and a drop of blood was spotted onto Whatman 903 filter paper cards.

In agreement with the guidelines of the Ministry of Health, antimalarial treatment with AS+AQ plus paracetamol was provided to patients with a positive *Plasmodium* RDT.

Identification of *Plasmodium* species and molecular assays. Giemsa-stained thick and thin blood films were examined and parasite densities recorded as the number of parasites per microliter of blood, assuming an average leukocyte count of 8,000/μl (all smears were examined against 500 leukocytes prior to being declared negative). *Plasmodium falciparum* asexual parasitemia was classified according to the following criteria: low (<800 parasites/μl), moderate (800 to 8,000 parasites/μl), and high (>8,000 parasites/μl).

Parasite genomic DNA was extracted from blood spots dried on filter paper (~50 μl) by using a phenol-chloroform technique (78). Detection and identification of *Plasmodium* species were carried out by nested PCR amplification of the small-subunit rRNA genes (79). *Plasmodium falciparum*-positive isolates (present in single or mixed infections) were then selected for further molecular characterization.

Analysis of drug resistance-associated genes. A nested PCR-restriction fragment length polymorphism (PCR-RFLP) method was used to analyze the presence/absence of mutations in CQ and SP resistance-associated genes, at codons 75 and 76 of the *Pfcrt* gene, codons 86 and 1246 of the *Pfmdr1* gene, codons 51, 59, 108, and 164 of the *Pfdhfr* gene, and codons 436, 437, 540, and 581 of the *Pfdhps* gene (80). Genomic DNAs from *P. falciparum* clones 3D7, Dd2, and K1 were used as positive controls. Restriction enzyme digests were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel and visualized under UV transillumination.

The genetic variability of the K13 gene was characterized using the primers and PCR conditions described by Ariey et al. (10). DNA fragments were purified using a QIAquick PCR purification kit (Qiagen), and samples were sequenced bidirectionally by Macrogen (Europe). Sequence reads were aligned to reference sequence PF3D7_1343700 (www.plasmodb.org) by using Jalview software (http://www.jalview.org/).

In order to have a timeline before and after the introduction of ACT therapy for the analysis of the K13 propeller domain, 58 randomly selected samples collected in 2005 were added to this study, including 29 from Ngonamanga and 29 from Miyobo. To estimate the *Pfmdr1* copy number, an additional 25 samples collected in 2005 in Ngonamanga were also randomly selected. The *Pfmdr1* copy number was estimated by real-time PCR following the method of Ferreira et al. (81).

The PROVEAN (Protein Variation Effect Analyzer) algorithm (http://provean.jcvi.org/) was used to predict the functional effects of protein sequence variations (single or multiple amino acid substitutions, insertions, and deletions). The possible neutral/deleterious effects caused by nonsynonymous SNPs were evaluated by the PROVEAN algorithm. The *Plasmodium falciparum* PF3D7_1343700 protein was used as a reference (www.plasmodb.org), with a cutoff score of −2.5.

Description of *P. falciparum* genetic diversity. Genetic diversity was evaluated through genotyping of the repeat region of the *Pfmsp2* gene according to the work of Snounou et al. (82). PCR products were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel and visualized under UV transillumination.

Genotyping of STRs flanking drug-associated genes and neutral STRs was also carried out. Microsatellites flanking the *Pfdhfr* (chromosome 4) and *Pfdhps* (chromosome 8) genes at the following positions were analyzed: for the *Pfdhfr* gene, loci located 0.3 kb, 4.4 kb, and 5.3 kb upstream of codon 108; and for the *Pfdhps* gene, loci located 0.8 kb, 4.3 kb, and 7.7 kb downstream of codon 437. The PCR conditions and primers are described in former reports (62, 65, 67). As a reference, the Southeast Asian *P. falciparum* K1 laboratory strain was used because its STR profile matches the East African *Pfdhps* double mutant (GE mutant) haplotype lineage SGE 1 (58).

To have a reference for genetic variation and linkage disequilibrium at neutral regions, the following nine additional STR loci were analyzed: TAA42, TAA81 (chromosome 5), TA1, TAA87, TAA109 (chromosome 6), ARA2 (chromosome 11), TA102, PfPK2, and Pfg377 (chromosome 12) (83, 84).

Amplified products were separated by capillary electrophoresis in an ABI3730 genetic analyzer (Applied Biosystems), and fragment sizes and genotypes were scored using the software GeneMarker 1.4 (Softgenetics).

For haplotype assembly, the resistance-specific polymorphic locus and the corresponding flanking microsatellite loci were considered according to a previous description (67). Isolates for which amplification failed for any of the loci or multiple peaks were generated were excluded from the haplotype grouping. Haplotypes were differentiated if they contained one or more allelic changes across the loci (62).

Whenever isolates presented multiple peaks (alleles) at any locus, only the peak with the highest amplification intensity was chosen for the subsequent analyses (85).

Statistical analysis. Comparisons based on gender, sites, age, and parasitemia levels were conducted using the chi-square test or Fisher's exact test, as appropriate. Nonparametric statistical tests (Mann-Whitney and Kruskal-Wallis tests) were used to test differences in multiplicity of infection (MOI) between areas, age groups, and parasitemia levels. SNP proportions were compared by using a *Z* test for comparison of two proportions.

The MOI, which was generated by *Pfmsp2* and neutral STR genotyping, was defined as the number of concurrent parasite clones per *P. falciparum*-positive host; the mean MOI for each site was obtained by dividing the total number of genotypes by the total number of isolates (86).

Genetic diversity was evaluated through the allelic richness (Rs) per locus (a measure of the number of alleles independently of sample size) and the expected heterozygosity (He). The genetic differentiation over loci was determined by calculating F_{ST}; significance levels were determined after 1,000 permutations, not assuming random mating within samples. Both results were obtained with the program FSTAT (version 2.9.3.2) (http://www2.unil.ch/popgen/softwares/fstat.htm).

For the genetic differentiation analysis, we compared wild-type and mutant genotypes at the *Pfdhps* locus, associated with SFX resistance, by defining subsamples as wild-type, single mutant (A437G mutant), or double mutant (GE mutant) samples. Considering the *Pfdhfr* gene, related to PYR resistance, the same comparisons were not possible because the number of single and double mutants was extremely low and only the triple mutant (IRN mutant) could have been analyzed. For genotype construction, mixed mutant/wild-type infections were scored as mutant to reflect the expected mutant pattern of the infection (87).

Linkage disequilibrium (LD) tests were performed with GENEPOP software (version 4.2) (http://genepop.curtin.edu.au/). Significance levels were adjusted using the sequential Bonferroni correction for multiple comparisons (88).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02556-15.

TEXT S1, PDF file, 0.4 MB.

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