

# Trends in Molecular Markers Associated with Resistance to Sulfadoxine-Pyrimethamine (SP) Among *Plasmodium falciparum* Isolates on Bioko Island, Equatorial Guinea: 2011–2017

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**Purpose:** Antimalarial drug resistance is one of the major challenges in global efforts to control and eliminate malaria. In 2006, sulfadoxine-pyrimethamine (SP) replaced with artemisinin-based combination therapy (ACT) on Bioko Island, Equatorial Guinea, in response to increasing SP resistance, which is associated with mutations in the dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes.

**Patients and Methods:** To evaluate the trend of molecular markers associated with SP resistance on Bioko Island from 2011 to 2017, 179 samples collected during active case detection were analysed by PCR and DNA sequencing.

**Results:** *Pfdhfr* and *Pfdhps* gene sequences were obtained for 90.5% (162/179) and 77.1% (138/179) of the samples, respectively. For *Pfdhfr*, 97.5% (158/162), 95.7% (155/162) and 98.1% (159/162) of the samples contained N51I, C59R and S108N mutant alleles, respectively. And *Pfdhps* S436A, A437G, K540E, A581G, and A613S mutations were observed in 25.4% (35/138), 88.4% (122/138), 5.1% (7/138), 1.4% (2/138), and 7.2% (10/138) of the samples, respectively. Two classes of previously described *Pfdhfr*-*Pfdhps* haplotypes associated with SP resistance and their frequencies were identified: partial (IRNI-SGKAA, 59.4%) and full (IRNI-SGEAA, 5.5%) resistance. Although no significant difference was observed in different time periods ( $p > 0.05$ ), our study confirmed a slowly increasing trend of the frequencies of these SP-resistance markers in Bioko parasites over the 7 years investigated.

**Conclusion:** The findings reveal the general existence of SP-resistance markers on Bioko Island even after the replacement of SP as a first-line treatment for uncomplicated malaria. Continuous molecular monitoring and additional control efforts in the region are urgently needed.

**Keywords:** malaria, drug resistance, dihydrofolate reductase, dihydropteroate synthase, sulfadoxine-pyrimethamine

## Introduction

According to the World Malaria Report 2019,<sup>1</sup> an estimated 228 million (95% confidence interval [CI]: 206–258 million) persons worldwide have malaria, with 405,000 deaths in 2018. Eighty per cent of global malaria deaths happened in 14 sub-Saharan African countries and India.<sup>2</sup> This mortality in sub-Saharan Africa is aggravated by the high proportion of deaths in under 5 years age groups (approximately 90% malaria-related deaths), more than any other malaria-endemic region.<sup>2</sup> In general, pregnant

women and children under the age of 5 years are the most vulnerable due to depressed immunity or the absence of partial immunity against the disease.<sup>3</sup> Thus, strategies for reducing infection and disease burden in pregnant women, infants and children, groups bearing the highest burden of the disease, are increasingly urgent. Intermittent preventive treatment (IPT) is one such strategy. The World Health Organization (WHO) recommends IPT in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) in all areas of Africa with moderate to high malaria transmission.<sup>4,5</sup> In sub-Saharan Africa, 39 countries adopted IPTp between 1993 and 2007.<sup>5</sup> In addition, the concept of IPT has been extended to include infants (IPTi) and children (IPTc; now referred to as SMC (seasonal malaria chemoprevention)).

The sub-Saharan African country Equatorial Guinea has a total population of 1.31 million (2018). According to WHO data, malaria causes 15% of all deaths in children under age 5 in Equatorial Guinea.<sup>4</sup> Bioko, an island of Equatorial Guinea off the coast of Cameroon, has historically high malaria transmission.<sup>6</sup> The most recent studies focused on detecting the prevalence of mutations in *Pfmdr1* and *Pfcr1* on Bioko Island.<sup>7,8</sup> SP has been used as a second-line therapy and IPTp on Bioko for several decades.<sup>8,9</sup> Although IPT (IPTp, IPTi, IPTc) as a malaria control strategy has been shown to have a positive impact in preventing disease-related death in IPT-treated individuals, there are challenges due to the emergence of resistance to the drugs used for IPT treatment. From 1992 to 1999, the Carlos III Institute of Health (Madrid, Spain), working in collaboration with Equatorial Guinea's Ministry of Health, conducted a study on Bioko Island and concluded that resistance to SP was approximately 25% among children under 5 years of age.<sup>10,11</sup> In 2006, following the WHO recommendation to use combination therapy for malaria treatment, artemisinin-based combination therapy (ACT) was adopted as a first-line treatment,<sup>12,13</sup> but SP is still used for IPT and in combination with artemisinin for the treatment of uncomplicated malaria, which may have led to *Plasmodium falciparum* isolates undergoing sustainable selection pressure.

Parasite resistance to SP is influenced by single-nucleotide polymorphisms (SNPs) of the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes, which encode proteins involved in the folate biosynthesis pathway.<sup>14</sup> Previous report revealed that the combination of *Pfdhfr*-*Pfdhps* mutations significantly correlated with the treatment failure.<sup>15</sup> For the *Pfdhfr* gene, mutations N51I, C59R, S108N and

I164L are associated with pyrimethamine resistance. Similarly, five mutations in *Pfdhps* (S436A, A437G, K540E, A581G and A613S/T) have been implicated in sulfadoxine resistance.<sup>16</sup> Evidence suggests that SP resistance tends to increase as the result of the stepwise accumulation of SNPs in *Pfdhps* and *Pfdhfr*.<sup>6</sup> Selective sweeps flanking *Pfdhps* and *Pfdhfr* have been shown in African populations as well.<sup>17–19</sup> Under SP pressure, negative selection would begin to reduce the prevalence of the resistant genotypes, while recombination and mutation would begin to increase heterozygosity in markers flanking the resistance-conferring genes, degrading the selective sweep.<sup>19</sup> All these observations underscore the need to monitor the distribution of parasite populations to mitigate the dispersal of resistant haplotypes in the region.

Unlike in other African countries, studies investigating *Pfdhfr* and *Pfdhps* mutations on Bioko Island are limited.<sup>10,12</sup> Our recent study revealed a high prevalence of *Pfdhfr* and *Pfdhps* mutations associated with SP resistance among Bioko *P. falciparum* isolates.<sup>20</sup> However, it remains unclear whether SP resistance has been decreasing or increasing in recent years since the Ministry of Health switched from SP to ACT for first-line treatment in 2006. Therefore, to ensure the prophylactic efficacy of IPT and to support the national antimalarial policy, the present study was carried out on Bioko Island at different time points to investigate the trend of molecular markers associated with SP resistance among *P. falciparum* isolates from Jan. 2011 to Dec. 2017. Additionally, further molecular evolutionary and population genetic approaches were applied to understand the evolution and spread of parasite drug resistance on Bioko Island.

## Patients and Methods

### Study Area

This study was carried out in the clinic of the Chinese medical aid team to the Republic of Equatorial Guinea, Malabo Regional Hospital of Bioko Island. Ethical approval was obtained from the Ethics Committee of Malabo Regional Hospital. Bioko is an island 32 km off the west coast of Africa and the northernmost part of Equatorial Guinea. The island has a population of 334,463 inhabitants (2015 census, of which approximately 90% live in Malabo, the capital city of Equatorial Guinea) and a humid tropical environment. Malaria due to *P. falciparum* is still the major public health issue on the island.<sup>21</sup> The Bioko Island Malaria Control Project

(BIMCP) has committed to reducing the burden of malaria on Bioko Island through methods such as concerted vector control, improved case management, and various educational interventions for the past 15 years. As a result of the vector control interventions and case management since 2004, the parasite prevalence on Bioko has decreased from over 45% in 2004 to 8.5% in 2016, and the entomological inoculation rate has decreased from more than 1000 before 2004 to 14 in 2015.<sup>22</sup>

## Sample Collection

A total of 179 blood spot samples were collected from patients with uncomplicated malaria from January 2011 to December 2017. The included patients were aged between 4 months and 80 years and were residents of Bioko Island, collected in outpatient service of Malabo Regional Hospital with active case detection. All patients confirmed *P. falciparum* malaria cases identified by microscopic examination and an immuno-colloidal gold test kit (ICT Diagnostics). The patients were classified into uncomplicated malaria states according to WHO criteria, which were defined as positive smears for *P. falciparum* and the presence of fever ( $\geq 37.5^{\circ}\text{C}$ ).

Verbal informed consent was obtained from all participating subjects or their parents, and this study, as well as the consent process, was approved by the Ethics Committee of Malabo Regional Hospital. Dried blood spots were collected on day zero of enrolment through finger prick, with the blood spotted onto Whatman 903<sup>®</sup> filter paper (GE Healthcare, Pittsburgh, USA). Laboratory screening for malaria was performed using rapid diagnostic tests and confirmed using microscopic examination of blood smears. For quality control, archived malaria-positive microslides were re-examined, and parasite density was recorded. *Plasmodium* species were identified by real-time PCR followed by high-resolution melting.<sup>23</sup> pGEM-T standard plasmids for four human *Plasmodium* species, including *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*, which were kindly provided by Dr. Cao J (Jiangsu Institute of Parasitic Diseases, Wuxi, Jiangsu Province, China), were used as controls.

## DNA Extraction and Sequencing

DNA was extracted from the dried blood spots following the Chelex-100 extraction procedure described in our previous report.<sup>7</sup> Mutation screening for *Pfdhfr* and *Pfdhps* was performed by nested PCR as previously reported, with minor modifications.<sup>20</sup> All primers were ordered from Genewiz

(Soochow, China). The PCR products were separated by 2% agarose gel electrophoresis and stained with Gel Red (Genewiz, Soochow, China). The correct genes were identified based on size using an ultraviolet transilluminator. Next, the PCR fragments were purified and sequenced using corresponding primers by Sanger sequencing (Genewiz, Soochow, China). The sequences were analysed using Lasergene analysis software (DNA Star, Madison, WI) with 3D7 *Pfdhfr* and *Pfdhps* sequences as references (GenBank accession numbers: XM\_001351443 and Z30654.1).

## Data Analysis

Samples with multiple peaks at any genotyped SNP codon (mixed genotype) were excluded from SNP and haplotype analyses. The prevalence of mutations and haplotypes between collection years were compared using the chi-square or Fisher's exact test, as appropriate (SPSS v.17 statistical software). Statistical analyses were performed at the 5% significance level and 95% CI. The inferred haplotype network was constructed by the median-joining network algorithm<sup>24</sup> with the POPART program.<sup>25</sup> *Pfdhfr* and *Pfdhps* sequences from 20 other countries (18 for *Pfdhps* and 18 for *Pfdhfr*) were downloaded from NCBI or our previous study<sup>26</sup> to analyse global genetic diversity.<sup>27–36</sup>

## Results

### General Characteristics

From January 2011 to December 2017, we enrolled 179 patients with uncomplicated malaria from Bioko Island, Equatorial Guinea. Among the cases, 90.5% (162/179) and 77.1% (138/179) of the samples collected were successfully amplified and sequenced for the *Pfdhfr* and *Pfdhps* genes, respectively. These nucleotide sequences have been deposited in GenBank under accession numbers MN598219–MN598518. Multiclonal samples were excluded from this study. The median (interquartile range [IQR]) age of the patients was 39 years (14 to 68), and 36.9% of them were women. The median (IQR) parasite density was 4650 parasites/ $\mu\text{L}$  (340 to 63,120). Because the percentages are low, samples with mixed genotypes were not included when calculating the allele prevalence and reconstructing the various haplotypes (data not shown).

### Frequency of *Pfdhfr* and *Pfdhps* Mutations

*Pfdhfr* and *Pfdhps* were examined to assess the frequency and variation trend of SP-resistance mutations in Bioko parasites from 2011 to 2017. The detailed information is

shown in [Tables 1](#) and [2](#). The three *Pfdhfr* mutations N51I, C59R and S108N were detected in 97.5%, 95.7% and 98.1% of the samples, respectively ([Table 1](#)). No other *Pfdhfr* mutations associated with SP resistance were observed. Five *Pfdhps* mutations associated with SP resistance were found. The mutation A437G was the most prevalent, being found in close to 90% of the samples (88.4%), and this frequency was significantly higher than the other mutations ( $p < 0.05$ ). Among the other four mutations, the frequency of S436A was relatively high (25.4%), followed by A613S (7.2%), K540E (5.1%) and A581G (1.4%) ([Table 2](#)). The frequency of most molecular markers in the *Pfdhfr* and *Pfdhps* genes exhibited a slowly increasing trend over the 7 years. However, there was no significant difference among the time periods ( $p > 0.05$ ).

## Frequency of *Pfdhfr* and *Pfdhps* Haplotypes

Four *Pfdhfr* and 10 *Pfdhps* haplotypes were obtained from Bioko parasites ([Table S1](#)). The frequency of the *Pfdhfr*

triple-mutant haplotype (IRNI) was significantly higher (95.7%, 155/162) than was that of double-mutant haplotypes (ICNI, 2.5%; NRNI, 0.6%) and wild-type haplotypes (NCSI, 1.8%) ( $p < 0.05$ ). This haplotype profiling was consistent with neighbouring countries, including Gabon,<sup>37</sup> Cameroon<sup>34</sup> and Nigeria<sup>38</sup> ([Figure 1](#)). IRNI and NRNI are the two most common *Pfdhfr* haplotypes worldwide. In the vast majority of Africa, IRNI is prevalent in many endemic regions, including Bioko Island, Gabon,<sup>37</sup> Cameroon,<sup>33</sup> Kenya<sup>36</sup> and Malawi.<sup>32</sup> NRNI also appears frequently, with a high percentage occurring in countries outside of Africa, such as Iran (92.3%),<sup>39</sup> Nepal (90.6%),<sup>40</sup> India (63.3%),<sup>34</sup> Indonesia (62.5%),<sup>31</sup> and Papua New Guinea (95.7%).<sup>30</sup>

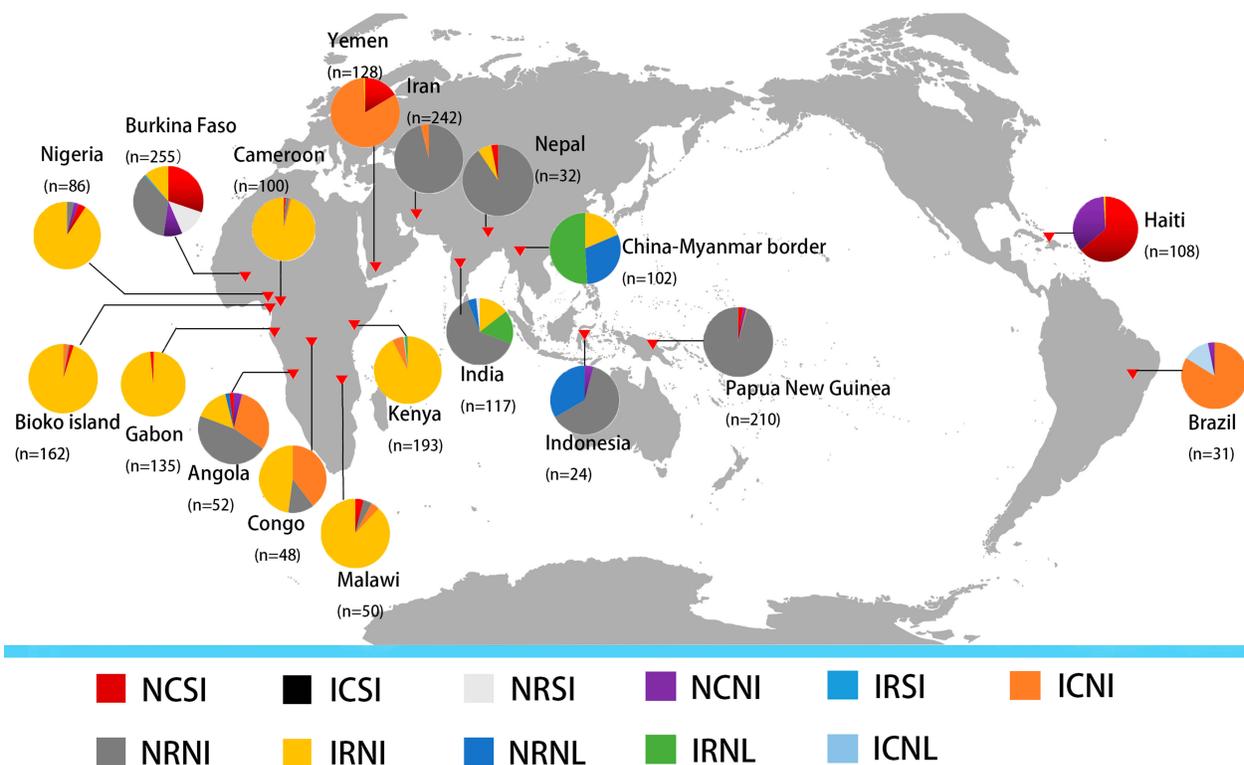
The frequency of the *Pfdhps* single-mutant haplotype (SGKAA) was predominant among the Bioko parasitic population (63.8%). Four haplotypes (SAKAS, SGKAS, SGKGS and AGKGS) were only found in one isolate. Interestingly, the *Pfdhps* haplotype diversity displays a slowly increasing trend since 2011 ([Table S1](#)). This haplotype profiling was consistent with neighbouring countries Angola<sup>35</sup> and Gabon<sup>37</sup> ([Figure 2](#)). A significant difference in haplotype

**Table 1** The Frequency and Variation Trend of SP-Resistance Mutations in *Pfdhfr* Among Bioko Parasites from 2011 to 2017

<i>Pfdhfr</i>	Amino Acid	2011–2012 (n=52)	%	2013–2014 (n=51)	%	2016–2017 (n=59)	%	Total (n=162)	%
N51I	I	48/52	92.3	51	100	59	100	158/162	97.5
	N	4/52	7.7	0	0	0	100	4/162	2.5
C59R	R	47/52	90.4	50/51	98	58/59	98.3	155/162	95.7
	C	5/52	9.6	1/51	2	1/59	2.7	7/162	4.3
S108N	N	49/52	94.2	51	100	59	100	159/162	98.1
	S	3/52	5.8	0	0	0	0	3/162	1.9

**Table 2** The Frequency and Variation Trend of SP-Resistance Mutations in *Pfdhps* Among Bioko Parasites from 2011 to 2017

<i>Pfdhps</i>	Amino Acid	2011–2012 (n=42)	%	2013–2014 (n=44)	%	2016–2017 (n=52)	%	Total (n=138)	%
S436A	A	14/42	33.3	10/44	22.7	11/52	21.1	35/138	25.4
	S	38/42	90.5	34/44	77.3	41/52	79.9	103/138	74.6
A437G	G	34/42	81	41/44	93.2	47/52	90.3	122/138	88.4
	A	8/42	19	3/44	6.8	5/52	9.6	16/138	11.6
K540E	E	0	0	1/44	2.3	6/52	11.5	7/138	5.1
	K	42/42	100	43/44	96.7	46/52	88.5	131/138	94.9
A581G	G	0	0	0	0	2/52	3.8	2/138	1.4
	A	42/42	100	44/44	100	50/52	96.2	136/138	98.6
A613S	S	2/42	4.8	4/44	9.1	4/52	7.7	10/138	7.2
	A	40/42	95.2	40/44	90.9	48/52	92.3	128/138	92.8



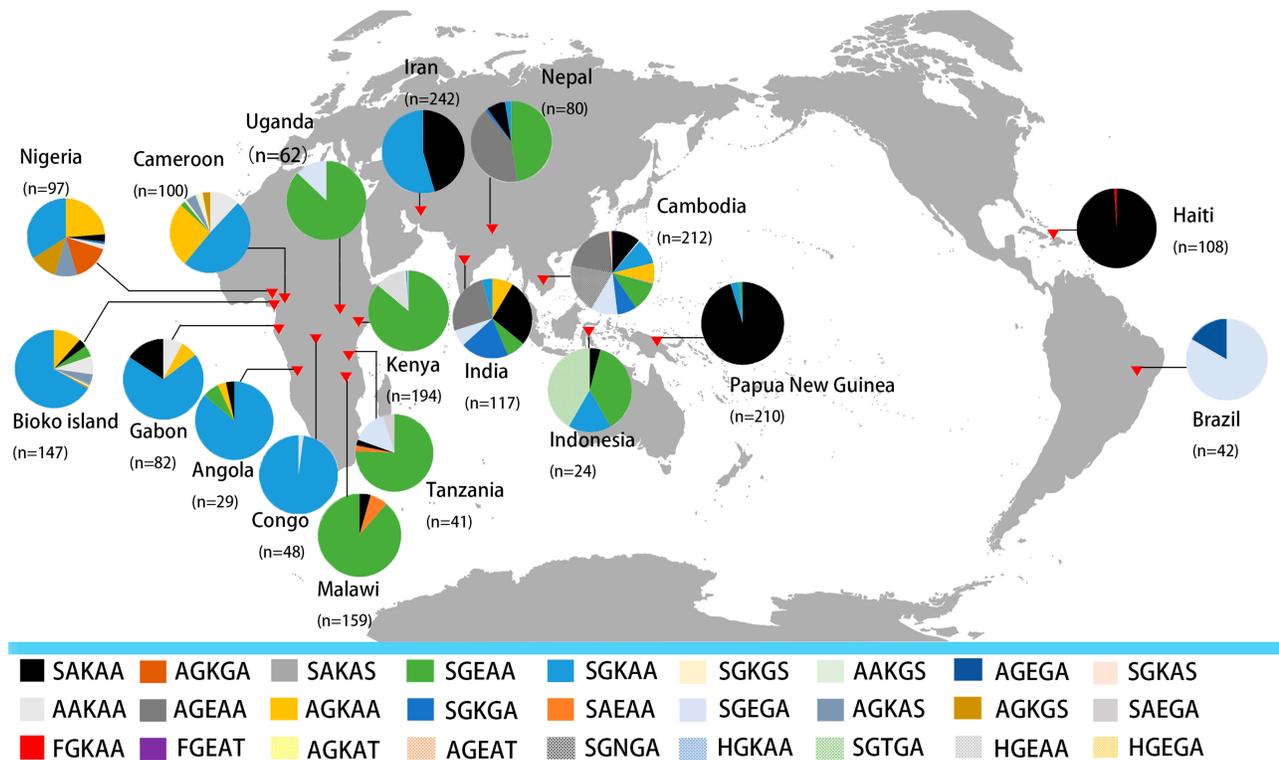
**Figure 1** Geographical distribution of *Pf dhfr* haplotype mutants from 18 countries and areas. The four capital letters denote amino acids at positions 51, 59, 108, and 164. Different haplotypes are identified by different colours.

profiles was found between East African countries and West African countries along the Atlantic Ocean (Figure 2). SGKAA has been reported to be the dominant haplotype in most African countries along the Atlantic Ocean, including Cameroon,<sup>33</sup> Nigeria,<sup>38</sup> Gabon,<sup>37</sup> Angola,<sup>35</sup> Democratic Republic of Congo<sup>41</sup> and Bioko Island, and SGEAA is the major haplotype in East African countries, such as Uganda,<sup>29</sup> Tanzania,<sup>29</sup> Kenya<sup>36</sup> and Malawi.<sup>32</sup> The wild-type haplotype (SAKAA) is rare in almost all African and Asian countries but prevalent in Haiti<sup>42</sup> and Papua New Guinea.<sup>30</sup>

The combination of 3 *Pf dhfr* and 5 *Pf dhps* haplotypes was found in 128 samples, and 14 *Pf dhfr*-*Pf dhps* haplotypes were determined; triple *Pf dhfr* and single *Pf dhps* mutations (IRNI-SGKAA) were most prevalent (59.4%, 76/128), followed by triple *Pf dhfr* and quintuple *Pf dhps* mutations (IRNI-AGKAA) (10.9%, 14/128) and triple *Pf dhfr* and single *Pf dhps* mutations (IRNI-AAKAA) (7.8%, 10/128). The wild-type haplotype (NCSI-SAKAA) was not found in this study (Table S2).

## Relationship Among *Pf dhfr* and *Pf dhps* Sequences Between Bioko and Global Parasites

To assess the relationship among *Pf dhfr* sequences between Bioko and global *Pfalciparum* isolates, 603 sequences, including 352 from this study and our previous study<sup>25</sup> and 251 of other African and Asian countries (India, Congo, and Nigeria) downloaded from NCBI,<sup>34,38,41</sup> were used to construct a haplotype network (Figure 3). For all SNPs (nonsynonymous and synonymous mutations) located in the *Pf dhfr* gene, 21 distinct haplotypes (H\_1 to H\_21) were found worldwide (Figure 3). H\_1, the most prevalent haplotype in African countries, is associated with Asian sequences through H\_3 and H\_2. In addition, the network suggested that the majority of rare African haplotypes may have originated from the H\_1 haplotype. Notably, approximately half of the *Pf dhfr* haplotypes only share sequences with Bioko parasites (10/21), indicating a high number of genetic polymorphisms of this gene in the Bioko parasitic population.



**Figure 2** Geographical distribution of *Pfdhps* haplotype mutants from 18 countries and areas. The five capital letters denote amino acids at positions 436, 437, 540, 581, and 613. Different haplotypes are identified by different colours.

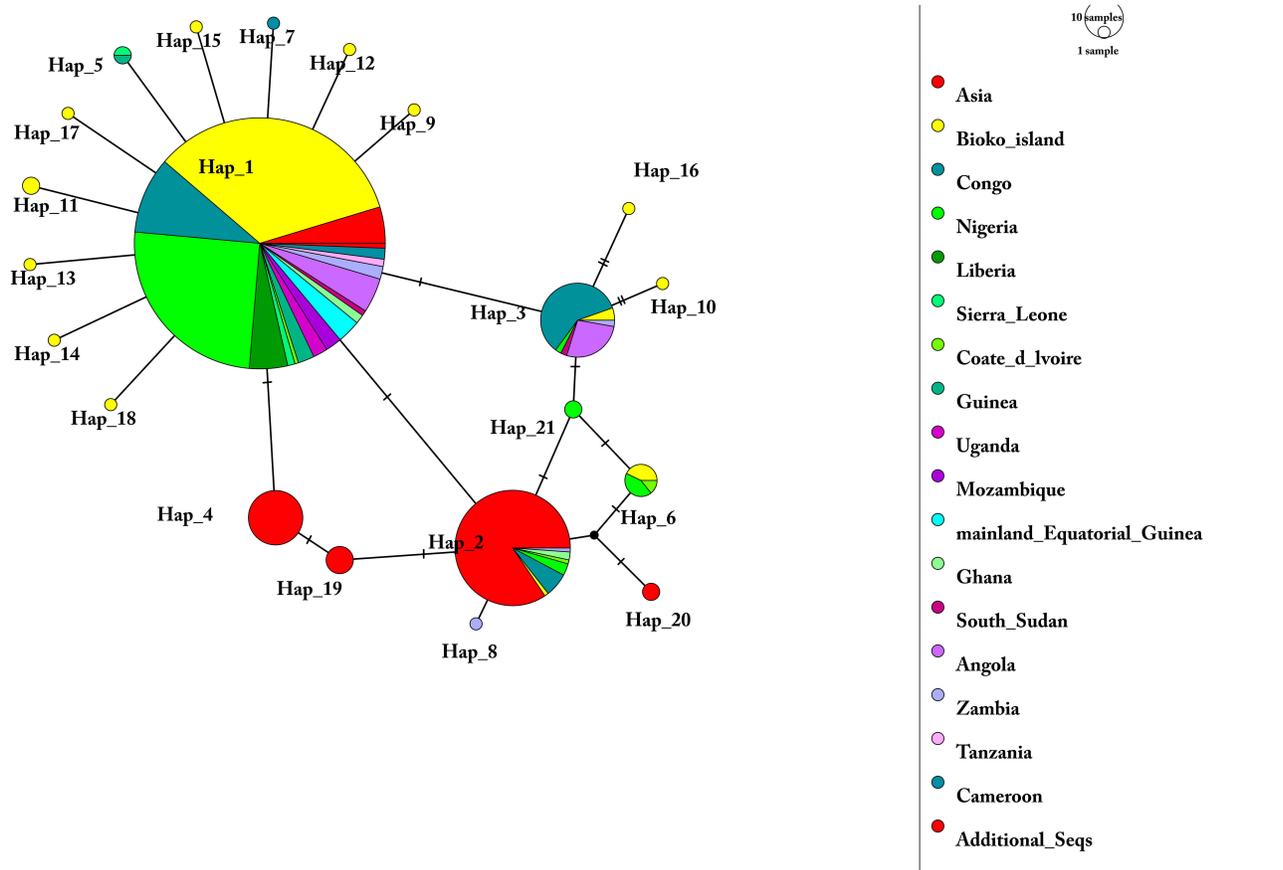
A total of 589 *Pfdhps* sequences were used to construct another haplotype network (Figure 4). These sequences included 327 from this study and our previous study<sup>25</sup> and 262 of other African and Asian countries (India, Congo, and Nigeria) downloaded from NCBI.<sup>34,38,41</sup> For all SNPs (non-synonymous and synonymous mutations) in the *Pfdhps* gene, a total of 23 distinct haplotypes (H\_1 to H\_23) were identified (Figure 4). Eight haplotypes are shared by *Pfdhps* sequences from at least two countries; 15 haplotypes were found for one sequence. Consistent with *Pfdhfr*, most of them were found to be distributed on Bioko (8/15), which also indicates rich genetic polymorphisms among Bioko parasites. The most prevalent haplotype, H\_1, with a percentage of 53.1%, has been found in Asia and 11 countries in Africa. Most haplotypes are shared with intercontinental *Pfdhps* sequences.

## Discussion

In the current study, a large amount of mutations in the *Pfdhfr* and *Pfdhps* genes related to SP resistance was found among samples collected on Bioko Island at different time periods. Though there is still a lack of direct evidence to demonstrate that the treatment failures linked to SP

resistance on Bioko Island, the frequencies of most mutations showed a slow and persistent increasing trend over the 7 years (2011–2017) investigated. When a country or region withdraws a given treatment due to drug resistance, the sensitive parasite population increases its presence with respect to the resistant population over a given time period. On Bioko, although the Ministry of Health withdrew SP as a first-line treatment for uncomplicated malaria after 2006,<sup>12</sup> SP is still used as an alternative drug for IPTp and as one of the compositions of ACT therapy, which may explain the increasing trend of molecular markers associated with SP resistance in the region.

Similar to previous reports,<sup>12,20</sup> two *Pfdhfr* mutations (N51I and S108N) in the Bioko parasite population kept its high frequency during the study period (2011–2017) and even reached 100% in recent years (2013–2017). This result might indicate that the two mutations have been fixed in the Bioko parasite population, which might influence the effectiveness of SP applied on Bioko because these mutations are related to pyrimethamine resistance. *Pfdhfr* I164L is an important mutation implicated in the effectiveness of SP as IPT, both in children under 5 years



**Figure 3** Network of *Pfdhfr* haplotypes for 603 *Plasmodium falciparum* isolates from 16 African countries and Asia. The network was constructed by using the POPART program with the median-joining algorithm. The size of each pie indicates the proportion of the haplotype frequencies. Different colours in each pie indicate different countries or regions. Additional\_Seqs stands for 3D7 isolates.

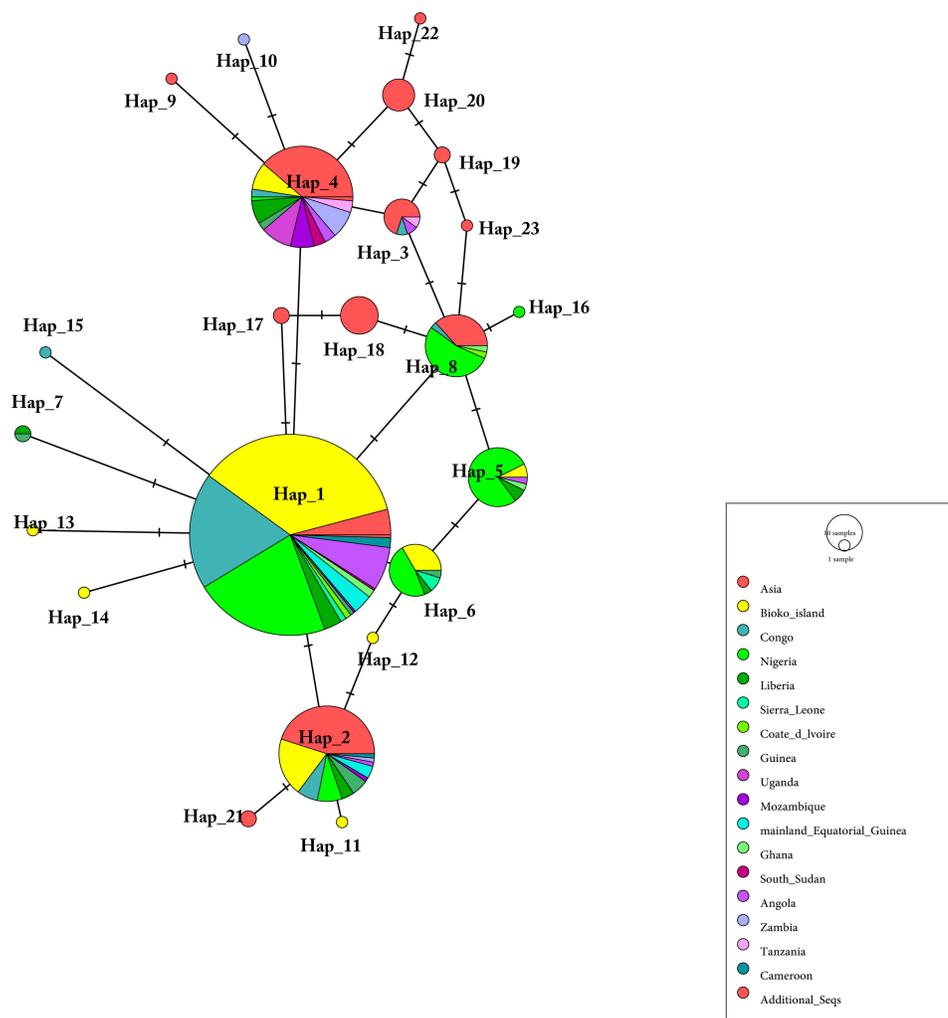
old and pregnant women,<sup>12</sup> and this mutation has appeared on the Equatorial Guinea mainland. Fortunately, this mutation was absent among Bioko isolates.

The current WHO recommendations suggest that SP-IPTp should be discontinued if the frequency of K540E is more than 50.0% and that of A581G is above 10.0%. Fortunately, the frequencies of these two mutations on Bioko Island did not reach the standard yet. For this reason, SP-IPTp should still be effective on Bioko Island. Nonetheless, the Bioko Malaria Control Program (BIMCP) and the Ministry of Health and Social Welfare of Equatorial Guinea should implement measures on the island to control the spread of these mutations. Moreover, it is important to prevent an increase in the frequency of these two mutations to avoid reducing the efficiency of IPT-SP.

Consistent with our previous finding (2013–2014), this study revealed an intense level of *Pfdhfr* triple mutants (IRNI) and a single-mutant *Pfdhps* haplotype (SGKAA) among Bioko parasites.<sup>20</sup> Global data<sup>27–36</sup> were also applied in the comparison analysis, and the patterns for

African countries in this study were in line with previous research.<sup>43</sup> High similarity of the distribution of *Pfdhfr* and *Pfdhps* haplotypes was found between Bioko Island and its neighbouring countries. Combined with information from the network analysis, this result indicates that the origin of the *Pfdhfr* and *Pfdhps* haplotypes in Bioko parasites is most likely related to the African mainland.

SP-resistant parasites can be classified into three groups: “partially resistant”, “fully resistant” and “super resistant” based on *Pfdhfr*-*Pfdhps* haplotypes. In particular, the combination of the triple *Pfdhfr* mutant (N51I, C59R, S108N; haplotype IRNI) and *Pfdhps* A437G (haplotype SGKAA) confers partial resistance; the combination of the triple *Pfdhfr* mutant (N51I, C59R, S108N; haplotype IRNI) and double *Pfdhps* mutant (A437G, K540E; haplotype SGEAA) confers full resistance; and the combination of the triple *Pfdhfr* mutant (N51I, C59R, S108N; haplotype IRNI) and triple *Pfdhps* mutant (A437G, K540E, A581G; haplotype SGEGA) confers super resistance.<sup>44</sup> These different combinations of mutations or haplotypes



**Figure 4** Network of *Pfdhps* haplotypes for 589 *Plasmodium falciparum* isolates from 16 African countries and Asia. The network was constructed by using the POPART program with the median-joining algorithm. The size of each pie indicates the proportion of the haplotype frequencies. Different colours in each pie indicate different countries or regions. Additional\_Seqs stands for 3D7 isolates.

might affect the results of IPT in infected pregnant women and children. However, our data, fortunately, demonstrated that partially SP-resistant (IRNI-SGKAA) parasite was moderately popular and fully SP-resistant (IRNI-SGEAA) parasite was still rare on Bioko Island. Indeed, no super SP-resistant parasites were found on Bioko Island. Based on current evidence, IPTp-SP remains effective in preventing the adverse consequences of malaria on maternal and foetal outcomes on Bioko Island.

## Conclusion

Historically, SP has been extensively used on Bioko by the Ministry of Health of Equatorial Guinea. Our findings reveal the general existence of SP-resistance markers on Bioko Island even after the replacement of SP for first-line treatment of uncomplicated malaria in 2006. Although no significant

difference was observed in different time periods ( $p > 0.05$ ), our study found a slow increasing trend of the frequencies of these SP-resistance markers among Bioko parasites over the 7 years evaluated. Therefore, it is very important to control the use of SP and to ensure that it is used exclusively for IPT in children under 5 years old and pregnant women. Furthermore, to halt the SP mutations, it is urgent to call for careful monitoring of genotypic resistance markers and in vivo validation of IPT efficacy. Nowadays, SP is not recommended for use as a daily treatment on Bioko Island, either alone or in combination with another treatment (artesunate+SP; AS+SP). This strategy will limit increases in the number of mutations, the occurrence of new mutations, and in particular, the spread of mutations to protect the population more effectively. In addition, continuous molecular monitoring and additional control efforts are urgently needed.

## Abbreviations

IPT, intermittent preventive treatment; IPTp, intermittent preventive treatment in pregnancy; SP, sulfadoxine-pyrimethamine; ACT, artemisinin-based combination therapy; SNP, single-nucleotide polymorphisms; *P.falciparum*, *Plasmodium falciparum*; *P.ovale*, *Plasmodium ovale*; *P.malariae*, *Plasmodium malariae*; *P.vivax*, *Plasmodium vivax*; *Pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *Pfdhps*, *Plasmodium falciparum* dihydropteroate synthase.

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## Disclosure

The authors declare that they have no competing interests in this work.

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