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Mapping antimalarial drug resistance markers in Plasmodium falciparum isolates across Benin

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Research Article

Keywords: Malaria, SP, drug resistance, Pfdhfr, Pfdhps, Pfmdr1, Pfcrt, PfK13, PfKelch, Illumina sequencing, mapping, chemoprevention, PMC

Posted Date: November 20th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11594721/v1

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Additional Declarations: No competing interests reported.

Mapping antimalarial drug resistance markers in *Plasmodium falciparum* isolates across Benin

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Abstract:

Background: Perennial malaria chemoprevention (PMC) with sulfadoxine-pyrimethamine (SP) is recommended for children less than two years of age. However, SP resistance, which is attributable to a combination of mutations in the *Pfdhps* and the *Pfdhfr* genes, reduces the duration of SP protective efficacy. The present study aims to provide geographically complete information on the status of

antimalarial drug resistance in *P. falciparum* across Benin to inform the subnational tailoring of antimalarial chemoprevention and treatment.

Method: We conducted cross-sectional sampling of parasites from each of the 12 administrative departments of Benin during 2023. In each department, health facilities where the Ministry of Health conduct routine TES were chosen as sites of collection. Finger-prick blood samples from consenting symptomatic malaria patients of all ages were collected and the MOSAIC Illumina next-generation sequencing platform used to analyse *Pfdhfr*, *Pfdhps*, *PfK13*, *Pfcrt* and *Pfmdr1*.

Results: In total, 1,731 samples were analysed. In the *Pfdhfr* gene, the triple mutant **IRN**I was detected at (> 90%) in all sites, and at *Pfdhps,* the single mutant 437G was recorded at >90% in all sites. The novel emerging *Pfdhps* haplotype **V**A**G**K**GS** (which combines mutations 431**V**, 437**G**, 581**G** and 613**S**) was detected at low prevalence in all sites, most commonly in Couffo (9%). The *Pfmdr1* haplotypes NFD and N**Y**D (codon 86-184-1246) were widespread with NFD in the majority. The *Pfcrt* CV**IET** mutant haplotype was significantly more common in the south of Benin. Finally, no *PfK13* WHO candidate or validated mutations were identified at any of the sites.

Conclusion: Our study captures the distribution of molecular markers of antimalarial drug resistance across Benin. The absence of *PfK13* WHO candidate or validated mutations in any region is reassuring for the continuing use of ACTs, however the emergence of *Pfdhps* 431**V** in the *Pfdhps* haplotype **V**A**G**K**GS** may present a challenge to the protective efficacy of SP-based chemoprevention in the future. Standardized maps of drug resistance in Benin provide support for sub-national tailoring of chemoprevention and treatment and a baseline for future surveillance.

Keywords: Malaria, SP, drug resistance, *Pfdhfr*, *Pfdhps*, *Pfmdr1*, *Pfcrt*, *PfK13, PfKelch, Illumina sequencing, mapping, chemoprevention, PMC.*

Introduction

In 2025, malaria was responsible for 610,000 deaths and 282 million cases globally **(1)**. The sub-Saharan African region bears the greatest burden, accounting for 94% of cases and 95% of deaths, primarily among children under five years of age **(1)**.

In Benin, malaria is the leading cause of morbidity and mortality among children under five years of age. According to the Ministry of Health's statistical report, malaria accounted for 49.4% of illnesses and 19.8% of deaths recorded during consultations and hospitalizations in 2022. Malaria burden in Benin is generally higher in northern regions than in the south, and the heterogeneity in transmission patterns necessitates region-specific strategies for effective control and potential elimination **(2–4)**. The Benin National Malaria Control Program (NMCP) implements multiple malaria control strategies nationally, including the use of long-lasting insecticide-treated nets, treatment of uncomplicated malaria cases with artemisinin-based combination therapies, and intermittent preventive treatment of pregnant women. A geographically stratified approach to malaria chemoprevention has been adopted in Benin, with seasonal malaria chemoprevention (SMC) implemented in five departments: Atakora, Alibori, Donga, Borgou and Collines. The programme was initially limited to Atakora and Alibori but has recently been expanded to include the three additional departments. Perennial malaria chemoprevention (PMC) is implemented in the south in Atlantique, Couffo, Littoral, Mono, Oueme, Plateau and Zou. For PMC, SP is administered to children under two years of age, based on an 8-contact model aligned with the vaccination schedule (at 10 weeks, 14 weeks, 9 months and 15 months) and Vitamin A supplement (at 6 months, 12 months, 18 months and 24 months) **(5)**.

All current chemoprevention interventions rely heavily on sulfadoxine-pyrimethamine (SP), with PMC and intermittent preventive treatment of malaria in pregnancy (IPTp) using SP alone, and SMC using a combination of SP with amodiaquine (AQ). The continued efficacy of SP is therefore critical to the success of these chemopreventive interventions, and timely surveillance of SP and AQ resistance across all administrative regions is essential to inform and support its stratified implementation **(6)**.

Drug resistance levels can vary significantly within a single country and geographically representative surveillance is increasingly recognized as essential. Studies in Tanzania **(7)**, Uganda **(8)**, Zambia **(9)** and the Democratic Republic of Congo (DRC) **(10)** have all demonstrated significant sub-national variation in resistance patterns. Standardized sampling across all regions during the same time period is crucial to ensure comparability across sites and to provide an accurate national picture which is adequate for the support of stratification.

Antimalarial drug resistance is driven by mutations in key *Plasmodium falciparum* genes. Resistance to SP is associated with accumulation of mutations in both the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) and the *P. falciparum* dihydropteroate synthase gene (*Pfdhps*), which confer resistance to pyrimethamine and sulfadoxine respectively. Commonly these mutations are in codons 51-59-108 and 164 of *Pfdhfr* and in codons 431-436-437-540-581 and 613 of *Pfdhps* **(11)**. In East and Southern Africa, the haplotype combination of *Pfdhfr* triple mutant (51I, 59R,108N, I164) with *Pfdhps* double mutant (I431-436A-437G-540E-581A-613A) **IRN**I-IS**GE**AA is strongly associated with SP treatment failure **(12)**. While in West and Central Africa **IRN**I-IS**G**KAA and **IRN**I-IA**GK**AA are common and associated with partial SP resistance **(13–17)**. New variants of *Pfdhps*, such as 431**V**-436**A**-437**G**-540K-581**G**-613A (**V**A**G**K**G**A) and 431**V**-436**A**-437**G**-540K-581**G**-613**S** (**V**A**G**K**GS**), are emerging in Nigeria and Cameroon **(15,18)**, but their impact on SP efficacy remains unclear.

Mutations in other genes are associated with resistance to other antimalarial drugs. The *P. falciparum* chloroquine transporter gene (*Pfcrt*) specifically SNPs in codons 74-75-76 are associated with chloroquine resistance (CQR). Two main haplotypes: the wild-type CVMNK and the CQR mutant CV**IET** are distributed across all African regions **(19)** and modify parasite susceptibility to both chloroquine and amodiaquine. Also contributing to resistance profiles of *P. falciparum* is multidrug resistance gene 1 (*Pfmdr1*). Mutations 86**Y**, 184**Y** and 1246**Y** have been associated to resistance to amodiaquine in therapeutic studies in Africa **(20)**.

The *P. falciparum* Kelch13 (*PfK13*) gene has been associated with delayed parasite clearance time following artemisinin-based combination therapy treatment **(21)**. Mutations in the propeller domain of *PfK13* have been explored in numerous studies and specific SNPs are experimentally confirmed as molecular markers of partial artemisinin-resistant malaria. The WHO has published a list of validated markers of artemisinin resistance, and classified others as candidate or potential markers of resistance **(22)**. On the sub-Saharan African continent, validated SNPs are predominantly emerging *de novo* in East and Southern Africa and currently include F446I, N458Y, C469Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y, R622I and A675V **(23)**. So far the reported prevalence of validated non-synonymous *PfK13* mutations in West Africa is rare **(24–26)**.

Previous studies have reported the prevalence of *Pfdhfr*, *Pfdhps*, *Pfcrt* and *Pfmdr1* mutations in some parts of Benin **(27–30)**, but there is so far no comprehensive understanding of the spatial distribution of markers of drug resistance across the whole country because studies were done separately and samples collected at different times. Knowing that resistance is a dynamic phenomenon we aimed to generate a national profile of drug resistance by sampling all sites at the same time and carrying out genotyping with a standardized methodology, and providing national maps of drug resistance to the Benin Ministry of Health.

Methodology

Study sites and sample collection.

Between August 2023 and January 2024, a total of 1,828 *P. falciparum*-positive blood samples were collected from malaria patients attending health facilities in 24 districts across all 12 departments of Benin. These included five departments in the north eligible for seasonal malaria chemoprevention (SMC) and seven departments in the south eligible for perennial malaria chemoprevention (PMC) (Table 1).

Sampling strategy

Samples were collected to determine the prevalence of circulating drug-resistance genotypes at the departmental level, the first administrative division in Benin. We estimated that 114 participants with

available haplotype data would be required to determine a 5% prevalence with 4% absolute precision and 95% confidence level. Assuming that 20-30% of samples may not be successfully genotype sequenced (or haplotype determined), we estimated that a sample size of approximately 150 dried blood spots (DBSs) per department was required. In each department, one to three public health facilities were chosen for sample collection. Public health facilities were selected within each department based on their reported patient attendance (at least 100 visits per month) and malaria caseload (at least 50 cases per month), using hospital records from 2021–2022.

Ethical approval

The study protocol was approved by the National Health Research Ethics Committee of the Ministry of Health of Benin (Reference; CNERS036/2023, session du 14 Juillet 2023), by the World Health Organization Research Ethics Review Committee (ERC Reference number 0003949), and by the LSHTM Research Ethics committee (LSHTM Reference number 29281).

Study design

Patients of all ages attending the selected health facilities during the study period were eligible for inclusion if they had a confirmed diagnosis of uncomplicated *Plasmodium falciparum* malaria by microscopy or rapid diagnostic test (RDT), according to the routine diagnostic practice at each facility. Following confirmation of the diagnosis, eligible patients were approached and invited to participate in the study and amples were collected from those who agreed to participate. Participants received a brief explanation of the study and age-appropriate informed consent procedures were used to explain risks and benefits to patients or caregivers. Informed consent was obtained from adult participants aged 18 years and above and from the parents or legal guardians of children aged under 18 years. Additional assent was obtained from children aged 10–17 years. An impartial witness was required if the patient or caregiver could not read or write. Those who agreed signed the consent form or provided a thumbprint if unable to write. Assenting participants completed a short questionnaire to confirm their understanding and

agreement. No personal information, including age and sex, was collected from patients. Participant information sheets and consent forms were provided in French. For participants who did not speak French, the information was verbally translated into Nfon, a widely spoken local language in Benin, during the informed consent process. This language is not commonly written, so we relied upon an oral translation and purposely recruited sample collectors who were proficient in the local language. Treatment for malaria was provided for all patients as per national guidelines whether or not they chose to participate in the study.

Sample storage and transportation

The data collected for each sample was limited to the following: 1. Name of study staff collecting sample. 2. Study site (Name of health facility and department). 3. Date and time of collection (dd/mm/yy and 00:00 am/ pm). 4. Diagnostic tests used and result (RDT or Microscopy) 5. Pre-generated barcode number from sticker, inclusive of country ID and sample ID number.

Dried blood spot (DBS) samples were individually enclosed cardboard covers and stored at room temperature in plastic boxes containing desiccant. Samples were registered in a central repository using a standardized spreadsheet template to record barcode numbers and the data fields described above. The samples were subsequently shipped by courier at ambient temperature to the London School of Hygiene & Tropical Medicine (LSHTM), London, UK, where they were logged into the laboratory information system and stored at room temperature, with desiccant.

DNA extraction and initial molecular analysis

A centralized "MOSAIC" genotyping workflow was implemented in accordance with Hansson *et al.* **(10)** to ensure standardized procedures and data comparability. DNA extraction and qPCR confirmation was performed at the London School of Hygiene & Tropical Medicine, and then sequencing and genotypic analyses were conducted at the University of Copenhagen, with the participation of visiting scientists from Benin.

Genomic DNA from *P. falciparum* dried blood spots was extracted using an automated high-throughput platform and multiplex qPCR screening performed according to published protocols **(31,32)**.

Targeted amplicon sequencing

Drug resistance genotyping was performed using multiplex amplicon-based Illumina deep sequencing with dual indexing. This approach targeted key resistance markers *Pfdhfr*, *Pfdhps*, *Pfcrt*, *Pfmdr1* and *Pfk13* and the methodology previously described **(10,33)**. Quality control of Illumina reads and the analyses of sequence data was carried out as described by Hansson *et al* **(10)**.

Data management and analysis

Data processing and analysis was carried out according to protocols described by Hansson *et al* **(10)**. The prevalence of mutations in the *Pfdhfr*, *Pfdhps*, *Pfcrt*, *Pfmdr1* and *PfK13* genes was recorded as being mutant (having only the mutant allele), or as wild type (having only the wild type allele), or as mixed (having both wild type and mutant sequence present in the same infection). The frequency of haplotypes for each gene was mapped across the twelve departments of Benin, using complete (fully sequenced) haplotypes, which were unmixed at the codons of interest. Data visualization was performed in R version 4.3.1. (**https://www.R-project.org/**) National boundaries for Benin were obtained from the global administrative areas database (GADM, version 4.0) (**https://gadm.org**).

Results

Of 1828 *P. falciparum* RDT-positive patient samples in the 12 administrative departments, 1731 samples (94.7%) were successful for analysis of molecular markers of drug resistance markers.

Prevalence of *Pfdhfr* and *Pfdhps* mutations in patient infections from the twelve departments.

Mutations in *Pfdhfr* at codons 51, 59 and 108 exceed 86% in all departments and approached 100% for the 59**R** and 108**N**, while only wild type was observed at codon 164 (Supplementary Table 2). Combining

193 *Pfdhfr* codons to construct haplotypes, we observed six different *Pfdhfr* haplotypes whose geographic
194 distribution is shown in Figure 1 (and full haplotype data presented in Supplementary Table 3). The triple
195 mutant **IRN**I haplotype was highly prevalent across all the twelve departments (> 90%) while the wild-
196 type haplotype NCSI was absent or are (<2%).

197 For *Pfdhps,* the 437**G** mutation exhibited a very high prevalence ranging from 91% to 100% among the
198 12 departments (Supplementary Table 2). The *Pfdhps* 581**G** mutation was comparatively rare, with
199 prevalences between 1.0% and 10.7%. The 613**S** mutant, excluding mixed 613A/**S** infections showed
200 moderate prevalence, from 5.0% to 24.0%, with higher proportions again recorded in the south, notably
201 reaching 24% in Littoral and 17.5% in Plateau. Similarly, the *Pfdhps* 431**V** mutation was detected in all
202 departments with frequencies ranging from 3% to 11% and again with higher prevalence in southern
203 departments, particularly in Couffo (11%) and Littoral, Mono, Oueme (10%) (Supplementary Table 2).
204 By contrast, the 540**E** genotype was rare, being identified in two unmixed samples originating from
205 Oueme, and two mixed samples one from Atakora and one from Couffo.

206 Combining *Pfdhps* codons 431, 436, 437, 540, 581 and 613 a very large number of haplotypes were
207 identified. Codon 436 is highly polymorphic, occurring in both resistant and susceptible wildtype *Pfdhps*
208 haplotypes. To simplify haplotype classification the 436 is shown as a"*" due to the multiple variations
209 presented (A, S and AS) and the speculative significance of this codon regarding SP susceptibility. The
210 distribution of eleven key haplotypes is shown in figure 2 (with full data in Supplementary Table 3). The
211 single-mutant haplotype (I-*-**G**-K-A-A haplotype) characterized by the presence of 437**G** only, was the
212 most common (> 90%). Haplotypes containing the 431**V** mutation included the *Pfdhps* **V**AAKAA,
213 **V**A**G**KAA, **V**A**G**KA**S** and **V**A**G**K**GS. The V**A**G**K**GS** haplotype was detected in all the 12 departments
214 at varying frequencies, with a trend toward higher frequencies in the southern departments (e.g. Couffo,
215 Littoral and Oueme at 8.6%, 4,9% and 5,5%, respectively) but there was not a statistically significant
216 difference between frequencies in the southern and the northern departments (χ^2= 0.02, p= 1).

217 **Prevalence of *Pfmdr1*, *Pfcrt*, and *Pfk13* mutations in patient infections from the twelve departments.**

In *Pfmdr1*, mutations were observed at codons 86, 184 and 1246, but not at codons 1034 and 1042. The 86**Y** mutant was present at low prevalence (< 11%) across all departments (details in supplementary table 2). The prevalence of 184**F** mutation ranged from 61.7% in Atlantique to 80.5% in Oueme and only two cases of the 1246**Y** were detected, both in Donga (Supplementary Table 2).

Four *Pfmdr1* haplotypes were identified and their distribution among the study sites is shown in Figure 3 (data in Table 3). The haplotype N**F**SND was most predominant, followed by the wild type haplotype NYSND. The **YF**SND haplotype was detected at low frequencies ranging from 0%-5%. The **YY**SND was present at low frequency (2%) in Atakora and Alibori and absent from other departments.

In Pfcrt three haplotypes were recorded, the mutant CV**IET** haplotype and wild type CVMNK and a single observation of CV**IN**T haplotpe in Atakora. The mutant CV**IET** haplotype was less common in the north (Donga 5% Alibori and Borgou 6%, Atakora 9% and Collines 20%) than in the south (Couffo 13%, Zou33%, Mono 37%, Plateau 40%, Atlantique 43%, Littoral 47%, and Oueme 53%) (Table 3; Figure 4). There was a statistically significant difference between regions (χ^2 =14,57, p= 0. 006).

Overall, the *Pfk13* profile was characterized by substantial genetic diversity, with a predominance of non-synonymous over synonymous variants, all listed in Table 4. Certain mutations were seen to occur in relatively high numbers, notably K189T (n=566), K189N (n=70), N119Q (n=49), R255K (n=36), G112Q (n=13), L258M (n=9), and H136N and N217H (n=8 each). Other non-synonymous mutations detected at low frequency, included premature stop codons observed at codon positions 290, 391, 470, 518, 611 and 619. Synonymous polymorphisms are listed in full in Supplementary table 4, the most commonly observed were C469C (n=29), V589V (n=7), G690G (n=5), T207T (n=4) and A676A (n=4).

Discussion

To support optimization of malaria chemoprevention strategies and ensure their sustained efficacy, continuous monitoring of the emergence and spread of resistance-associated mutations is essential. This

study was part of the Unitaid-funded Plus Project evaluation of SP suitability for PMC, and aimed to provide up to date maps of antimalarial drug resistance across all administrative departments of Benin. We provided maps of *Pfdhfr* and *Pfdhps* which are relevant to SP efficacy, as well as *Pfcrt*, *Pfmdr1* and *Pfk13* which are important to Artemisinin and the partner drugs used in firstline treatment.

Our analysis revealed a largely homogeneous distribution of molecular resistance markers across the country. Susceptible genotypes including *Pfcrt*-CVMNK, *Pfdhps*-SAKAA and *Pfdhfr*-NCSI were rare or absent across Benin, although slightly more frequent in northern departments such as Alibori and Borgou. As expected, the triple mutant *Pfdhfr* **IRN**I haplotype was highly prevalent across all study sites, consistent with previous reports from Benin and neighboring countries **(12, 16, 33, 34, 35)**, thus departmental variability in SP resistance is almost exclusively mediated by the differences in *Pfdhps* haplotypes present and their frequency **(36)**.

The prevalence of single mutant *Pfdhps* 437**G** was high across the country while *Pfdhps* 540**E** was virtually absent, with only two cases detected in Oueme. These findings are consistent with previous studies in Benin **(29)** and aligns with broader regional patterns in Africa, where 540**E** remains uncommon in the West compared to East Africa **(12, 33)**. A key finding, relevant to chemoprevention with SP, was the presence of emerging haplotypes carrying the *Pfdhps* 431**V** mutation in combination with 581**G** and 613**S,** resulting in the *Pfdhps* **V**A**G**K**GS** haplotype. First identified in Atakora in 2019 **(30)** the *Pfdhps* **V**A**G**K**GS** haplotype was seen in every department of Benin in the current study. While the effect of this particular haplotype on SP efficacy remains unclear, its expansion in West and Central Africa suggests that it is under drug selection **(18, 37)**. The continued administration of SP in malaria chemoprevention for IPTp, PMC, and SMC may contribute to selection of this haplotype, and persistence may increase due to increasing prophylactic use of SP and thus, increasing drug pressure. The non-significant trend for higher prevalence of VAGKGS in the South of Benin could reflect differing drug pressure or greater connectivity with neighbouring regions where VAGKGS is common **(18).** An analysis of *Pfdhps* mutant

prevalence studies across Africa by Okell et al **(36)** found that *Pfdhps* mutation prevalence tends to be similar within a 300km range, likely reflecting connectivity among parasite populations.

A significant difference between parasites in the north and south was found with respect to *Pfcrt*. The CV**IET** mutant haplotype, which is associated with chloroquine and amodiaquine resistance, was lower in the north (range 5-20%) than in the south (range 13-53%). The reasons why prevalence of the CV**IET** haplotype was still high in the southern departments, suggests ongoing selection pressure and/or persistence of resistant parasites following withdrawal of chloroquine as a first line treatment for malaria in 2004.

Mutant haplotypes in *Pfmdr1* are associated with resistance to a range of different antimalarial drugs **(38,39)**. The N**F**SND, associated with reduced susceptibility to lumefantrine **(40,41)** was detected at a high prevalence throughout all the departments. In the same gene, haplotypes YFSND and YYSND, containing 86Y which is associated with resistance to chloroquine, were rare (less than 5%), suggesting limited selection of these variants.

We show a very high variability of *Pfk13* propeller domain, but importantly no validated or candidate mutations associated with partial artemisinin resistance. This finding supports the expectation of current and continued efficacy of artemisinin-based combination therapies in Benin at this time. Monitoring this gene will be helpful in supporting future decision-making around national treatment guidelines and is a valuable baseline for continued monitoring and surveillance.

Conclusions

This study provides the first comprehensive map of the national distribution of antimalarial drug resistance across all twelve departments of Benin. The absence of *Pfk13* confirmed markers of artemisinin resistance, is reassuring for the continued efficacy of first-line ACTs. The distribution of SP resistance was broadly homogeneous, with *Pfdhps* single mutant 437G haplotypes at 76% - 98% and the *Pfdhfr* triple

mutant haplotype near fixation in every department. The 431**V** was present in all departments with prevalence ranging between 2% and 14%, it was generally more common in the south of the country which could be due to connectivity with neighboring regions or countries, such as Nigeria, where reported prevalence of 431V is significantly higher. However, the presence of the *Pfdhps* 431V mutation and the VAGKGS haplotype may present problems for SP based chemoprevention in future, and we advocate for repeated national surveillance to monitor changes.

Ethics approval and consent to participate

All participants enrolled by our research team were consented using the protocol approved by relevant ethics committees. The study protocol IRB00006860 MS IRB #1-CNPERS was approved by the National Research Ethics Committee of Benin (Reference; CNERS036/2023, session du 14 Juillet 2023), by the World Health Organization Research Ethics Review Committee (ERC Reference number 0003949) and by the LSHTM Research Ethics committee (LSHTM Reference number 29281)

Consent for publication

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request. Data will be available on LSHTM Data Compass (https://datacompass.lshtm.ac.uk/) after publication.

Competing interests

The authors declare no competing interests

Funding

This work was supported by funding from the UNITAID Plus Project (Grant number 101150IC). The UNITAID project staff Ambachew Medhin Yohannes and others were consulted on Plus Project sampling collection protocol which was reviewed and approved by WHO Ethical Review Committee; (ERC Reference 0003949). Funders had no role in the genotyping analysis, or preparation of the manuscript.

Author contributions

All authors read and approved the final manuscript.

Acknowledgements

As part of the Plus project, we are very grateful to all participants and colleagues involved in this work.

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Table1: Sample collection at Department and District level across Benin

Department	**Alibori**	**Atakora**	**Atlantique**	**Borgou**	**Collines**	**Couffo**	**Donga**	**Littoral**	**Mono**	**Ouémé**	**Plateau**	**Zou**
Region	**North**	**North**	**South**	**North**	**North**	**South**	**North**	**South**	**South**	**South**	**South**	**South**
Number of selected health facilities	2	2	1	2	3	3	2	3	1	1	2	2
Health facility District	Gogounou & Kandi	Natitingou	Akassato	Bembereke &Sinende	Glazou &Dassa	Klouekanme Tchikpe & Adjahonme	Djougou	Gbegamey Ayelawadje &Cotonou	Come	Ongala	Takon , &Sakete-Ifangni	Bohicon, &Wassaho
Number of RDT+ samples collected	151	154	150	150	150	160	150	152	151	154	151	155
Number of samples sequenced	151	154	143	150	150	159	150	124	151	154	90	155

Supplementary Table2: Prevalence of SNPs in *Pfdhfr*, *Pfdhps* and *Pfmdr1* in the twelve departments of Benin.

For each codon position the number (and percentage) of samples which were non-mutant, mutant, and mixed. N shows number of samples successfully sequenced / the total amount of samples run.

Pfdhfr		**Alibori n (%)**	**Atakora n (%)**	**Atlantique n (%)**	**Borgou n (%)**	**Collines n (%)**	**Couffo n (%)**	**Donga n (%)**	**Littoral n (%)**	**Mono n (%)**	**Ouémé n (%)**	**Plateau n (%)**	**Zou n (%)**
51	N51	9 (9,2)	8 (7,0)	9 (7,0)	8 (6,0)	3 (3,0)	2 (2,0)	2 (3,0)	1 (2,0)	8 (6,0)	1 (1,2)	1 (2,0)	5 (4,0)
	51**I**	84 (85,7)	108 (91,0)	123 (92,0)	120 (88,0)	87 (96,0)	124 (98,0)	54 (92,0)	61 (98,0)	132 (92,0)	78 (96,3)	63 (98,0)	122 (95,0)
	N51I-Mix	5 (5,1)	2 (2,0)	2 (1,0)	8 (6,0)	1 (1,0)	0 (0,0)	3 (5,0)	0 (0,0)	3 (2,0)	2 (2,5)	0 (0,0)	1 (1,0)
	(N)	98/151	118/154	134/143	136/150	91/150	126/159	59/150	62/124	143/151	81/154	64/90	128/155
59	C59	1 (1,0)	0 (0,0)	0 (0,0)	2 (1,5)	3 (3,0)	0 (0,0)	1 (2,0)	0 (0,0)	2 (1,4)	1 (1,2)	0 (0,0)	1 (1,0)
	59**R**	95 (97,0)	118 (100,0)	133 (99,0)	130 (95,5)	88 (97,0)	126 (100,0)	58 (98)	62 (100,0)	140 (98)	78 (96,3)	64 (100)	126 (98,0)
	C59R-Mix	2 (2,0)	0 (0,0)	1 (1,0)	4 (3,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	1 (0,6)	2 (2,5)	0 (0,0)	1 (1,0)
	(N)	98/151	118/154	134/143	136/150	91/150	126/159	59/150	62/124	143/151	81/154	64/90	128/155
108	S108	1 (1,0)	0 (0,0)	0 (0,0)	2 (1,5)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	108**N**	96 (98,0)	112 (97,0)	133 (99,0)	131 (96,3)	133 (100,0)	126 (100,0)	116 (100,0)	62 (100,0)	143 (100,0)	81 (100,0)	64 (100,0)	128 (100,0)
	108T	0 (0,0)	1 (1,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	S108N-Mix	1 (1,0)	2 (**2**,0)	1 (**1**,0)	3 (**2**,2)	0 (0,0)	0 (0,0)	0 (0,0)	0 (**0**,0)	0 (**0**,0)	0 (**0**,0)	0 (**0**,0)	0 (0,0)
	(N)	98/151	115/154	134/143	136/150	133/150	126/159	116/150	62/124	143/151	81/154	64/90	128/155
164	I164	98 (100,0)	115 (100,0)	134 (100,0)	136 (100,0)	133 (100,0)	126 (100,0)	116 (100,0)	62 (100,0)	143 (100,0)	81 (100,0)	64 (100,0)	128 (100,0)
	164**L**	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	I164L-Mix	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	98/151	115/154	134/143	136/150	133/150	126/159	116/150	62/124	143/151	81/154	64/90	128/155

Pfdhps		**Alibori n (%)**	**Atakora n (%)**	**Atlantique n (%)**	**Borgou n (%)**	**Collines n (%)**	**Couffo n (%)**	**Donga n (%)**	**Littoral n (%)**	**Mono n (%)**	**Ouémé n (%)**	**Plateau n (%)**	**Zou n (%)**
431	I431	75 (91,5)	105 (95,0)	91 (92,0)	124(94,0)	87 (98,0)	104 (86,0)	58 (97,0)	52 (89.7)	127 (90,0)	86 (88,0)	48 (94.1)	97 (97,0)
	431**V**	6 (7,3)	4 (4,0)	4 (4,0)	5(4,0)	1 (1,0)	13 (11,0)	2 (3,0)	6 (10,3)	9 (6,0)	10 (10,0)	3 (5.9)	3 (3,0)
	I431V-Mix	1 (1,2)	1 (1,0)	4 (4,0)	3(2,0)	1 (1,0)	4 (3,0)	0 (0,0)	0 (0,0)	5 (4,0)	2 (2,0)	0 (0,0)	0 (0,0)
	(N)	82/151	110/154	99/143	132/150	89/150	121/159	60/150	58/124	141/151	98/154	51/90	100/155
436	S436	32 (48,5)	48 (48,0)	65 (78,0)	53(42,4)	61 (71,0)	65 (61,0)	23 (49,0)	31 (61,0)	99 (73,0)	74 (77,0)	29 (71,0)	53 (64,0)
	436**A**	26 (39,3)	33 (33,0)	14 (17,0)	55(44,0)	20 (23,0)	35 (33,0)	18 (38,0)	19 (37,0)	29 (21,0)	21 (22,0)	9 (22,0)	21 (25,0)
	S436A-Mix	8 (12,2)	17 (17,0)	4 (5,0)	17 (13 ,6)	5 (6,0)	6 (**6**,0)	6 (13,0)	1 (**2**,0)	8 (6,0)	1 (**1**,0)	3 (**7**,0)	9 (11,0)
	436F	0 (0,0)	3 (3,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	66/151	101/154	83/143	125/150	86/150	106/159	47/150	51/124	136/151	96/154	41/90	83/155
437	A437	5 (7,5)	7 (6,8)	0 (0,0)	10(7,8)	1 (1,0)	1 (1,0)	0 (0,0)	0 (0,0)	0 (0,0)	4 (4,0)	0 (0,0)	1 (1,0)
	437**G**	61 (91,0)	93 (91,2)	84 (100,0)	117 (90,7)	84 (97,0)	104 (98,0)	49 (100,0)	49 (100,0)	134 (99,0)	90 (95,0)	42 (100,0)	83 (99,0)
	A437G-Mix	1 (1,5)	2 (2,0)	0 (0,0)	2(1,5)	2 (2,0)	1 (1,0)	0 (0,0)	0 (0,0)	1 (1,0)	1 (1,0)	0 (0,0)	0 (0,0)
	(N)	67/151	102/154	84/143	129/150	87/150	106/159	49/150	49/124	135/151	95/154	42/90	84/155
540	K540	84 (100,0)	110 (99,0)	98 (100,0)	133(100,0)	131 (100,0)	121 (99,0)	115 (100,0)	52 (100,0)	136 (100,0)	95 (98,0)	51 (100,0)	98 (100,0)
	540**E**	0 (0,0)	0 (0,0)	0 (0,0)	0(0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	2 (2,0)	0 (0,0)	0 (0,0)
	K540E-Mix	0 (0,0)	1 (1,0)	0 (0,0)	0(0,0)	0 (0,0)	1 (1,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	84/151	111/154	98/143	133/150	131/150	122/159	115/150	52/124	136/151	97/154	51/90	98/155
581	A581	78 (91,7)	109 (97,0)	92 (92,0)	127(95,5)	128 (97.7)	109 (89,3)	113 (97,0)	49 (91,0)	131 (94,0)	90 (93,0)	52 (93,0)	104 (99,0)
	581**G**	4 (5,0)	2 (2,0)	4 (4,0)	4(3)	2 (1,5)	9 (7,4)	2 (2,0)	4 (7,0)	6 (4,0)	7 (7,0)	4 (7,0)	1 (1,0)
	A581G-Mix	3 (3,3)	1 (1,0)	4 (4,0)	2(1,5)	1 (0.8)	4 (3,3)	1 (1,0)	1 (2,0)	3 (2,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	85/151	112/154	100/143	133/150	131/150	122/159	116/154	54/124	140/151	97/154	56/90	105/155
613	A613	74 (86,0)	92 (82,0)	83 (83,0)	117(88,0)	111 (85,0)	93 (76,0)	107 (92,0)	40 (74,0)	117 (83,0)	81 (84,0)	41 (72,0)	92 (87,5)
	613**S**	9 (10,5)	8 (7,0)	11 (11,0)	8(6,0)	13 (10,0)	19 (16,0)	6 (5,0)	13 (24,0)	16 (11,0)	13 (13,0)	10 (17,5)	8 (7,5)
	A613S-Mix	3 (3,5)	12 (11,0)	6 (6,0)	8(6,0)	7 (5,0)	10 (8,0)	3 (3,0)	1 (2,0)	8 (6,0)	3 (3,0)	6 (10,5)	5 (5,0)
	(N)	86/151	112/154	100/143	133/150	131/150	122/159	116/154	54/124	141/151	97/154	57/90	105/155

Pfmdr1		**Alibori n (%)**	**Atakora n (%)**	**Atlantique n (%)**	**Borgou n (%)**	**Collines n (%)**	**Couffo n (%)**	**Donga n (%)**	**Littoral n (%)**	**Mono n (%)**	**Ouémé n (%)**	**Plateau n (%)**	**Zou n (%)**
86	N86	93 (95,0)	104 (89,0)	132 (99,0)	127 (94,1)	89 (99,0)	96 (97,0)	59 (97,0)	61 (100,0)	141 (98,0)	87 (100,0)	64 (100,0)	125 (97,0)
	86**Y**	3 (3,0)	6 (5,0)	1 (1,0)	6 (4,4)	1 (1,0)	3 (3,0)	1 (1,5)	0 (0,0)	3 (2,0)	0 (0,0)	0 (0,0)	2 (1,5)
	N86Y-Mix	2 (2,0)	7 (6,0)	0 (0,0)	2 (1,5)	0 (0,0)	0 (0,0)	1 (1,5)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	2 (1,5)
	(N)	98/151	117/154	133/143	135/150	90/150	99/159	61/154	61/124	144/151	87/154	64/90	129/155
184	Y184	36 (37,5)	23 (20,0)	51 (38,3)	27 (20,0)	40 (30,5)	30 (31,0)	31 (26,0)	22 (37,0)	43 (30,0)	17 (19,5)	22 (34,0)	34 (26,0)
	184**F**	48 (50,0)	71 (61,0)	65 (49,0)	91 (67,0)	78 (59,5)	61 (63,0)	74 (62,0)	33 (55,0)	86 (60,0)	64 (73,5)	34 (53,0)	85 (66,0)
	Y184F-Mix	12 (12,5)	22 (19,0)	17 (12,7)	17 (13,0)	13 (10,0)	6 (6,0)	14 (12,0)	5 (8,0)	15 (10,0)	6 (7,0)	8 (13,0)	10 (8,0)
	(N)	96/151	116/154	133/143	135/150	131/150	97/159	119/154	60/124	144/151	87/154	64/90	129/155
1034	S1034	78 (100,0)	109 (100,0)	128 (100,0)	127 (100,0)	88 (100,0)	126 (100,0)	57 (100,0)	62 (100,0)	72 (100,0)	92 (100,0)	49 (100,0)	113 (100,0)
	1034**C**	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	S1034C-Mix	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	78/151	109/154	128/143	127/150	88/150	126/159	57/154	62/124	72/151	92/154	49/90	113/155
1042	N1042	78 (100,0)	109 (100,0)	128 (100,0)	127 (100,0)	88 (100,0)	126 (100,0)	57 (100,0)	62 (100,0)	72 (100,0)	92 (100,0)	49 (100,0)	113 (100,0)
	1042**D**	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	N1042D-Mix	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	78/151	109/154	128/143	127/150	88/150	126/159	57/154	62/124	72/151	92/154	49/90	113/155
1246	D1246	75 (100,0)	110 (100,0)	128 (100,0)	127 (100,0)	127 (100,0)	126 (100,0)	110 (98,0)	59 (100,0)	70 (100,0)	92 (100,0)	46 (100,0)	114 (100,0)
	1246**Y**	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	2 (2,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	D1246Y-Mix	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
	(N)	75/151	110/154	128/143	127/150	127/150	126/159	112/154	59/124	70/151	92/154	46/90	114/155

Table 3. Frequency of haplotypes in *Pfdhfr*, *Pfdhps, Pfmdr1* and *Pfcrt* in samples from the twelve departments across Benin.

Site	**Alibori**	**Atakora**	**Atlantique**	**Borgou**	**Collines**	**Couffo**	**Donga**	**Littoral**	**Mono**	**Oueme**	**Plateau**	**Zou**
Number of selected health facilities	**2**	**2**	**1**	**2**	**3**	**3**	**2**	**3**	**1**	**1**	**2**	**2**
N	**151**	**154**	**143**	**150**	**150**	**159**	**150**	**124**	**151**	**154**	**90**	**155**
Pfdhfr												
I-R-N-I	83 (90,0)	92 (92,0)	123 (93,2)	119 (94,0)	84 (93,4)	124(98,0)	53(95,0)	56 (98,0)	129(93,0)	74 (99,0)	63 (98,0)	120(95,0)
N-**R-N-**I	8 (9,0)	7 (7,0)	7 (6,8)	6 (5,0)	3 (3,3)	2 (2,0)	2 (3,0)	1 (2,0)	8 (6,0)	0	1 (2,0)	5 (4,0)
N-C-S-I	1 (1,0)	0	0	2 (1,0)	0	0	0	0	0	0	0	0
I-C-**N-I**	0	0	0	0	3 (3,3)	0	1 (2,0)	0	2 (1,0)	0	0	1 (1,0)
N-C-**N-**I	0	0	0	0	0	0	0	0	0	1 (1,0)	0	0
N-C-**T**-I	0	1 (1,0)	0	0	0	0	0	0	0	0	0	0
N	**92**	**100**	**132**	**127**	**90**	**126**	**56**	**57**	**139**	**75**	**64**	**126**
Pfdhps												
I-*-A-K-A-A (I-**A**-A-K-A-A I-S-A-K-A-A)	5 (7,8)	3 (3,3)	0	7 (5,8)	1 (1,3)	1 (1,1)	0	0	0	4 (4,4)	0	1 (1,3)
I-***-G-**K-A-A (I-**A-G**-K-A-A I-S-**G**-K-A-A I-SA-**G**-K-A-A I-x-**G**-K-A-A)	52 (81,2)	79 (87,7)	70 (88,6)	101 (84,8)	68 (86,1)	74 (79,6)	46 (97,9)	31 (75,6)	110 (88,7)	72 (79,1)	29 (82,8)	72 (91,1)
I-*-**G**-K-A-**S** (I-**A-G**-K-A-**S** I-S-**G**-K-A-**S** I-SA-**G**-K-A-**S)**	2 (3,1)	2 (2,2)	6 (7,6)	4 (3,4)	8 (10,1)	7 (7,5)	0	7 (17,1)	6 (4,8)	5 (5,5)	5 (14,3)	3 (3,8)
I-**F-G**-K-A-**S**	0	1 (1,1)	0	0	0	0	0	0	0	0	0	0
I-**A-G**-K-**G-S**	0	0	0	2 (1,7)	1 (1,3)	0	0	0	0	0	0	0
I-S-**G-E**-A-A	0	0	0	0	0	0	0	0	0	2 (2,2)	0	0
I-F-A-K-A-**S**	0	2 (2,2)	0	0	0	0	0	0	0	0	0	0
V-A-A-K-A-A	0	1 (1,1)	0	3 (2,5)	0	0	0	0	0	0	0	0

V-A-G-K-**G-S**	4 (6,3)	1 (1,1)	3 (3,8)	2 (1,7)	1 (1,3)	8 (8,6)	1 (2,1)	2 (4,9)	5 (4,0)	5 (5,5)	1 (2,8)	1 (1,3)
V-A-G-K-A-A	1 (1,6)	1 (1,1)	0	0	0	1 (1,1)	0	1 (2,4)	0	2 (2,2)	0	1 (1,3)
V-A-G-K-A-**S**	0	0	0	0	0	2 (2,1)	0	0	3 (2,4)	1 (1,1)	0	1 (1,3)
n	**64**	**90**	**79**	**119**	**79**	**93**	**47**	**41**	**124**	**91**	**35**	**79**
Pfmdr1												
N-F-S-N-D	33 (54,1)	61 (72,6)	58 (53,2)	78 (70,9)	45 (60)	57 (66,3)	28(59,5)	25 (55,5)	41 (67,2)	56 (77,7)	25 (67,6)	73(71,6)
N-**Y**-S-N-D	26 (42,6)	18 (21,4)	50 (45,9)	26 (23,6)	29 (38,7)	26 (30,2)	18(38,4)	20 (44,4)	20 (32,8)	16 (22,2)	12 (32,4)	28(27,4)
Y-F-S-N-D	1 (1,6)	3 (3,6)	1 (1)	6 (5,4)	1 (1,3)	3 (3,5)	1 (2,1)	0	0	0	0	1 (1)
Y-Y-S-N-D	1 (1,6)	2 (2,4)	0	0	0	0	0	0	0	0	0	0
n	**61**	**84**	**109**	**110**	**75**	**86**	**47**	**45**	**61**	**72**	**37**	**102**
Pfcrt												
C-V-M-N-K	73 (93,6)	100 (90,1)	60 (56,6)	119 (94,4)	65 (80,2)	106(86,9)	56(94,9)	31 (53,4)	80 (63,5)	24 (47,1)	36 (60,0)	56(67,5)
C-V-**I-E-T**	5 (6,4)	10 (9,0)	46 (43,4)	7 (5,6)	16 (19,8)	16 (13,1)	3 (5,1)	27 46,6	46 (36,5)	27 (52,9)	24 (40,0)	27(32,5)
C-V-**I-N-T**	0	1 (0,9)	0	0	0	0	0	0	0	0	0	0
n	**78**	**111**	**106**	**126**	**81**	**122**	**59**	**58**	**126**	**51**	**60**	**83**

Frequency of haplotypes in ***Pfdhfr*, *Pfdhps***, ***Pfmdr1*** and ***Pfcrt*** among samples from each of the 12 departments of Benin. Mutant amino acid marked in bold type.
The frequencies are based on proportion of samples with haplotype among all complete haplotypes.
Due to the multiple variations at codon 436, samples A or S or AS are pooled and the 436 was shown by" *"
in the haplotype nomenclature (eg. I-*-**G**-K-A-A)

Supplementary Table 4. Number of mutation detected in the K13 from all the samples.

K13	N	AA 3D7	CODON	AA MMUT	SYN/NON SYN
S30N	1	S	30	N	non-syn
K33R	1	K	33	R	non-syn
N45Y	1	N	45	Y	non-syn
Q57K	1	Q	57	K	non-syn
M81I	1	M	81	I	non-syn
V83I	2	V	83	I	non-syn
L84I	1	L	84	I	non-syn
D85G	2	D	85	G	non-syn
P96L	1	P	96	L	non-syn
S100P	1	S	100	P	non-syn
R101G	1	R	101	G	non-syn
M106E	2	M	106	E	non-syn
S107F	1	S	107	F	non-syn
N110D	1	N	110	D	non-syn
G112Q	13	G	112	Q	non-syn
N119F	1	N	119	F	non-syn
N119Q	49	N	119	Q	non-syn
K123N	3	K	123	N	non-syn
D125N	1	D	125	N	non-syn
T126A	1	T	126	A	non-syn
T128I	1	T	128	I	non-syn
N131D	2	N	131	D	non-syn
N131S	3	N	131	S	non-syn
H136N	8	H	136	N	non-syn
Q143L	1	Q	143	L	non-syn
Q143N	1	Q	143	N	non-syn
T144A	1	T	144	A	non-syn
T149S	2	T	149	S	non-syn

M157I	1	M	157	I	non-syn
N158D	1	N	158	D	non-syn
D180G	1	D	180	G	non-syn
S182P	1	S	182	P	non-syn
G183R	1	G	183	R	non-syn
G183V	1	G	183	V	non-syn
L187F	1	L	187	F	non-syn
K189N	70	K	189	N	non-syn
K189T	566	K	189	T	non-syn
T192P	3	T	192	P	non-syn
T199I	1	T	199	I	non-syn
E206D	1	E	206	D	non-syn
A212T	1	A	212	T	non-syn
N217H	8	N	217	H	non-syn
G220S	1	G	220	S	non-syn
E254G	1	E	254	G	non-syn
R255K	36	R	255	K	non-syn
R257Q	1	R	257	Q	non-syn
L258M	9	L	258	M	non-syn
L262I	1	L	262	I	non-syn
Q271E	1	Q	271	E	non-syn
Q271H	1	Q	271	H	non-syn
D281G	1	D	281	G	non-syn
Q290Stop*	1	Q	290	Stop*	non-syn
K292R	1	K	292	R	non-syn
V319A	1	V	319	A	non-syn
N333D	1	N	333	D	non-syn
M351I	1	M	351	I	non-syn
I354V	1	I	354	V	non-syn
G357D	1	G	357	D	non-syn
G382E	2	G	382	E	non-syn
R383K	1	R	383	K	non-syn

Q391Stop*	2	Q	391	Stop*	non-syn
G392R	1	G	392	R	non-syn
E401K	1	E	401	K	non-syn
T415I	1	T	415	I	non-syn
P419L	1	P	419	L	non-syn
E424G	1	E	424	G	non-syn
A427T	2	A	427	T	non-syn
F442L	1	F	442	L	non-syn
P443L	1	P	443	L	non-syn
N458D	1	N	458	D	non-syn
W470Stop*	1	W	470	Stop*	non-syn
T474A	1	T	474	A	non-syn
T474I	1	T	474	I	non-syn
S485N	2	S	485	N	non-syn
V487E	1	V	487	E	non-syn
V494I	1	V	494	I	non-syn
W518Stop*	1	W	518	Stop*	non-syn
R529S	1	R	529	S	non-syn
C532S	1	C	532	S	non-syn
R561S	1	R	561	S	non-syn
P570L	1	P	570	L	non-syn
A578S	3	A	578	S	non-syn
A582T	2	A	582	T	non-syn
K586R	1	K	586	R	non-syn
G591S	1	G	591	S	non-syn
R597G	1	R	597	G	non-syn
W611Stop*	1	W	611	Stop*	non-syn
Q613H	3	Q	613	H	non-syn
A617V	1	A	617	V	non-syn
L619Stop*	1	L	619	Stop*	non-syn
A627T	1	A	627	T	non-syn
G639D	1	G	639	D	non-syn

G665S	4	G	665	S	non-syn
K670R	1	K	670	R	non-syn
N672D	2	N	672	D	non-syn
I684V	1	I	684	V	non-syn
T685A	1	T	685	A	non-syn
G686E	1	G	686	E	non-syn
F698L	1	F	698	L	non-syn

K13	**N**	**AA 3D7**	**CODON**	**AA MMUT**	**SYN/NON SYN**
N27N	2	N	27	N	Syn
S38S	1	S	38	S	Syn
L48L	1	L	48	L	Syn
K92K	1	K	92	K	Syn
K118K	1	K	118	K	Syn
I127I	1	I	127	I	Syn
N142N	1	N	142	N	Syn
Q152Q	2	Q	152	Q	Syn
S161S	1	S	161	S	Syn
K167K	2	K	167	K	Syn
E169E	1	E	169	E	Syn
N170N	1	N	170	N	Syn
L172L	3	L	172	L	Syn
I178I	1	I	178	I	Syn
N186N	1	N	186	N	Syn
T199T	1	T	199	T	Syn
T207T	4	T	207	T	Syn
F237F	1	F	237	F	Syn
K241K	2	K	241	K	Syn
K249K	2	K	249	K	Syn
L258L	2	L	258	L	Syn

E270E	1	E	270	E	Syn
H274H	1	H	274	H	Syn
N286N	1	N	286	N	Syn
K293K	1	K	293	K	Syn
E297E	1	E	297	E	Syn
I313I	1	I	313	I	Syn
K315K	1	K	315	K	Syn
K340K	1	K	340	K	Syn
T350T	1	T	350	T	Syn
E362E	1	E	362	E	Syn
L368L	1	L	368	L	Syn
Q370Q	2	Q	370	Q	Syn
R383R	1	R	383	R	Syn
T387T	2	T	387	T	Syn
G392G	1	G	392	G	Syn
L396L	3	L	396	L	Syn
P413P	2	P	413	P	Syn
L422L	1	L	422	L	Syn
L429L	1	L	429	L	Syn
L440L	2	L	440	L	Syn
G449G	1	G	449	G	Syn
S459S	1	S	459	S	Syn
C469C	29	C	469	C	Syn
T474T	1	T	474	T	Syn
T478T	1	T	478	T	Syn
Y493Y	2	Y	493	Y	Syn
Y500Y	1	Y	500	Y	Syn
E509E	1	E	509	E	Syn
V510V	2	V	510	V	Syn
L524L	1	L	524	L	Syn
T535T	2	T	535	T	Syn
R539R	1	R	539	R	Syn

I543I	1	I	543	I	Syn
D559D	1	D	559	D	Syn
E567E	3	E	567	E	Syn
F583F	1	F	583	F	Syn
V589V	7	V	589	V	Syn
S600S	1	S	600	S	Syn
I601I	1	I	601	I	Syn
A626A	1	A	626	A	Syn
N629N	2	N	629	N	Syn
E651E	1	E	651	E	Syn
E668E	1	E	668	E	Syn
G674G	1	G	674	G	Syn
A676A	4	A	676	A	Syn
G690G	5	G	690	G	Syn
F698F	1	F	698	F	Syn

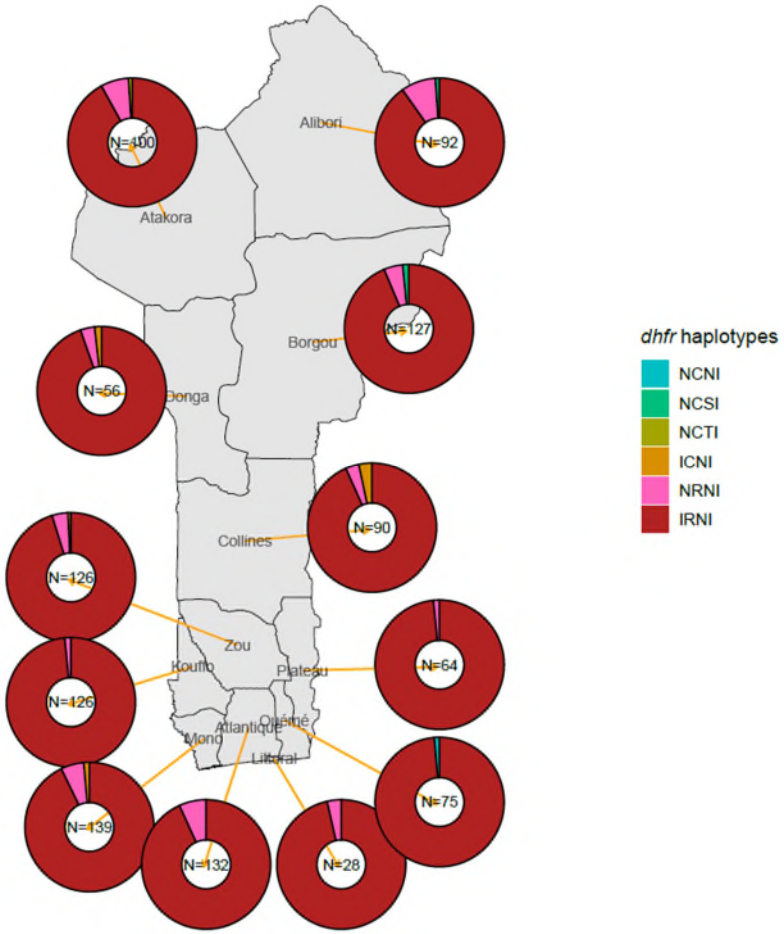

Figure 1: **Frequency of *Pfdhfr* haplotypes across Benin.** Frequencies are based on *P. falciparum* positive samples collected in each Department of Benin within the Plus Project. The haplotypes consist of amino acid changes at codon positions 51, 59,108, and 64. We define frequency as the number with each haplotype divided by the total number of samples successfully genotyped and which are not mixed at any of the above loci.

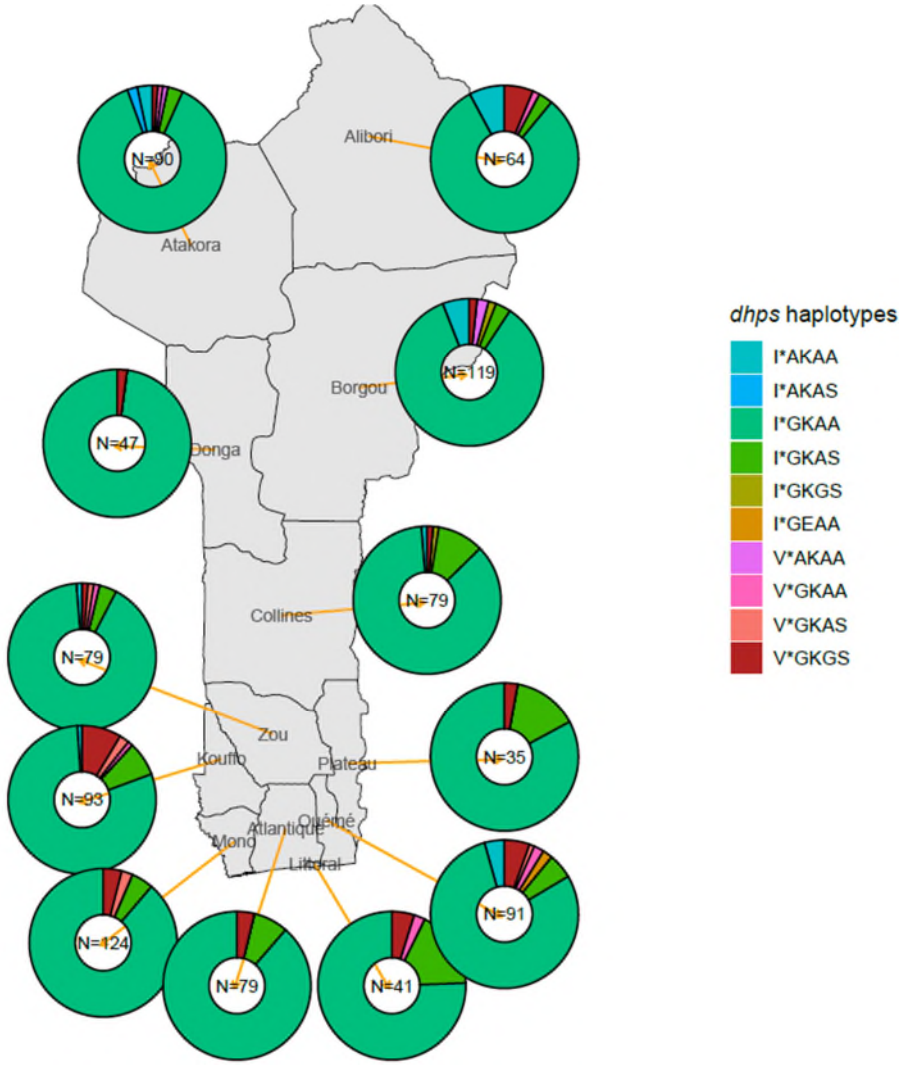

Figure 2: **Frequency of *Pfdhps* haplotypes across Benin.** Frequencies are based on *P. falciparum* positive samples collected in each Department of Benin within the Plus Project. The haplotypes consist of amino acid changes at positions 431, 436 (as "*"), 437, 540, 581, and 613. We define frequency as the number with each haplotype divided by the total number of samples successfully genotyped and which are not mixed at 431,437,540,581 or 613.

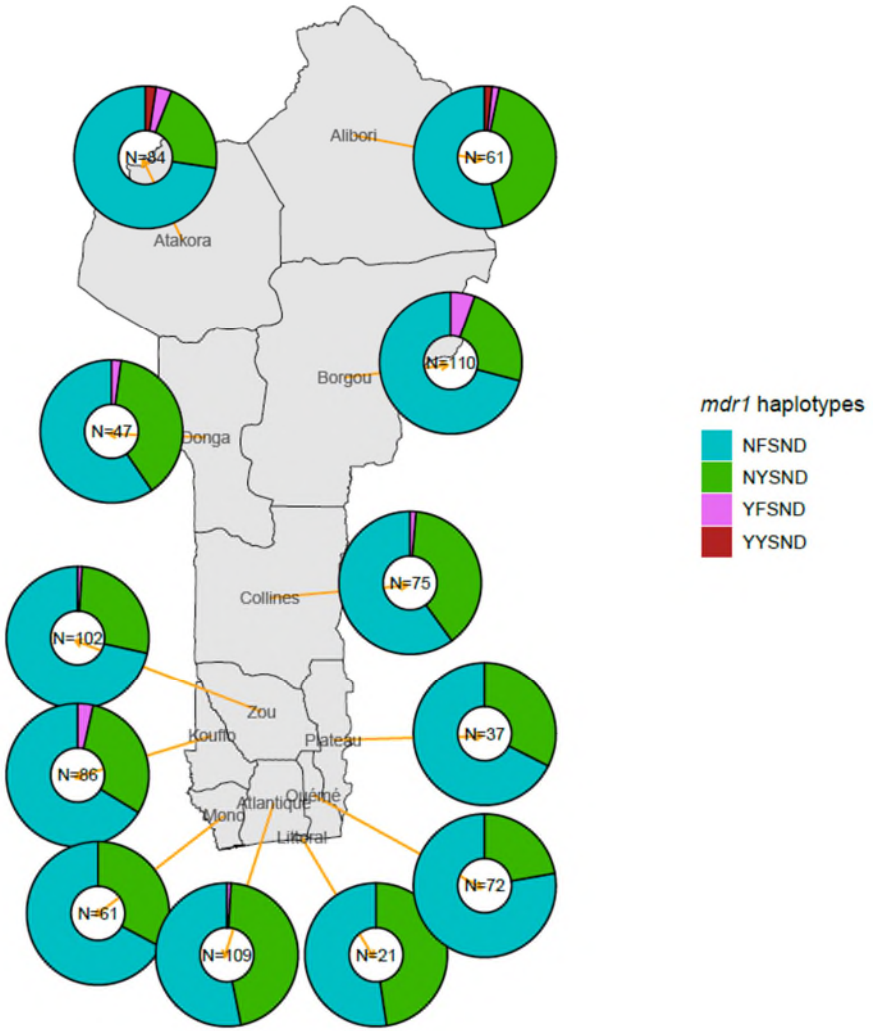

Figure 3: **Frequency of *Pfmdr1* haplotypes across Benin.** Frequencies are based on *P. falciparum* positive samples collected in each Department of Benin within the Plus Project. The haplotypes consist of amino acid changes at positions 86, 184, 1034, 1042, and 1246. We define frequency as the number with each haplotype divided by the total number of samples successfully genotyped and which are not mixed at any of the above loci.

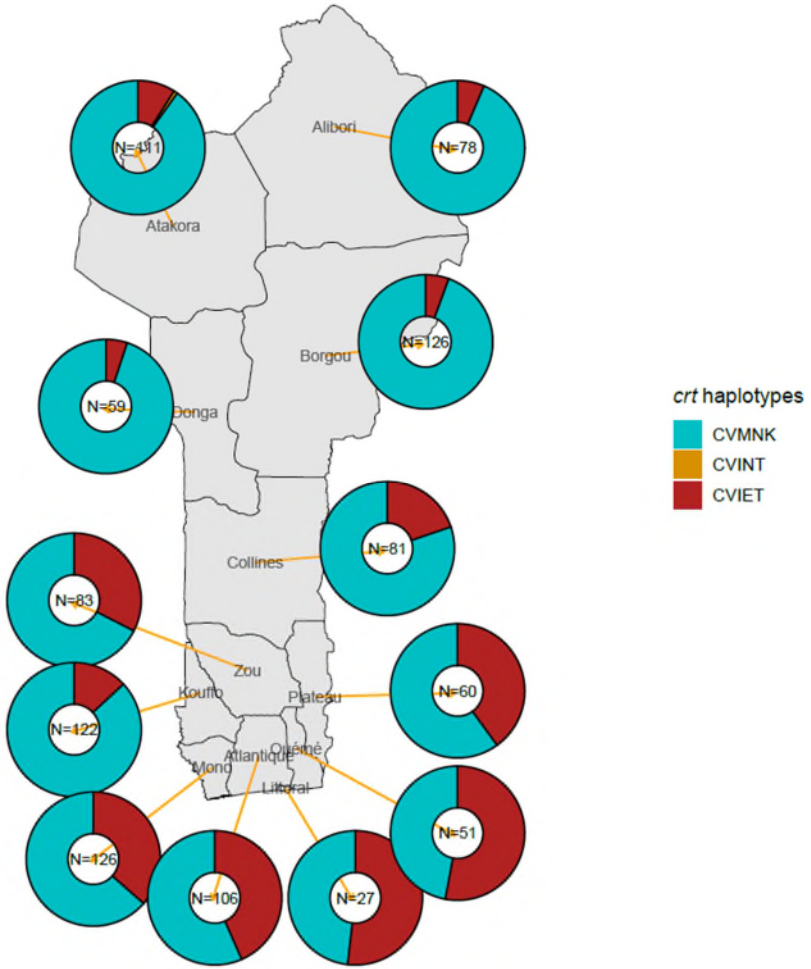

Figure 4: **Frequency of *Pfcrt* haplotypes across Benin.** Frequencies are based on *P. falciparum* positive samples collected in each Department of Benin within the Plus Project. The haplotypes consist of amino acid changes at positions 72, 73, 74, 75, and 76. We define frequency as the number with each haplotype divided by the total number of samples successfully genotyped and which are not mixed at any of the above loci.