Molecular profile of malaria drug resistance markers of *Plasmodium falciparum* in Suriname

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Abstract

In Suriname, an artesunate monotherapy therapeutic efficacy trial was recently conducted to evaluate partial artemisinin resistance emerging in *Plasmodium falciparum*. We genotyped the *PfK13* propeller domain of *P. falciparum* in forty samples as well as other mutations proposed to be associated with artemisinin resistant mutants. We did not find any mutations previously associated with artemisinin resistance in Southeast Asia but we found fixed resistance mutations for chloroquine and sulfadoxine-pyrimethamine. Additionally, the *Pfcrt* C350R mutation, associated with reversal of CQ resistance and piperaquine selective pressure was present in 62% of the samples. Our results from neutral microsatellite data also confirmed a high parasite gene flow in the Guiana Shield. Although recruiting participants for therapeutic efficacy studies in very low malaria endemic areas is challenging due to the low number of malaria cases reported, conducting these studies along with molecular surveillance remains essential to monitor artemisinin resistant alleles and to characterize the population structure *P. falciparum* in areas targeting malaria elimination.

Introduction

In 2004, artemisinin combination therapy (ACT) was adopted in Suriname as part of its malaria control program. Currently, Suriname uses artemether-lumefantrine (AL) plus primaquine (PQ) as the first-line regimen for treatment of *Plasmodium falciparum* malaria, and mefloquine (MQ) as prophylaxis for travelers and treatment for pregnant women. Suriname successfully reduced the number of malaria cases from 11361 in 2000 to 374 in 2014. In 2014, only 160 *P. falciparum* cases were reported (1). Most of the malaria patients were gold miners or their relatives working in bordering countries, who then sought treatment in Suriname. Since most of the *P. falciparum* cases in Suriname are imported, continuous monitoring of the treatment efficacy is necessary to guide treatment recommendations.

The World Health Organization (WHO) currently recommends monitoring the efficacy of artemisinin combination therapy (ACT) every 2 years in falciparum-endemic countries. The therapeutic efficacy study (TES) conducted in Suriname in 2005-2006, revealed a 98% efficacy (2). Subsequently, another TES conducted in 2011 showed an adequate clinical and parasitological response but a day 3 positivity rate of 31% (3). The proportion of patients who are parasitemic on day 3 is a key indicator for routine monitoring to identify suspected artemisinin resistance in *P. falciparum*. According to the WHO, if ≥ 10% of patients show persistent parasitemia by microscopy on day 3 after treatment with ACT or artesunate monotherapy, then partial artemisinin resistance is are suspected (2). To further investigate this possibility, from July 2013 until July 2014, a TES was conducted in Suriname consisting of 3 days of artesunate monotherapy followed by mefloquine and primaquine to determine the efficacy of artemisinin, without confounding partner drugs. In that study, the day 3 positivity rate for *P. falciparum* was 10%, and at least 17.9 % of the samples exhibited a parasite half-life ≥ 5 hours, suggesting suspected partial artemisinin resistance (unpublished data).

Additionally, WHO recommends conducting molecular surveillance to detect mutations in the K13 propeller domain as a complementary tool in assessing the presence of artemisinin resistance in endemic countries. Currently, eight K13 mutations: P441L, F446I, S449A, N458Y, P553L, V568G, P574L, and L675V have been associated with delayed parasite clearance. In addition, five K13 mutations: Y493H, R539T, I543T, R561H, and C580Y have been confirmed as K13 resistance mutations by *in vivo* and *in vitro* data (2). Studies conducted in Southeast Asia using whole genome analysis identified polymorphisms in other genes such as *fd* (ferredoxin), *mdr2* (multidrug resistance protein 2) and *crt* (chloroquine resistance transporter) associated with the resistance-causing K13 propeller mutations (4). It remains to be further validated if these proposed molecular markers are relevant for monitoring artemisinin resistance in other geographical regions including South America.

Recently, Pelleau *et al*. described a new mutation at codon C350R in the *Pfcrt* gene that was found to revert the CQ resistant phenotype parasites with **S**VMN**T** genotypes to a CQ sensitive phenotype. Further, it was proposed that this genetic change also impaired susceptibility to piperaquine (PPQ), a drug commonly used by migrant workers in the Guiana shield (5). Although AL combination therapy was officially introduced in 2008, illegal gold miners are known to self-medicate, creating a drug selective pressure on the parasite. We recently showed that 5.1% of the blood samples collected in Guyana in 2010 had the C580Y mutation. These mutant parasites shared a common haplotype based on K13 flanking microsatellites, which were different from those reported in Southeast Asia. Based on this finding it was proposed that the C580Y allele found in Guyana had emerged independently in this region (6). In the current study, the 41 samples collected in Suriname from the most recent therapeutic efficacy study were used to determine the presence of any mutations associated with resistance to artemisinin and other antimalarial drugs. In addition, we characterized the population structure of these isolates using neutral microsatellite markers and compared them with isolates found in other countries within the Guiana Shield.

Materials and Methods

Study site

Plasmodium isolates

We tested 41 *Plasmodium falciparum* blood samples collected from July 2013 to June 2014 for artesunate-based monotherapy efficacy trial. The study was approved by the national ethics committee (CMWO) of the Ministry of Health in Suriname. Patients and/or their legal guardians provided written consent to participate in the study. Patients with uncomplicated falciparum malaria, who met the study inclusion criteria, were enrolled at the Tourtonne laboratory, a malaria diagnostic and treatment facility in the north of Paramaribo. Mostgold miners dwell in this neighborhood while in the capital. The participants were febrile persons of ages $\geq$ 2 years with microscopically confirmed uncomplicated *P. falciparum* infection presenting at the clinic.

DNA isolation and genotyping methods

Genomic DNA was isolated from blood spots taken at enrollment (Day 0) using the QIAamp DNA mini kit (QIAGEN, Valencia, CA). Samples were screened using the multiplex PET-PCR (7). For each sample, duplicate PET-PCR reactions were run with 5 µL of DNA template used in the PCR reaction. All assays were performed using Agilent Mx3005pro thermocyclers (Agilent technologies, Santa Clara, CA, USA). As previously established, a CT value of 40 was considered the cut-off value to score a reaction as positive, samples above 40 were considered to be negative. The confirmed *P. falciparum* samples were used to amplify the K13-propeller domain using previously described methods (6). PCR amplifications of specific codons in *Pfcrt* (codons 350 and 356), *Pffd* (codon 139) and *Pfmdr2* (codon 484) were carried out in 20 µl volume reactions using 20 ng of total genomic DNA, 1X PCR buffer with $MgCl_2$, 0.2 mM of dNTPs, 0.75 µM of each forward and reverse primers: PF535 (5'-CCATATAATTTTTCATTTTC-3') and PF536 (5'-GTTCTCTTACAACATCAC-3') for *Pfcrt;*

PF11723 (5'-TTGTTAGAATCATGAATATTG-3') and PF11724 (5'-GATTGAGGACAAATTACATG-3') for *Pffd;* PF10283 (5'-GCAAAAGGATAGATATGAAAG-3') and PF10284 (5'-CCTATAAATAATACACTACC-3') for *Pfmdr2,* and 0.6 U/µl of High fidelity Taq polymerase (Expand high fidelity PCR system, Roche). The cycling conditions were as follows: an initial denaturation step at 94°C for 5 min; 35 cycles of: denaturation at 94°C for 30s, annealing at: 52°C for *Pfcrt*; 46°C for *Pffd;* and 50°C for *Pfmdr2* for 30s and extension at 68°C for 1 min; followed by a final extension step at 68°C for 10 min. PCR products were confirmed after ExoSAP clean up using a 1.8% agarose gel electrophoresis and Gel Red (Biotium, Hayward, CA USA). We also include the artemisin-resistant laboratory control samples: 3D7, 7G8, W2, HB3 and Dd2 for comparison.

The samples were also genotyped by direct sequencing for *Pfcrt* (codons 72-76), *Pfdhfr* (codons 50, 51, 59, 108 and 164) and *Pfdhps* (codons 436, 437, 540, 581 and 613) using an Applied Biosystems 3130 capillary sequencer. In addition, *Pfmdr1* copy number and codon mutations (86, 184, 1034, 1042 and 1246) were evaluated. The PCR primers and conditions for these *Pfcrt*, *Pfdhps*, *Pfdhfr* and *Pfmdr1* codons have been previously described (8, 9). *Pfmdr1* copy number was determined by TaqMan real-time PCR (Stratagene MX3005P; Agilent Technologies, La Jolla, CA) using a previously described protocol (9).

Microsatellite analysis

Seven neutral microsatellites (TA1, Polyα, PfPK2, TA109, C2M34, C3M69, 2490) located in chromosomes 2, 3, 4, 6 and 12 were PCR amplified using previously published methods for analyzing *Plasmodium* population structure (10,11). Fluorescently labeled PCR products were separated on an Applied Biosystems 3130 capillary sequencer and scored using Gene Marker v1.95 (SoftGenetics LLC). The discovery of one or more additional alleles in a single locus was interpreted as a co-infection with two or more genetically distinct clones in the same isolate. Missing data (no amplifications) were observed for some loci but not considered for defining haplotypes. Neutral microsatellite data from previously published data in Suriname (12) were also included to evaluate any changes in the haplotypes

144 circulating in the country. Moreover, results from samples collected from 2003-2004 in Venezuela
145 (132), 2010 in Guyana (6) and 1983-1999 in Brazil (14), were included to compare historical haplotypes
146 circulating in this region.

147 We used Structure v2.1 (15) to test whether *P. falciparum* samples from different countries clustered as
148 a single population. This Bayesian clustering approach assigns isolates to *K* populations or clusters
149 characterized by the allele frequencies at each locus. The sample assignment was evaluated at
150 different *K* values (*K*=2 to 10). Given that this algorithm relies on stochastic simulations, each *K* value
151 was run independently ten times with a burn-in period of 10,000 iterations followed by 50,000 iterations.
152 The admixture model was used allowing for the presence of individuals with ancestry in two or more of
153 the *K* populations. Heterozygosity (He) and the fixation index (FST) were calculated using Arlequin
154 3.5(16). FST values were classified as follows: <0.05 = little genetic differentiation, 0.05-0.15 =
155 moderate genetic differentiation, 0.15-0.25 = great genetic differentiation and >0.25 = very great genetic
156 differentiation (17).

157 **Results**

158 A total of 40 out of 41 samples were positive for *P. falciparum* according to our PET-PCR results; 38 of
159 these samples successfully amplified for all genes. From these samples, 7 displayed a mixed *P.*
160 *falciparum* infection as represented by different *P. falciparum* neutral microsatellite haplotypes in the
161 sample. No mutations in the K13 propeller domain were found. In addition, the recently reported
162 polymorphisms in *Pffd* (N193Y), *Pfmdr2* (T484I) and *Pfcrt* (I356T) associated with artemisinin
163 resistance alleles in Southeast Asia (4) were not found in these samples (Figure 1) but only in lab
164 controls W2 and Dd2. Instead, we found nonsynonymous mutations in position 105 (A/T) of *Pffd* and in
165 codons 423 (F/Y) and 429 (I/V) of *Pfmdr-2* in all samples. These mutations were also found in the South
166 American 7G8 laboratory strain (Table 1).

Also, the *Pfcrt* mutation I356L was found in all isolates from Suriname, as well as an insertion of four AT repetitive motifs in positions 2477 to 2485. These samples also exhibited the *Pfcrt* double mutant C72S/K76T, the *Pfdhps* triple mutant A437G/K540E/A581G and the *Pfdhfr* triple mutant C50R/N51I/S108N (Table 2). More importantly, the *Pfcrt* C350R mutation, recently described as a reverse phenotypic mutation associated with CQ sensitivity and associated with piperaquine resistance, was found in 62% of the samples. We also found two *Pfmdr1* mutant genotypes, Y184F/N1042D/D1246Y (triple mutant) and Y184F/S1034C/N1042D/D1246Y (quadruple mutant). The *Pfmdr1* copy number determination indicated that only a single sample had 2 gene copies. The frequency of mutations and the copy number results are shown in Figure 2.

Neutral microsatellite analysis

We found at least 12 different neutral microsatellite haplotypes from Suriname and its bordering regions in these Surinamese samples (Table 3). By doing a comparative analysis of our data with previously reported data from Suriname (12) and Guyana (6), we found at least two *Plasmodium* populations represented by the red and green clusters (Figure 3a). Each cluster is composed of highly similar *P. falciparum* haplotypes. Moreover, using tests of genetic differentiation between sampling populations (FST), we found that there was little genetic differentiation between Venezuela-Guyana, Venezuela-Suriname, Venezuela-Brazil, Guyana-Brazil and Brazil-Suriname (Figure 3b; Table 4), as represented by similar Plasmodium haplotypes circulating in all of these countries (clusters blue, red and green were found in all sampling populations). No significant genetic difference was observed between samples from Guyana and Suriname given that similar *P. falciparum* haplotypes (represented by the clusters red and green) were shared by both countries.

190 **Discussion**

191 Historically, resistance to widely used first line antimalarial drugs such as CQ and sulfadoxine-
192 pyrimethamine (SP) evolved at about the same time in both South America and Southeast Asia and
193 spread to other regions. Artemisinin resistance as defined by delayed parasite clearance has been well
194 documented in Southeast Asia but it has not been definitively confirmed in South America. Although the
195 overall efficacy of ACT remains high in Suriname, the day 3 positivity rate for the *in vivo* study
196 conducted in 2011 and the parasite half-life results from 2014, showed evidence of suspected
197 artemisinin resistance in this population (3). Due to previous evidence for the independent emergence of
198 the K13 C580Y allele in Guyana (6), it was important to assess the molecular profile of the 2014
199 samples from Suriname to further determine if artemisinin resistance associated alleles were present in
200 these samples. The limited number of samples available from the 2014 TES study showed no evidence
201 of K13 resistance alleles including those samples with slow parasite clearance rates. Although several
202 mutations in K13 have been shown to be strongly associated with artemisinin resistance, some parasites
203 with K13 wild type alleles in Southeast Asia also exhibited a delayed parasite clearance phenotype (18,
204 19). The lack of association between resistance phenotypes and K13 polymorphisms in some field
205 isolates suggests that additional genes may be involved in the development of artemisinin resistance in
206 *P. falciparum*. Another explanation may be that the limited sample size for this study may have failed to
207 detect low prevalent K13-resistant alleles. The high level of sequence conservation of the Kelch
208 propeller domain in *Plasmodium* and the limited spread of artemisinin resistance-causing K13
209 mutations, imply that there is a substantial fitness cost in the absence of sustained drug pressure. These
210 fitness costs may be compensated by other genetic variants, either in kelch 13 or elsewhere in the
211 Plasmodium genome (20).

212 We further investigated mutations in gene markers suchs as *Pfcrt*, *Pffd* and *Pfmdr2* reported to be
213 associated with the artemisinin resistance phenotype in Southeast Asia (4). Lack of these mutations in

Suriname samples and the presence of different non-synonymous mutations suggests that parasite isolates in Suriname are evolving independently. Moreover, K13-wild type laboratory strains of *P. falciparum* from Southeast Asia also presented the mutations previously associated with artemisinin resistance, which suggests a very particular *P. falciparum* genetic profile present on that part of the world. It is possible that K13-resistant mutants in South America arise with a different background and increase in frequency due to a greater selective pressure. However, the potential role of background mutations and their association with artemisinin resistance needs further validation in South America.

Additionally, most of the Suriname samples (62%) had the *Pfcrt* C350R mutation, which has been associated with a CQ phenotypic reversion and a strong piperaquine drug selective pressure (5). Given that gold miners living in the forest are known to self-medicate with dihydroartemisinin-piperaquine-trimethoprim tablets, it is not surprising that this *Pfcrt* mutation is found in high frequency in this study samples, especially if we consider that 87% of the patients came from artisanal gold mining areas in French Guiana. In addition, *dhfr* and *dhps* mutations associated with SP resistance were fixed in these samples.

Pfmdr1 alleles N86Y, Y184F, and D1246Y are common in *P. falciparum* populations in Africa; however, reduced susceptibility to lumefantrine has been linked to haplotypes harboring the N86, 184F, D1246 residues and to the K76 residue in *Pfcrt* (18). In Suriname, this particular profile was not found. The majority of the isolates displayed the Y184F/N1042D/D1246Y mutant while Y184F/S1034C/N1042D/D1246Y was found in less frequency. These *Pfmdr1* mutants have also been found in other countries of the Guiana Shield such as Guyana (6), Venezuela (8) and Brazil (22). The different results in genotype prevalence and gene copy number could be attributed to a different target population. Most of the recent samples from 2014 originated from small scale gold miners coming from French Guiana, while the previous samples collected in 2010, mainly corresponded to people working or living in the villages in the interior, so most of the drug resistance profile results in this study

corresponded to imported malaria cases. Changes in prevalence of these alleles could also indicate selection by a partner drug. A decrease in the partner drug's efficacy could facilitate the emergence of new foci of resistance to artemisinin, as observed in the Mekong region (21).

Furthermore, in the illegal gold mining areas of the Guiana Shield, non-recommended treatments including artemisinin monotherapy and non-registered artemisinin derivatives are available through the informal sector (23). Indeed, infected gold miners could reintroduce malaria in areas where competent vectors exist, possibly resulting in the spread of artemisinin resistant parasites. Moreover, our results from cluster analyses, which included data from Venezuela, Suriname, Brazil and Guyana, reflect the high parasite gene flow in the Guiana Shield. In particular, *P. falciparum* isolates from Guyana and Suriname are highly genetically related and behaved as a single parasite population.

Although K13 genotypes associated with artemisinin resistance were not detected, our findings highlight the presence of multidrug resistance genotypes in Suriname. Given that the prevalence of *P. falciparum* in Suriname has been dramatically reduced in the last years, it has become more challenging to conduct *in vivo* studies to assess the therapeutic efficacy of artemisinin. Therefore, molecular surveillance continues to be an important method for monitoring changes in prevalence of drug resistance genotypes.

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Figures

Figure 1. Genes related to artemisinin resistance and mutations found within these genes.

Figure 2. Percentage of *Pfmdr1* mutants (NFSDY and NFCDY) and gene copy number found in Suriname samples. Previous reported data collected in 2005 and 2010 from Adhin et al., 2013 were also included.

Figure 3. Population structure using neutral microsatellite loci. Each sample is represented by a vertical line which is partitioned into *K* color segments that represent the individual's estimated membership fraction in each of the *K* clusters. A) Clustering (*K*=2: colors red and green) per year using Structure v2.3. B) Clustering (*K*=3: colors blue, red and green) including other countries.

Tables

Table 1. Aminoacid mutations found in genomic markers associated with K13 resistance mutations. Genetic background mutations associated with artemisinin resistance previously found in Southeast Asia are in bold and underscored.

	PfCRT		**PfMDR2**			**PfFD**	
	C350R	**I356T/L**	**F423Y**	**T484I**	**I492V**	**A105T**	**D193Y**
SURINAME	C/R	L	Y	T	V	A	D
3D7	C	I	F	T	V	T	D
7G8 (Brazil)	C	L	Y	T	V	A	D
W2 (Indochine)	C	**T**	Y	**I**	I	T	**Y**
HB3 (Honduras)	C	I	Y	T	I	T	D
Dd2 (Indochine)	C	**T**	Y	**I**	I	T	**Y**

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279 **Table 2.** Molecular profile of drug resistance genes for Suriname

Locus	Genotype						
Pfcrt	C72S	73V	M74I	N75E	K76T	C350R	%
	S	V	M	N	**T**	C	38
	S	V	M	N	**T**	**R**	62
Pfmdr1	N86Y	Y184F	S1034C	N1042D	D1246Y		
	N	**F**	S	**D**	**Y**		61
	N	**F**	**C**	**D**	**Y**		39
Pfdhfr	C50R	N51I	C59R	S108N	I164L		
	R	**I**	C	**N**	I		100
Pfdhps	S436A	A437G	K540E	A581G			
	S	**G**	**E**	**G**			100

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Table 3. *Plasmodium falciparum* haplotypes found in Suriname using neutral microsatellites.

Haplotype	TA1	Polyα	PfPK2	TA109	2490	C2M34	C3M69	Frequency
Haplo-1	172	185	172	176	84	238	134	0.18
Haplo-2	172	185	172	176	84	226	134	0.15
Haplo-3	172	183	172	176	84	226	134	0.15
Haplo-4	172	185	172	176	84	238	151	0.15
Haplo-5	172	183	172	176	84	238	134	0.15
Haplo-6	172	183	172	176	84	238	151	0.06
Haplo-7	140	183	172	176	84	238	151	0.03
Haplo-8	172	150	172	176	80	238	134	0.03
Haplo-9	172	185	172	176	84	226	151	0.03
Haplo-10	172	183	172	176	84	226	151	0.03
Haplo-11	172	150	172	176	84	238	134	0.03
Haplo-12	172	183	172	176	84	238	138	0.03

Table 4. Population genetic differentiation using six neutral microsatellite loci (microsatellite results for at least 80% of the population with a clear pattern were included) A) Genetic diversity between sampling populations B) FST comparisons between sampling populations.

A)

Population	N	Collection year	Haplotypes	Private hap	He	SD
Venezuela	54	2003-2004	22	15	0.4946	0.0624
Guyana	65	2010	32	21	0.4481	0.0618
Suriname	43	2009-2011	24	13	0.3640	0.0946
Brazil	122	1983-1999	61	52	0.5115	0.0472

He: Heterozygosity

SD: Standard deviation

B)

Population	Venezuela	Guyana	Suriname
Guyana	0.044*		
Suriname	0.043*	0.009	
Brazil	0.044*	0.027*	0.019*

*Significant ($p<0.05$)

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FIGURE 1 Genes related to artemisinin resistance and mutations found within these genes.

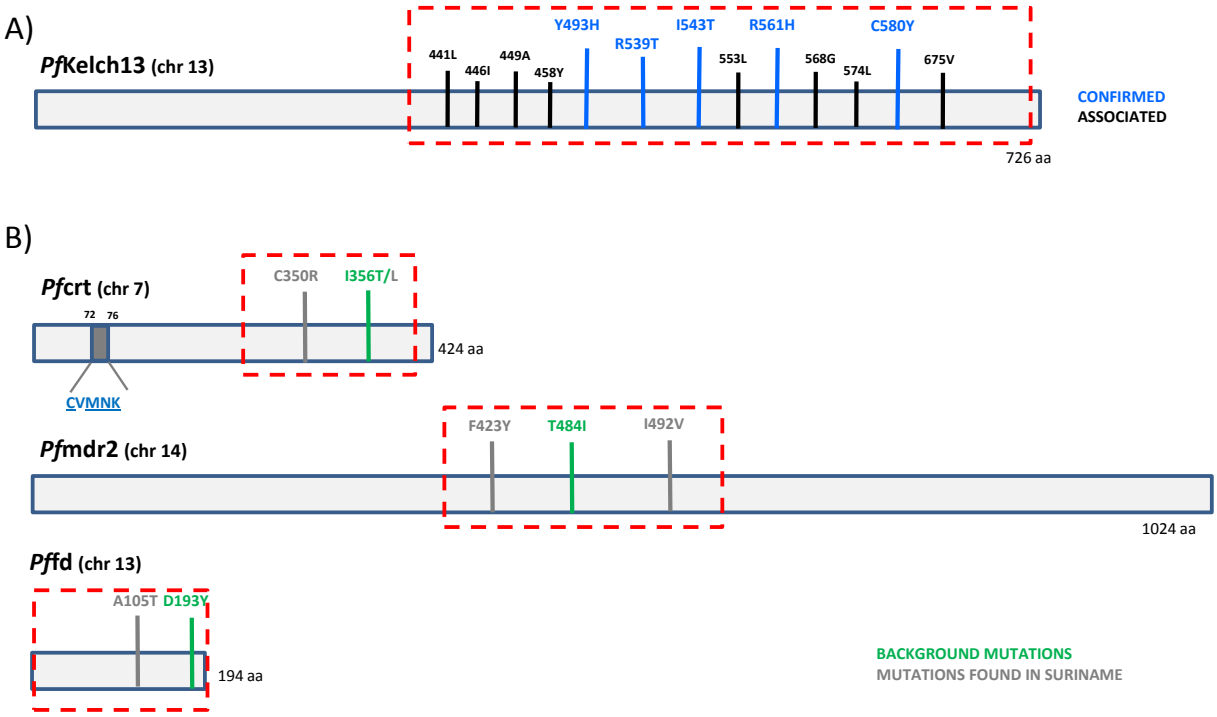


FIGURE 2 Percentage of *Pfmdr1* mutants (NFSDY and NFCDY) and gene copy number found in Suriname samples. Previous reported data collected in 2005 and 2010 from Adhin et al., 2013 were also included.

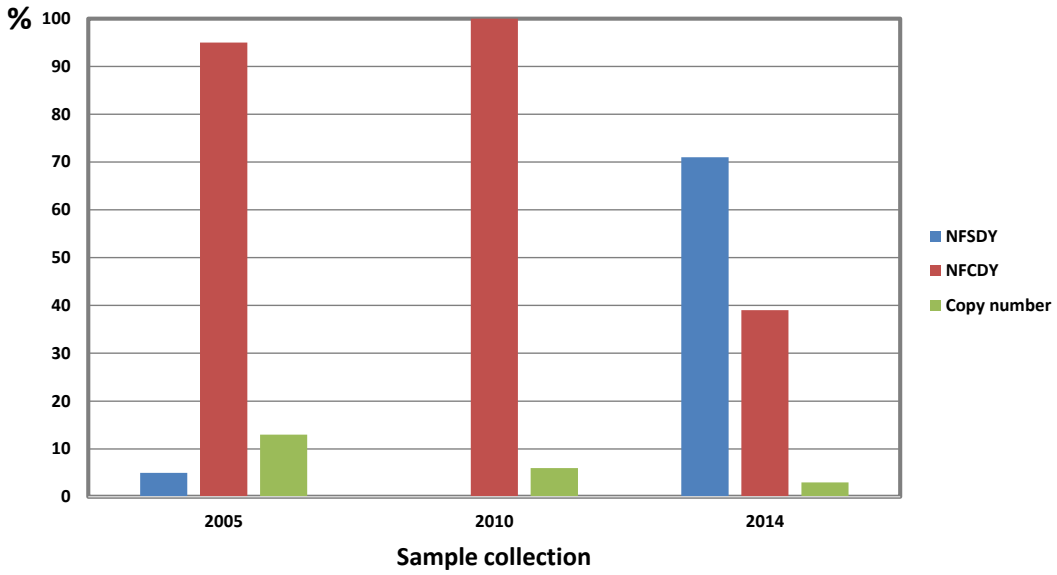
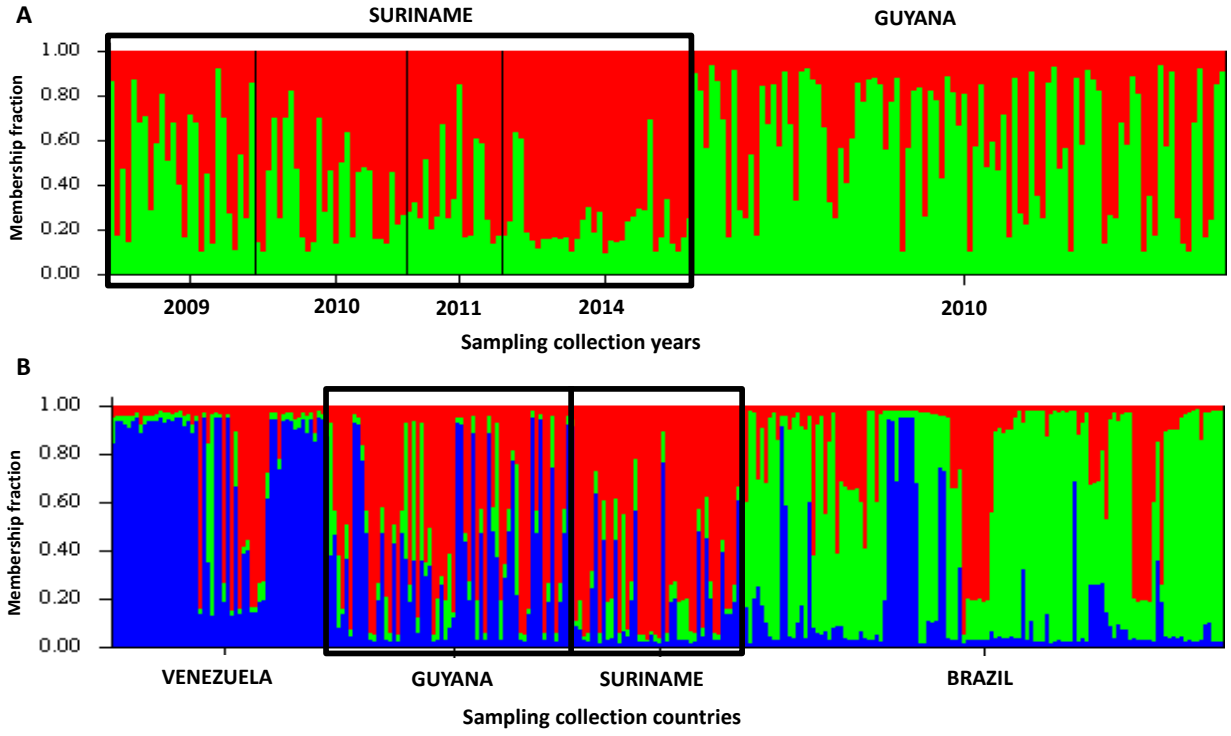


FIGURE 3

Population structure using neutral microsatellite loci. Each sample is represented by a vertical line which is partitioned into *K* color segments that represent the individual's estimated membership fraction in each of the *K* clusters. A) Clustering (*K*=2: colors red and green) per year using Structure v2.3. B) Clustering (*K*=3: colors blue, red and green) including other countries.



K= *Plasmodium* populations obtained by Bayesian inference.

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