Global analysis of *Plasmodium falciparum* Na$^+$/H$^+$ exchanger (*pfnhe-1*) allele polymorphism and its usefulness as a marker of *in vitro* resistance to quinine

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ABSTRACT

The aim of this study was to provide a comprehensive analysis of the worldwide genetic polymorphism of ms4760 alleles of the *pfnhe-1* gene and to discuss their usefulness as molecular marker of quinine resistance (QNR). A new numbering of ms4760 allele, classification grouping ms4760 alleles according to the number of DNNND and DDNHNDNHNND repeat motifs in blocks II and V was also proposed.

A total of 1508 ms4760 sequences from isolates, culture-adapted parasites or reference strains from various geographical regions were retrieved from GenBank (last update on 15th June 2012) or from publications and were used for genetic analyses. The association of different alleles of *pfnhe-1* with resistance to quinoline antimalarial drugs showed marked geographic disparities.

The validity and reliability of candidate polymorphisms in *pfnhe-1* gene as molecular markers of QNR appeared restricted to endemic areas from South Asia or possibly East African countries and needs to be confirmed.

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1. Introduction

Quinine (QN), a natural compound found in *Cinchona* bark, has been used for centuries in malaria endemic regions (Baird, 2005). It is currently recommended for treating severe malaria cases, malaria in pregnant women or as second-line therapy in combination with antibiotic for uncomplicated malaria (World Health Organization, 2010a). Though clinical failures have been reported in Asia and South America in the 1960s and later on, although more rarely in Africa, resistance to QN (QNR) remains particularly punctual and rare (Chongsuphajaisiddhi et al., 1983; Pukrittayakamee et al., 1994, 2000; de Vries et al., 2000; McGready et al., 2000, 2005; Rahman et al., 2001; Adam et al., 2005; Adegnika et al., 2005; Achan et al., 2009; World Health Organization, 2010a,b).

QN, a quinoline derivative, is a monoprotic weak base that accumulates within the low pH environment of the parasite digestive vacuole of *Plasmodium falciparum*. QN presumably acts by interference with the detoxification of heme produced during hemoglobin degradation by *P. falciparum* asexual blood stages, leading to toxic degradation by-products (Hawley et al., 1998). However, the mechanism of QNR is not well known. Several reports have documented associations between *in vitro* susceptibility to QN with other structurally related drugs such as amino-4-quinolines (chloroquine, amodiaquine) or aryl-amino-alcohol (mefloquine, halofantrine), suggesting that a common genetic determinant may affect the parasite response to these antimalarials (Simon et al., 1986; Warsame et al., 1991; Basco and Le Bras, 1992; Brasseur et al., 1992). Particularly, QNR has been associated with mutations in the *P. falciparum multidrug resistance 1* gene (*pfmdr-1*) and the *P. falciparum chloroquine resistance transporter* gene (*pfcrt*) (Wongsrichanalai et al., 2002; Valderramos and Fidock, 2006). More recently, other genetic polymorphisms, such as mutations in the *P. falciparum*

multi-resistance protein 1 gene (*pfmrp-1*) have been suggested (Mu et al., 2003), but not confirmed (Anderson et al., 2005). However, PfMRP knock-out parasite lines displayed increase susceptibly to several antimalarial drugs, including chloroquine, QN and artemisinin derivatives (Raj et al., 2009). The degree of implication or linkage of the three genes in QNR remains uncertain, probably because additional genes are involved. In 2004, by using quantitative trait loci (QTL) analysis on the genetic cross of the HB3 and Dd2 clones, Ferdig et al. (2004) identified genes associated with QN reduced susceptibility (Ferdig et al., 2004), namely *pfmdr-1* on chromosome 5, *pfcrt* on chromosome 7 and *pfnhe-1* (*P. falciparum* Na$^+$/H$^+$ exchanger-1) on chromosome 13. To test for an association of QN response with this latter gene, *pfnhe-1* was resequenced from the HB3 and Dd2 parents and the identified coding frame polymorphisms were surveyed in 71 *P. falciparum* culture-adapted isolates and reference lines from South-East Asia, Africa and Central and South America. Sequences of *pfnhe-1* showed multiple and complex variations. Three point polymorphisms at three separate codons (790 gtc/ttc, 894 aat/aaa, 950 ggg/gtg) and microsatellite variations in three different repeat sequences (msR1, ms3580 and ms4760) were observed (Fig. 1). Moreover, there was a significant association between variations in ms4760 and *in vitro* QN response. One of the eight ms4760 profiles, ms4760-1, was relatively frequent in lines with reduced susceptibility to QN (i.e. higher IC$_{90}$), but it was also present in fully susceptible parasites. More interestingly, the authors reported that presence of more than 2 DNNND repeat motifs in block II was associated with higher *in vitro* IC$_{90}$ for QN compared with presence of only one repeat (Ferdig et al., 2004).

The physiological role of PfNHE-1 is still debated. In all living organisms, the fundamental homeostatic mechanisms are ubiquitous and vital. These physiological processes which regulate cellular
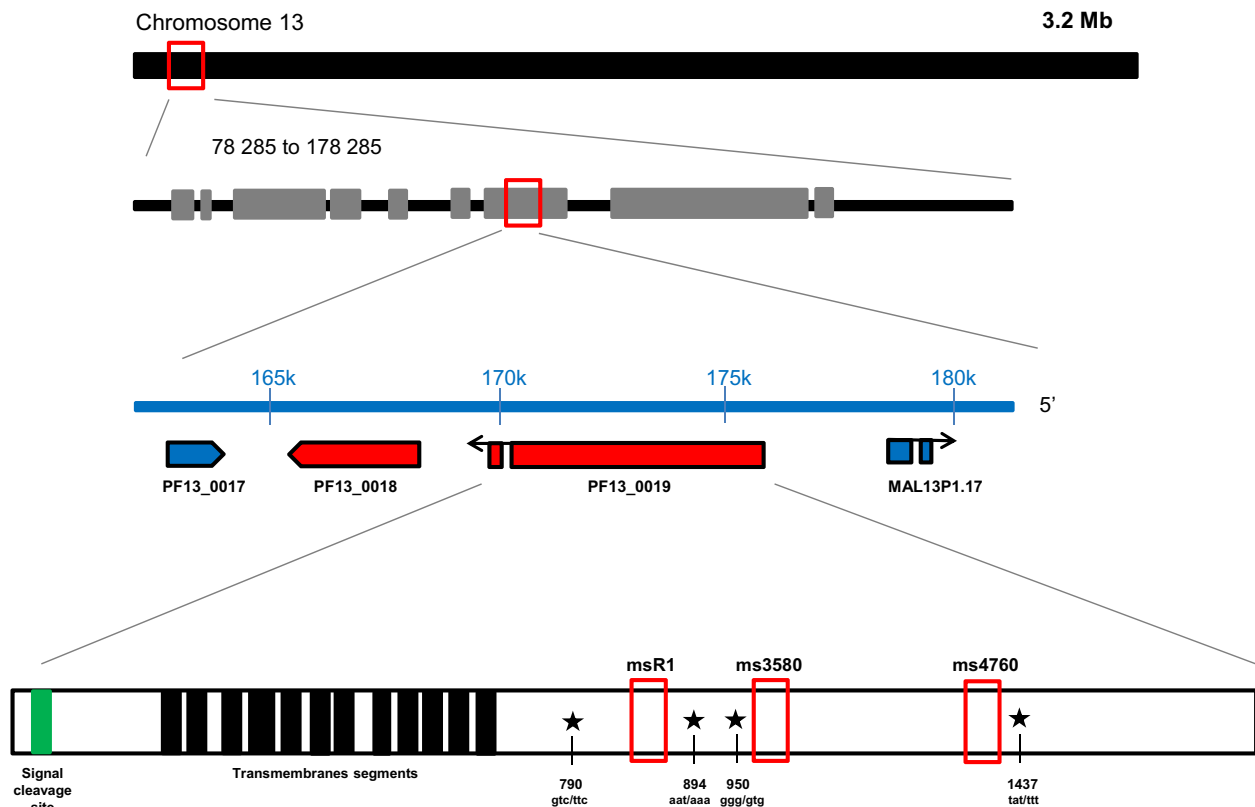

Fig. 1. Schematic representation of *pfnhe-1* gene (PF13_0019) on chromosome 13 and positions of codons polymorphisms (790, 894, 950 and 1437) and microsatellite variations (msR1, ms3580 and ms4760).

pH, volume, and ion composition are supported by transmembrane exchange of cations implying several transporters like the family of Na$^+$/H$^+$ exchangers (NHEs) (Pouyssegur et al., 1984; Putney and Barber, 2003). Investigations performed in 1993 by the group of Ginsburg have shown that the major role of *P. falciparum* Na$^+$/H$^+$ exchanger was to increase the cytosolic pH (pH$_{cyt}$) and to compensate acidosis caused by anaerobic glycolysis (Bosia et al., 1993). PfNHE-1, a 226 kDa protein with 12 predicted trans-membrane segments (Gardner et al., 2002; Ferdig et al., 2004), is supposed by some authors to reside in the parasite's plasma membrane (Bosia et al., 1993; Bennett et al., 2007) but others underlined that the subcellular localization of this protein is not established (Nkrumah et al., 2009). Saliba and Kirk (1999) demonstrated that *P. falciparum* maintains its pH$_{cyt}$ by using mainly a V-type H1-ATPase, which serves as the major route for the efflux of H$^+$ ions (Saliba and Kirk, 1999). Later, in 2007, Bennett et al. (2007) showed that high level of QNR was correlated to an increased PfNHE-1 activity which determines pH$_{cyt}$. They also demonstrated that antimalarial drug resistances were related to modifications of ion transport across plasma (pH$_{cyt}$) and digestive vacuole (pH$_{DV}$) membranes and concluded that pairwise interactions of genetic determinants located on chromosome 13 and chromosome 9 affecting pH$_{cyt}$ and PfNHE-1 were involved in QNR (Bennett et al., 2007). However, using the protocols of Bennett et al., Spillman et al. (2008) showed that the Na$^+$-dependent efflux of H$^+$ from parasites acidified using nigericin/BSA was attributable to Na$^+$/H$^+$ exchange via residual nigericin remaining in the parasite plasma membrane, rather than to endogenous transporter activity (Spillman et al., 2008). Likewise, Nkrumah et al. (2009) were unable to reproduce the Na$^+$/H$^+$-exchanger activity observed by Bennett et al., (Nkrumah et al., 2009) but they provided evidences that PfNHE-1 expression levels influenced QN sensitivity in concert with additional parasite genetic factors such as PfCRT, PfMDR1 and possibly additional yet unidentified parasite proteins. However, variations in PfNHE-1 expression levels did not impact on pH$_{cyt}$.

Since the seminal work by Ferdig et al. (2004), several studies have been conducted in different countries to evaluate the *pfnhe-1* polymorphisms and its association with *in vitro* QN susceptibility (Vinayak et al., 2007; Henry et al., 2009; Andriantsoanirina et al., 2010, 2012; Baliraine et al., 2010; Briolant et al., 2010, 2011; Meng et al., 2010; Okombo et al., 2010; Pelleau et al., 2011; Sinou et al., 2011). Conflicting data have been reported, likely due to the different geographical origin of parasites (implying different genetic backgrounds), the type of parasites used (fresh isolates, culture-adapted strains and reference lines) and the method used to assess *in vitro* QN susceptibility (Okombo et al., 2011; Pelleau et al., 2011). Thus, the implication of PfNHE-1 polymorphisms in QNR remains to be studied in detail.

The aim of this study was to provide a comprehensive analysis of the worldwide genetic polymorphism of ms4760 alleles of the pfnhe-1 gene and to discuss their usefulness as molecular marker of quinine resistance.

2. Plasmodium falciparum Na+/H+ exchanger (Pfnhe-1) allele polymorphism

Following the initial work of Ferdig et al. (2004) on genetic polymorphism of ms4760 within the *pfnhe-1* gene, a total of 1508 ms4760 sequences from isolates, culture-adapted parasites or reference strains from various geographical regions were retrieved from GenBank (last update on 15th June 2012) or from publications (Table 1) and were used for genetic analyses. A new numbering of ms4760 allele according to the chronological order of the data of the publication was conducted. Classification grouping ms4760 alleles according to the number of DNNND and

DDNHNDNHNND repeat motifs in blocks II and V was also performed.

Ms4760 sequences were aligned and compared using the Clustal W multiple alignment algorithm in BioEdit Sequence Alignment editor (Hall, 1999). Genetic diversity was assessed by Nei's unbiased expected heterozygosity (He) from haploid data and calculated as He = [n/(n − 1)][1 − pi] (n = the number of isolates sampled; pi = the frequency of the ith allele) (Nei, 1978). Population genetic differentiation was measured using Wright's F statistics (Wright, 1965); population genetic parameters were computed with FSTAT software, v2.9.4 (Goudet, 1995).

The Mann-Whitney *U* test or Kruskal–Wallis method were used for non-parametric comparisons, and Student's *t* test or one-way analysis of variance for parametric comparisons. For categorical variables, Chi-squared or Fisher's exact tests were used to assess significant differences in proportions.

All reported *P*-values are two-sided and were considered statistically significant if less than 0.05.

2.1. Global genetic polymorphism of ms4760 in pfnhe-1 gene

Amongst the 1508 studied sequences, 101 different *ms4760* alleles were observed. Ms4760 alleles were renumbered according to the chronology of the publication of the studies (ranging from ms4760-1 to ms4760-101) and are presented in Table 2. Alignment of sequences of blocks I–VI in ms4760 are displayed in Fig. 2.

2.1.1. Geographical distribution of ms4760 alleles: between continents

According to the location of the sample collection, 39 ms4760 alleles were observed in Asia (*n* = 398), 74 in Africa (*n* = 1070), 5 in South America (*n* = 17), 2 in Papua New Guinea (*n* = 5) and one in Haiti (*n* = 1). Five alleles were globally distributed (ms4760-1, ms4760-3 ms4760-5, ms4760-6, ms4760-7) while others were exclusively found in Asia and in Africa (*n* = 24, ms4760-2, ms4760-8, ms4760-9, ms4760-12, ms4760-14, ms4760-15 and ms4760-18 to ms4760-35) or only in Asia (*n* = 10, ms4760-4, ms4760-10, ms4760-11, ms4760-13, ms4760-16, ms4760-17 and ms4760-98 to ms4760-101) or only in Africa (*n* = 45, from ms4760-36 to ms4760-64, from ms4760-66 to ms4760-173 & ms4760-90 to ms4760-97). Seventeen alleles have unknown origins (ms4760-65 & ms4760-74 to ms4760-89). Data are presented in Table 2 and Fig. 3.

2.1.2. Geographical distribution of ms4760 alleles: within continents and between regions

Amongst the 39 alleles found in Asia, 16 (41%) were shared between South East Asia (Thailand, Cambodia, Vietnam, Malaysia and Myanmar/China border) and Central Asia (India), whereas 23 were specific to South East Asia. In Africa, the 74 observed alleles were distributed as follows: three were shared by all regions (West Africa, East Africa, Central Africa, South Africa, Indian Ocean Islands), seven were present in all regions except South Africa, five alleles were shared by West Africa, East Africa and Central Africa, four by East Africa, Central Africa and Indian Ocean Islands and 27 by East Africa and Central Africa. Thirteen alleles were found only in Central Africa and fifteen in Indian Ocean Islands only. Among the five alleles described in South America, only two were shared by Western countries (Honduras, Colombia, Ecuador and Peru) and Eastern country (Brazil). Two were specific from Western countries and one from Eastern countries (Table 2 and Fig. 3).

2.2. ms4760 profiles: geographical distribution and prevalence

According to the number of repeats in block II (DNNND) and block V (DDNHNDNHNND) which have been associated with modulation of *in vitro* QNR (Henry et al., 2009; Andriantsoanirina et al.,

Table 1

Summarized findings of the studies describing relationships between polymorphisms in *pfnhe-1*, *pfcrt*, *pfmdr-1* and *pfmrp* genes and *in vitro* susceptibility to quinine.

References	No. and type of parasites tested	Origin of parasites	*pfnhe*–ms4760				*pfcrt* polymorphism	*pfmdr-1* polymorphism	*pfmrp* polymorphism	GenBank accession No.
			No. of alleles	Association between No. of DNNND repeats and *in vitro* QN susceptibilty	Association between No. of DDNHNDNHNND repeatsand *in vitro* QN susceptibilty	Association between ms4760 allele and *in vivo* response	Association with *in vitro* QN susceptibilty	Association with *in vitro* QN susceptibilty	Association with *in vitro* QN susceptibilty	
Ferdig et al. (2004)	71 *P. falciparum* lines	South-east Asia ($n = 21$), Africa ($n = 34$), Central and South America ($n = 16$)	8	IC_{90}QN: 1 repeat vs. ⩾2 repeats ($P < 0.05$ in Asia and South America clones)	NA	NA	NA	NA	NA	NA
Vinayak et al. (2007)	244 *P. falciparum* isolates	India (5 different regions)	16	NA	NA	NA	NA	NA	NA	EF123065, EF123066 DQ864466–DQ864485 EF442125–EF442130
Henry et al. (2009)	6 reference strains and 17 culture-adapted isolates	South-east Asia ($n = 5$), Africa ($n = 17$), South America ($n = 1$)	8	IC_{50}QN: 1 repeat (154 ± 110 nM) vs. 2 repeats (548 ± 253 nM) vs. 3 repeats (764 ± 332 nM). A greater number of repeat was significantly associated with increased IC_{50}QN, $P < 0.0007$	IC_{50}QN: 1 repeat (673 ± 295 nM) vs. 2 repeats (222 ± 190 nM) vs. 3 repeats (58 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P = 0.002$	NA	No significant association (M74I, N75E, K76T, A220S, Q271E/V, I356T/L, 1371R), except S326D ($P = 0.0019$)	No significant association (N86Y, Y184F, S1034C, N1042D, D1246Y)	No significant association (H191Y, S437A)	NA
Andriantsoanirina et al. (2010)	83 clinical isolates	Madagascar ($n = 40$), Africa mainland ($n = 43$)	19	No significant association	IC_{50}QN: 1 repeat (1117 nM) vs. 2 repeats (192 nM). A greater number of repeat w as significantly associated with increased IC_{50}QN, $P = 0.02$)	NA	Significant association: IC_{50}QNK76 (121 nM and T76 (242 nM), $P = 0.001$	NA	NA	FJ947067–FJ947073.1
Okombo et al. (2010)	29 adapted isolates	Kenya	10	IC_{50}QN: 1 repeat (60 nM) vs. 2 repeats (227 nM) vs. 3 repeats (45 nM). The increase in the IC_{50}QN w as observed only for parasites with 2 repeats ($P < 0.05$) but not with parasites with 3 repeats ($P < 0.01$)	NA	NA	No significant association (K76T)	No significant association (N86Y), but a trend toward a decrease in IC_{50}QN in 86Y parasites (208 nM) vs. Wild-type (74 nM).	NA	HM210746–HM210771
Briolant et al. (2010)	8 reference strains and 15 culture-adapted isolates	South-east Asia ($n = 5$), Africa ($n = 15$), South America ($n = 3$)	8	No significant association	NA	NA	Significant association (M74I, N75E, K76T, A220S, I371R), except C72S, Q271E/V, I356T/L,	No significant association (N86Y, Y184F, S1034C, N1042D, D1246Y)	Significant association (H191Y, S437A)	NA
Meng et al. (2010)	60 culture-adapted	China–Myanmar	10	IC_{50}QN: 1 repeat (254 ± 89 nM) vs. 2 repeats	IC_{50}QN: 1 repeat (624 ± 337 nM) vs. 2	NA	No significant association (K76T,	No significant association (N86Y, Y184F, S1034C,	NA	NA

isolates	border		(453 ± 239 nM) vs. 3 repeats (674 ± 365 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P < 0.0045$), except for parasites with 4 repeats (462 ± 123 nM)	repeats (374 ± 244 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P < 0.0045$		A220S)	N1042D, D1246Y)		
Baliraine et al. (2010)	240 clinical isolates	Uganda	40	No significant association	No significant association	No significant association	NA (99.4% of 76T)	No significant association (N86Y, Y184F, D1246Y), but a trend toward a decrease in IC_{50}QN with increased number of mutations (wild-type, 65 nM; 1 mutation, 65 nM; 2 mutations, 101 nM and 3 mutations, 312 nM, $P < 0.02$)	NA	HQ412347–HQ412386
Briolant et al. (2011)	74 clinical isolates	Republic of Congo	27	No significant association	No significant association	NA	NA	NA	NA	FJ392810–FJ392827
Sinou et al. (2011)	79 clinical isolates	Vietnam	10	IC_{50}QN: 0–1 repeat (300 nM) vs. ⩾2 repeats (682 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P = 0.015$).	IC_{50}QN: 1 repeat (704 nM) vs. 2 repeats (375 nM). A greater number of repeat was significantly associated with decreased IC_{50}QN, $P < 0.01$.	NA	NA (84% of 76T)	NA (>95% of wild-type pfmdr-1)	NA	GQ845119–GQ845119–GQ465284
Pelleau et al. (2011)	90 *P. falciparum* isolates and 95 culture adapted isolates	Africa (n = 85), Indian Ocean (n = 36), Asia (n = 38), South America (n = 20), Unknown origin (n = 2)	32	Field isolates: no significant association. Culture-adapted isolates: IC_{50}QN: 0–1 repeat (set#1: 45 nM and set#2: 49 nM) vs. ⩾2 repeats (set#1:98 nM and set#2: 543 nM). A greater number of repeat was significantly associated with increased IC_{50}QN ($P < 0.01$).	Field isolates: no significant association. Culture adapted isolates: IC_{50}QN: For set#2, 1 repeat (557 nM) vs. 2 repeats (270 nM) vs. (118 nM) A greater number of repeat was significantly associated with decreased IC_{50}QN ($P = 0.01$).	NA	Field isolates: no significant association. Culture adapted isolates: Significant association (K76T) in set#1 (K76: 38 nM and T76: 142 nM) and set#2 (K76: 204 nM and T76: 543 nM).	Field isolates: no significant association. Culture adapted isolates: Significant association: S1034C in set#1 (S1034: 55 nM and C1042: 148 nM) and set#2 (S1034: 302 nM and C1042: 780 nM), N1042D in set#1 (N1042: 51 nM and D1042: 144 nM) and set#2 (N1042: 290 nM and D1042: 607 nM), D1246Y in set#1 (D1246: 58 nM and Y1246: 144 nM).	NA	GQ496590–GQ496601 FJ266461–FJ266471
Poyomtip et al. (2012)	85 *P. falciparum* culture-adapted isolates	Thai–Myanmar (n = 37) and Thai–Cambodia (n = 48)	?	No significant association	No significant association	NA	NA	No significant association with pfmdr-1 copy number. Significant association: N86Y (N86: 216.5 nM and Y86: 138.3 nM, $P = 0.02$), Y184F (Y184: 160.4 nM and F184: 228.3 nM, $P = 0.01$) & N1042D (N1042: 185.8 nM and D1042: 270.5 nM, $P = 0.01$)	NA	NA
Andriantsoanirina et al. (2012)	595 *P. falciparum* isolates	Madagascar (n = 345), Comoros Islands (n = 250)	29	NA	NA	NA	NA	NA	NA	JX472441–JX472448

NA, Not available; QN, Quinine; IC_{50}, 50% inhibitory concentration; IC_{90}, 90% inhibitory concentration; Significant associations are shown in red.

Table 2
Classification of *pfnhe-1* sequences in 101 different alleles according to the geographical location of the isolates.

Continent	ASIA							AFRICA																											SOUTH AMERICA						OTHER		
Region	South East				Central		Total	West Africa													East Africa						Central Africa			South Africa	Indian Ocean Islands		Total	West			East		Total			
Country	THA	KHM	VNM	MYS	CHN/MMR	IND		MRT	SEN	GMB	MLI	GNB	SLE	LBR	CIV	GHA	TGO	BEN	BFA	NER	SDN	KEN	UGA	DJI	CAF	TCD	COG	COD	ZAF	MOZ	COM	MDG		HND	COL	ECU	PER	BRA		PNG	HTI	?
No. of isolates	8	4	81	1	60	244	398	1	5	12	21	2	2	5	4	1	4	4	6	4	32	241	1	3	1	76	3	2	1	255	386	1070	1	1	1	5	8	17	5	1	23

(Rows ms4760-1 through ms4760-101, with grey boxes meaning "ms-4760 haplotype never detected" and black boxes meaning "ms-4760 haplotype detected at least once".)

Countries are designed by the 3-letters codes used by the United Nations: THA, Thailand; KHM, Cambodia; VNM, Vietnam; MYS, Malaysia; CHN, China; MMR, Myanmar; IND, India; MRT, Mauritania; SEN, Senegal; GMB, Gambia; MLI, Mali; GNB, Guinea-Bissau; SLE, Sierra Leone; LBR, Liberia; CIV, Côte d'Ivoire; GHA, Ghana; TGO, Togo; BEN, Benin; BFA, Burkina Faso, NER, Niger; SDN, Soudan; KEN, Kenya; UGA, Uganda; DJI, Djibouti; CAF, Central African Republic; TCD, Chad; COG, Congo; COD, Congo, the Republic Democratic of the; ZAF, South Africa; MOZ, Mozambique; COM, Comoros; MDG, Madagascar; HND, Honduras; COL, Colombia; ECU, Ecuador; PER, Peru; BRA, Brazil; PNG, Papua New Guinea; HTI, Haiti. Grey box means "ms-4760 haplotype never detected" and black box means "ms-4760 haplotype detected at least once".
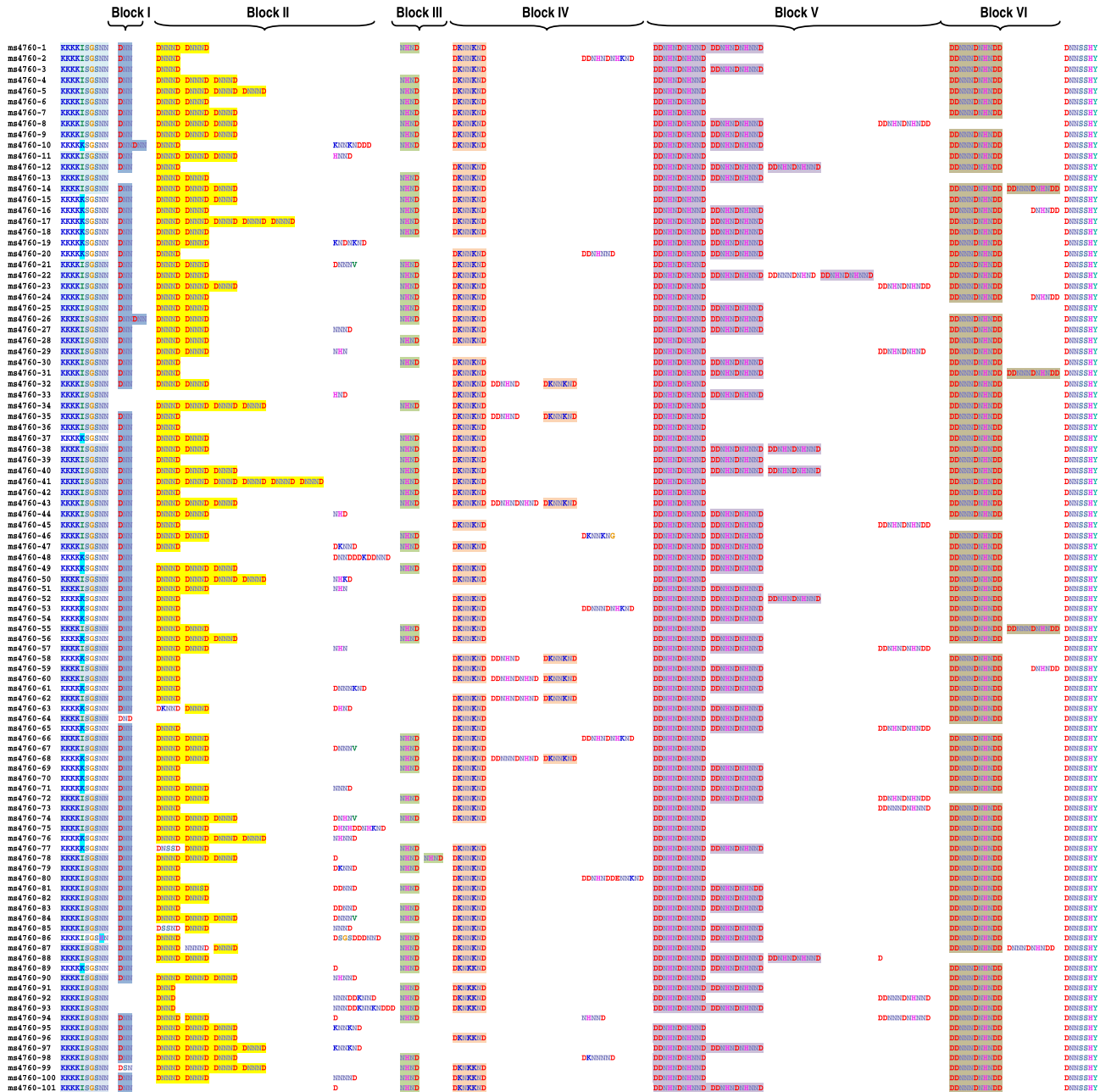
Fig. 2. Alignment of the 101 ms4760 protein sequences found in Genbank and in publications.

2010), ms4760 alleles were grouped in 15 allele profiles (from ms4760-A to ms4760-O) presented in Table 3. The number of repeats in block II (DNNND) varied from 0 (ms-4760-A) to 6 (ms4760-O) while the number of repeats in block V (DDNHNDNHNND) from 1 (ms4760-B, ms4760-E, ms4760-I, ms4760-L, ms4760-O) to 4 (ms4760-H). Sixty-six percent of ms-4760 alleles were grouped in 4 profiles: ms-4760-C [(DNNND)$_1$; (DDNHNDNHNND)$_2$] (23%), ms-4760-F [(DNNND)$_2$; (DDNHND NHNND)$_2$] (15%), ms4760-E [(DNNND)$_2$; (DDNHNDNHNND)$_1$] (14%) and ms-4760-I [(DNNND)$_3$; (DDNHNDNHNND)$_1$] (14%). Four profiles were globally distributed (ms-4760-C, ms-4760-E, ms-4760-F and ms-4760-L), five were observed in both Asia and Africa (ms-4760-A, ms-4760-B, ms-4760-D, ms-4760-H and ms-4760-J), two were only found in Asia (ms-4760-I, ms-4760-N) and four only in Africa (ms-4760-G, ms-4760-K, ms-4760-M and ms-4760-O) (Fig. 3).

The mean number of DNNND repeats was significantly higher in Asia (2.30, SD = 0.78) compared to Africa (2.06, SD = 0.90, $P < 0.001$). Inversely, the number of DDNHNDNHNND repeats was significantly lower in Asia (1.34, SD = 0.51) compared to Africa (1.72, SD = 0.54, $P < 0.001$). Consequently, the mean ratio of DNNND/DDNHNDNHNND repeats was significantly higher in Asia (2.03 ± 0.98 vs. 1.46 ± 1.05, $P < 0.001$).

The prevalence of the *pfnhe-1* ms4760 profiles according to the geographical location of the isolates (continent & country) significantly differed between continents ($P < 0.0001$, Table 4 and Fig. 4). In both continents (Asia & Africa), 11 profiles had a low prevalence (<10%). Three profiles were predominant in Africa (ms-4760-F, 32.9%; ms-4760-C, 21.3% and ms-4760-I, 15.5%), and four in Asia (ms-4760-I, 37.1%; ms-4760-E, 28.1%; ms-4760-C, 14.3% and ms-4760-F, 10.9%). Interestingly, the prevalence of the ms-4760-C (1 DNNND repeat) decreased from Central Africa (38%) to East Africa
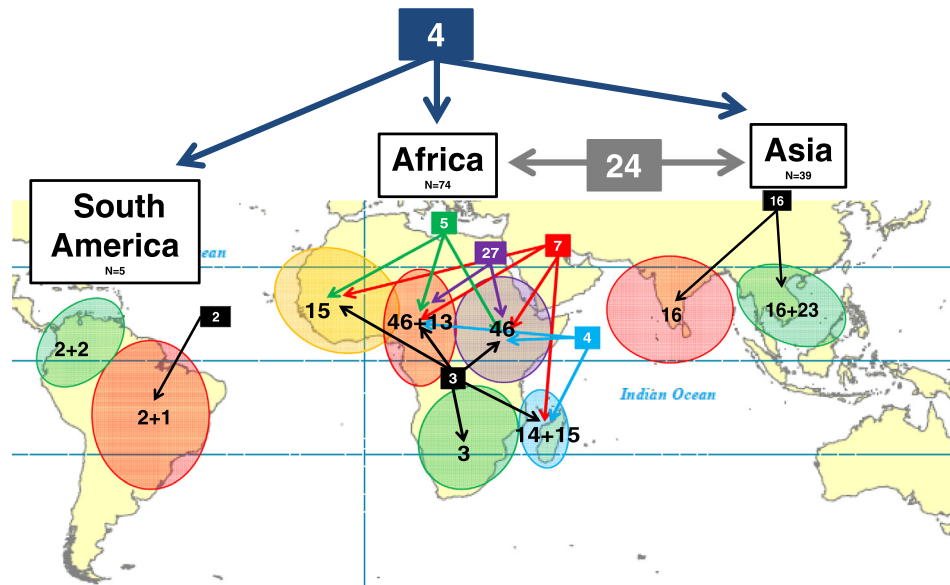
Fig. 3. Geographical distribution of the *pfnhe-1* ms4760 profile between and with continents. Numbers in boxes indicate shared ms4760 profiles between continents (blue and grey boxes) and regions (black, green, red, purple and light blue boxes). For each region, numbers of ms4760 profiles are split into shared profiles (first number) and local profiles (second number) (i.e., Madagascar: 14 shared profiles and 15 local profiles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Phne-1 ms4760 profile groups according to the number of repeat in block II (DNNND) and block V (DDNHNDNHNND) and the geographical location of the isolates.

ms4760 alleles	ms4760 profiles	No. of haplotypes	Block II DNNND	Block V DDNHNDNHNND	Asia	Africa	South America
33, 48, 64, 89, 91 92, 93, 101	ms4760-A	8	0	2	■	■	
2, 35, 36, 42, 58, 62, 79, 80	ms4760-B	8	1	1	■	■	■
3, 10, 20, 30, 31, 39, 45, 47, 53, 54, 59, 60, 61, 63, 65, 69, 70, 73, 77, 81, 83, 85, 86	ms4760-C	23	1	2	■	■	■
12, 52	ms4760-D	2	1	3	■	■	
6, 21, 24, 28, 29, 32, 37, 55, 66, 67, 68, 75, 94, 100	ms4760-E	14	2	1	■	■	■
1, 13, 16, 18, 19, 25, 26, 27, 44, 46, 51, 57, 71, 72, 82, 87	ms4760-F	16	2	2	■	■	■
38, 88	ms4760-G	2	2	3		■	
22	ms4760-H	1	2	4		■	
4, 7, 11, 14, 15, 23, 43, 74,78, 84, 90, 95, 96, 98	ms4760-I	14	3	1	■	■	
8, 9, 49, 56	ms4760-J	4	3	2	■	■	
40	ms4760-K	1	3	3		■	
5, 34, 50, 76, 99	ms4760-L	5	4	1	■	■	
97	ms4760-M	1	4	2	■		
17	ms4760-N	1	5	2		■	
41	ms4760-O	1	6	1			■

For each profile (A to O) and continent (Asia, Africa and South America), white box means "ms-4760 profile never detected" and black box means "ms-4760 profile detected at least once".

(21%), Indian Ocean (19%), Central Asia (19%) and South East Asia (6%) whereas the prevalence of the ms-4760-I (3 DNNND repeats) increased along the same west to east axis (7%, 14%, 17%, 23% and 61%).

2.3. Genetic diversity and genetic differentiation between parasite populations

Genetic diversity, assessed by Nei's unbiased expected heterozygosity (*He*) was significantly higher in Africa (Congo = 0.7649, Uganda = 0.7975, Kenya = 0.6582), Indian Ocean (Madagascar = 0.8053, Comoros Islands = 0.7946) or India (0.6807) compared to China/Myanmar (0.6807, $P = 0.04$) or Vietnam (0.4981, $P < 0.0001$) (Table 4).

The degree of genetic differentiation of the ms4760 profiles within parasite populations, estimated by *Fst* values, indicated a large divergence between Asian populations and African populations (Table 5). The highest differences were observed between populations from Vietnam or China/Myanmar and populations from Kenya (*Fst* = 0.319 and 0.183), Congo (*Fst* = 0.291 and 0.176), Uganda (*Fst* = 0.219 and 0.121), Madagascar (*Fst* = 0.202 and 0.111), Comoros Islands (*Fst* = 0.171 and 0.083) and India (*Fst* = 0.171 and 0.069). On the other hand, populations from Africa (Congo, Uganda, Kenya and Madagascar) showed very low divergence or were similar (*Fst* from 0.0001 to 0.076) and population from India was intermediate (*Fst* from 0.070 to 0.163).

3. Discussion

The biostatistical analyses performed in this study showed a large global genetic polymorphism of ms4760 in *pfnhe-1* gene. The African continent displayed the highest number of different alleles, followed by Asia and South America. While a few alleles were shared by three continents, others appeared restricted to Asia and/

Table 4
Prevalence and expected heterozygosity of the *pfnhe-1 ms4760* groups according to the geographical location of the isolates (continent & country)

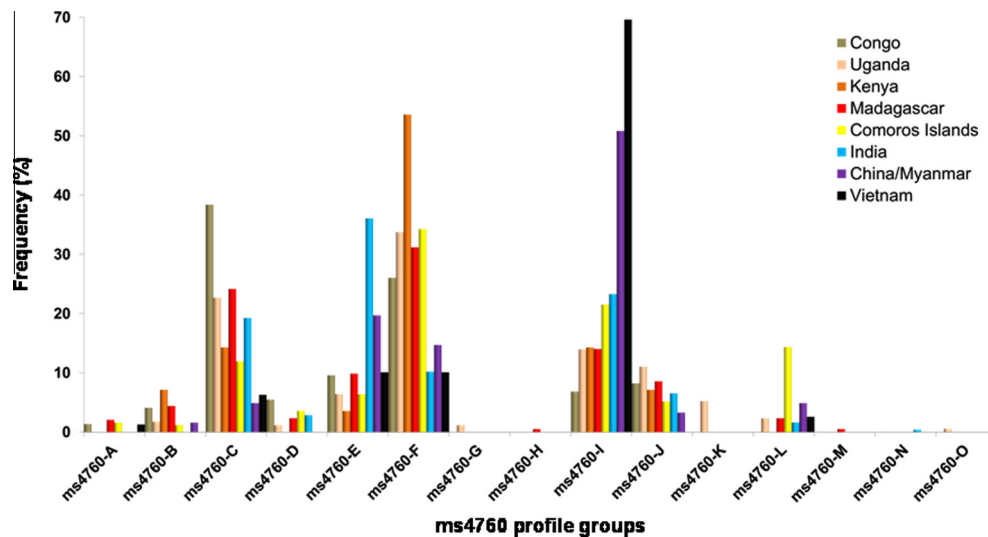| ms4760 profiles (%)	Prevalence by Continent/Country								Total
	Africa					Asia			
	Congo	Uganda	Kenya	Madagascar	Comoros Islands	India	China/Myanmar	Vietnam	
	n = 74	*n* = 172	*n* = 29	*n* = 386	*n* = 251	*n* = 244	*n* = 60	*n* = 79	
ms4760-A	1.3	0.0	0.0	2.0	1.5	0.0	0.0	1.3	1.1
ms4760-B	4.1	1.7	7.1	4.4	1.1	0.0	1.6	0.0	2.2
ms4760-C	38.3	22.6	14.2	24.1	11.9	19.2	4.9	6.3	19.2
ms4760-D	5.4	1.1	0.0	2.3	3.5	2.8	0.0	0.0	2.3
ms4760-E	9.5	6.3	3.5	9.8	6.3	36.0	19.7	10.1	13.9
ms4760-F	26.0	33.7	53.5	31.1	34.2	10.2	14.7	10.1	26.3
ms4760-G	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1
ms4760-H	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.1
ms4760-I	6.8	13.9	14.2	14.0	21.5	23.2	50.8	69.6	21.9
ms4760-J	8.2	11.0	7.1	8.5	5.1	6.5	3.3	0.0	7.0
ms4760-K	0.0	5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.7
ms4760-L	0.0	2.3	0.0	2.3	14.3	1.6	4.9	2.6	4.5
ms4760-M	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.1
ms4760-N	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.1
ms4760-O	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.1
He	0.765	0.797	0.658	0.805	0.794	0.766	0.680	0.493	

He: expected heterozygosity.

Fig. 4. Prevalence of the *pfnhe-1 ms4760* profile groups according to the geographical location of the isolates (continent and country). *Phne-1 ms4760* profile groups are described in Table 3 (according to the number of repeat in block II, DNNND and block V, DDNHNDNHNND).

Table 5
Degree of genetic differentiation of the ms4760 profile groups within parasite populations (African and Asian countries), estimated by pairwise population genetic distances (*FST*).

	Uganda	Kenya	Madagascar	Comoros Islands	India	China/Myanmar	Vietnam
Congo	0.01739	0.07610	0.01025	0.06382	0.08763	0.17689	0.29186
Uganda		0.01959	0.00011	0.01911	0.08776	0.12179	0.21948
Kenya			0.02770	0.02876	0.16355	0.18367	0.31962
Madagascar				0.02089	0.07075	0.11195	0.20280
Comoros Islands					0.09344	0.08370	0.17157
India						0.06970	0.17199
China/Myanmar							0.02694

or Africa. Similarly, within continents, some alleles were shared between various regions while others appeared restricted to specific areas as Central Africa or Indian Ocean. The geographical distribution and prevalence of ms4760 profiles, defined by variations in the number of repeats in block II and V, differed also significantly between continents. Interestingly, the mean ratio of DNNND/DDNHNDNHNND repeats, proposed by some authors as associated with *in vitro* QNR, was significantly higher in Asia than

in Africa. The predominant ms4760 profiles in Africa were the same that in Asia, except for the ms-4760-E which was predominant only in Asia. Some data suggested a geographical diffusion of some alleles. The prevalence of a particular profile, the ms-4760-C (1 DNNND repeat), decreased from Central Africa to East Africa, Indian Ocean, Central Asia and South East Asia while the prevalence of another "opposite" profile, the ms-4760-I (3 DNNND repeats), increased along the same west to east axis. Genetic anal-

ysis using expected heterozygosity H_e showed a higher genetic diversity in parasites from Africa (including Indian Ocean Islands) than in those from Asia. In addition, F_{st} values indicated a large divergence between Asian and African populations. These data showed that the Asian and African populations were clearly differentiated.

Several studies have described the relationships between polymorphisms in *pfnhe-1* and *in vitro* susceptibility to quinine (Table 1). Some of these studies have also considered the association of *in vitro* susceptibility to quinine with polymorphisms in *pfcrt*, *pfmdr-1* and *pfmrp* genes, with conflicting results for *pfcrt* and *pfmrp*, and no association (except in one study) with SNPs polymorphisms of *pfmdr-1* (Table 1).

Following the inaugural study of Ferdig et al. (2004), Henry et al. (2009) investigated a series of 23 culture-adapted isolates or reference strains. The relationship between the number of DNNND repeats and the inhibitory concentration 50% values (IC_{50}) to QN was confirmed and an increased number of the DDNHNDNHNND repeat motif was associated with decreased IC_{50}s to QN. A limitation of these studies was that the *in vitro* QN susceptibility and polymorphisms determinations were performed on culture-adapted cloned isolates or reference strains, which could lead to biased results due to accumulated mutations selected by *in vitro* conditions or to selection of specific alleles during the culture. Indeed, a recent study showed an association between *pfnhe-1* polymorphism and in vitro QN response on cultured adapted isolates but not in field isolates (Pelleau et al., 2011).

An increased number of DNNND repeats was positively associated with *in vitro* QNR in six studies (Ferdig et al., 2004; Henry et al., 2009; Meng et al., 2010; Okombo et al., 2010; Pelleau et al., 2011; Sinou et al., 2011) (Table 1). All these studies used culture-adapted parasites, except study from Sinou et al. (2011). It is worth noting that Ferdig et al. (2004) used IC_{90}s rather than IC_{50}s in the other studies (Ferdig et al., 2004). Parasites having 2 or more repeats had higher IC_{90}s than parasites having 1 repeat ($P < 0.05$ for Asia and South America lines). Okombo et al. (2010) observed this association only for parasites from Kenya having 2 repeats compared to 1 repeat ($P < 0.05$) (Okombo et al., 2010). Meng et al. (2010) reported a strong positive association in a series of 60 adapted isolates from the China–Myanmar border (Meng et al., 2010). Sinou et al. (2011) also reported a positive association in a series of 51 clinical fresh isolates from Vietnam: isolates with two or more DNNND motifs were less susceptible to QN than those harbouring zero or one DNNND repeats (Sinou et al., 2011). Discordant results were reported in a Thai study by Poyomtip et al. (2012) who did not observe an association between the number of DNNND repeats and *in vitro* QNR in a series of 81 culture-adapted isolates obtained from the Thai–Myanmar border and the Thai–Cambodia border (Poyomtip et al., 2012).

Pradines and colleagues (2009–2011) published 3 studies of *pfnhe-1* polymorphisms (Henry et al., 2009; Briolant et al., 2010, 2011). In the first one, Henry et al., 2009 including 23 reference strains or culture-adapted isolates of various geographic origin, found a positive association between the number of DNNND repeats and IC_{50}s (Henry et al., 2009). In another study Briolant et al. (2010, 2011) including 23 reference strains or culture adapted isolates of similar geographic origin did not found any association (Briolant et al., 2010). Lastly, in a series of 74 clinical isolates from Republic of Congo, Briolant et al. (2011) did not found an association either (Briolant et al., 2011). Two other studies including respectively 83 and 172 clinical isolates from African countries did not find any association between an increased number of repeats in DNNND and *in vitro* QNR (Andriantsoanirina et al., 2010; Baliraine et al., 2010). The studies conducted in Asian areas reported an overrepresentation of the *ms4760*-7 allele, harboured by 49.2% and 68.3% of isolates in China–Myanmar border and Viet-

nam, respectively (Meng et al., 2010; Sinou et al., 2011). In the study by Meng et al. (2010), ms4760-7 isolates were among those having the lowest *in vitro* susceptibility to QN but other ms4760-7 isolates of that series displayed perfect susceptibility (Meng et al., 2010). The ms4760-7 allele was also overrepresented in the study by Henry et al. (2009) including 4 Asian isolates with high IC_{50}s, ranging from 599 to 1310 nM (Henry et al., 2009). The presence of the *ms4760*-7 allele was not rare in other areas, in particular in African countries (Ferdig et al., 2004; Andriantsoanirina et al., 2010, 2012; Okombo et al., 2010; Briolant et al., 2011) but without obvious association with QNR, many isolates harbouring this allele showing full *in vitro* susceptibility. Thus, further studies are needed to confirm whether the *ms4760*-7 allele is necessary for the emergence QN resistance and can be used in monitoring the QNR spread in South East Asia.

The number of DDNHNDNHNND repeat motif was associated with reduced *in vitro* susceptibility to QN ($P < 0.01$) in one study of 83 clinical isolates obtained in African countries (Andriantsoanirina et al., 2010). Conversely, an increased number of DDNHNDNHNND repeats was associated with higher *in vitro* susceptibility to QN in studies of isolates from the China–Myanmar border (Meng et al., 2010) or Vietnam (Sinou et al., 2011). The same association was observed in one study (Henry et al., 2009) but not confirmed in 2 subsequent studies by the same team (Briolant et al., 2010, 2011). Three other studies did not find this association either (Baliraine et al., 2010; Okombo et al., 2010; Poyomtip et al., 2012).

The usefulness of PfNHE-1 polymorphisms as marker of *in vitro* QNR may be inferred from some publications. In parasites from Asian areas, the number of DNNND repeats has been positively associated with *in vitro* QNR in culture-adapted isolates from the China–Myanmar border (Meng et al., 2010) and in isolates from Vietnam (Sinou et al., 2011) but not in culture-adapted isolates from the Thai–Myanmar border and the Thai–Cambodia border (Poyomtip et al., 2012). In parasites from Africa, the existing data show no evidence of association of the number of DNNND repeats and QN susceptibility, excluding its use as a molecular marker of QNR (Andriantsoanirina et al., 2010; Baliraine et al., 2010; Briolant et al., 2010, 2011). This may change in the future and the situation could be particular in Kenya (Okombo et al., 2010) as resistance genotypes originating from the South-East Asia may have reached this country as it was the case in the past for chloroquine and antifolates resistant *P. falciparum*.

The number of DDNHNDNHNND repeats does not seem correlated with *in vitro* QNR or contributing to QNR in Asian areas, so it does not appear as an interesting marker. Choudhary and Sharma, 2009 studied the polymorphisms in flanking microsatellites of the *pfnhe-1* gene in 108 Indian isolates (Choudhary and Sharma, 2009). They observed an expected heterozygosity of 10 flanking microsatellites in the vicinity of ±40 kb of *pfnhe-1* gene comparable to any other neutral loci. Thus, no selective sweep or valley of reduced variation around ±40 kb of this gene was observed, indicating that there was no strong selection pressure on the *pfnhe-1* gene. In addition, these authors did not find an association between DNNND repeat polymorphisms and microsatellite alleles.

The association of PfNHE-1 polymorphism and clinical resistance remains to be evaluated. Currently, only 2 cases of clinical failures have been reported. Pradines and colleagues (2011) studied a QN treatment failure in a traveller from Senegal, and observed the association of two repeats of DNNND with a reduced in vitro susceptibility ($IC_{50} = 829$ nM) (Pradines et al., 2011). The second case, a QN treatment failure in a traveller from French Guiana, did not show this association as the ms4760 microsatellite showed 1 repeat of DNNND and 2 repeats of DDNHNDNHNND, though the isolate had a reduced susceptibility to QN ($IC_{50} = 1019$ nM) (Bertaux et al., 2011).

The finding of association of polymorphisms in putative genes with clinical failures and/or *in vitro* susceptibility constitutes a pivotal step in the development of tools for the surveillance of emergence and spreading of *P. falciparum* resistant strains. Such associations must be verified on numerous isolates originating from various geographical areas and supported by molecular studies to specifically assess the involvement of the candidate genes in drug resistance. Recent genetic and physiological studies reinforced the conclusion that QNR is a complex trait requiring multiple actors (Nkrumah et al., 2009). Several transporters have been identified as determinants of resistance to quinoline antimalarial drugs. The available data on molecular surveys of potential contributors to QN resistance do not allow to propose a simple molecular typing methodology of global application. It is possibly a consequence of the multigene nature of the QNR trait, which involves multiple gene interactions. Such gene interactions depend on the alleles at play in each genetic background and likely show substantial geographic variations. In particular *pfcrt* and *pfmdr1* known to contribute to QN susceptibility have different alleles in different geographic settings (Wellems et al., 2009). For example, CQR *P. falciparum* strains have originated from at least six different geographic locations spread across Southeast Asia, Latin America and the Pacific region (Wootton et al., 2002; Wellems et al., 2009). African CQR strains have their origins in a single foundation event, a strain apparently imported from Southeast Asia. In the case of QN (and of most other antimalarial drugs), the drug pressure that selected for resistance varied considerably with respect to intensity and time in the different geographic areas. As a result, the association of different alleles of transporters with resistance to quinoline antimalarial drugs may show geographic disparities. Likewise, the amplification of *pfmdr1*, associated with *in vitro* resistance to QN, mefloquine, and halofantrine is frequent in Asia (Price et al., 2004) but rare in the African continent. Analogous processes may have occurred for *pfnhe-1*. However, the absence of selective sweep in 108 Indian *P. falciparum* isolates and the lack of association of microsatellite markers with DNNND repeats, possibly indicates that there is no strong selection pressure on the *pfnhe-1* gene (Choudhary and Sharma, 2009). Studies summarized in this paper do not exclude a potential role for PfNHE-1 in QNR in a strain-dependent manner.

In this context, the validity and reliability of candidate polymorphisms in *pfnhe-1* gene as molecular markers of QNR appears restricted to endemic areas from South Asia or possibly East African countries and needs to be confirmed.

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Conflict of interest

None declared.

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