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# Multiple Genetic Backgrounds of the Amplified *Plasmodium falciparum* Multidrug Resistance (*pfmdr*1) Gene and Selective Sweep of 184F Mutation in Cambodia

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## Abstract

**Background—**The emergence of artesunate-mefloquine (AS+MQ)–resistant *Plasmodium falciparum* in the Thailand-Cambodia region is a major concern for malaria control. Studies indicate that copy number increase and key alleles in the *pfmdr*1 gene are associated with AS+MQ resistance. In the present study, we investigated evidence for a selective sweep around *pfmdr*1 because of the spread of adaptive mutation and/or multiple copies of this gene in the *P. falciparum* population in Cambodia.

**Methods—**We characterized 13 microsatellite loci flanking (± 99 kb) *pfmdr*1 in 93 single-clone *P. falciparum* infections, of which 31 had multiple copies and 62 had a single copy of the *pfmdr*1 gene.

**Results—**Genetic analysis revealed no difference in the mean (± standard deviation) expected heterozygosity ($H_e$) at loci around single (0.75 ± 0.03) and multiple (0.76 ± 0.04) copies of *pfmdr*1. Evidence of genetic hitchhiking with the selective sweep of certain haplotypes was seen around mutant (184F) *pfmdr*1 allele, irrespective of the copy number. There was an overall reduction of 28% in mean $H_e$ (± SD) around mutant allele (0.56 ± 0.05), compared with wild-type



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allele (0.84 ± 0.02). Significant linkage disequilibrium was also observed between the loci flanking mutant *pfmdr*1 allele.

**Conclusion—**The 184F mutant allele is under selection, whereas amplification of *pfmdr*1 gene in this population occurs on multiple genetic backgrounds.

The Thailand-Cambodia border has been an epicenter for drug-resistant malaria and is where resistance to chloroquine (CQ) emerged in the early 1960s [1]. Sulfadoxine-pyrimethamine (SP) officially became the first-line treatment for falciparum malaria in 1989, followed by mefloquine (MQ) in 1994, although both drugs were likely available in the private sector much earlier. Resistance to MQ exceeded 25% (RI–RIII) in western Cambodia in the mid-1990s, and artemisinin-based combination therapy (ACT) consisting of artesunate (AS) and MQ, was adopted as the first-line treatment against *Plasmodium falciparum* countrywide in 2000 [2]. There is growing evidence that efficacy of AS+MQ is decreasing along the Thailand-Cambodia border [3–7], as well as in southwestern Cambodia [8].

One of the most widely used molecular markers for predicting MQ or AS+MQ early treatment failure and/or recrudescence is an increase in copy number in the *pfmdr*1 gene [3, 8–10]. The *pfmdr*1 gene amplification has been found to be associated with resistance to MQ, AS, quinine (QN), and halofantrine (HAL) in in vitro–selected parasite lines [11–13] and genetically modified parasites [14], as well as in clinical isolates [9, 15–18]. In addition to gene amplification, *pfmdr*1 alleles 86N, 1034S, 1042N, and 1246D have also been associated with in vitro resistance to MQ and AS in genetically modified parasite lines [19, 20] and clinical isolates [17, 21]. Field studies have also shown selection of the 86N allele in recurrent infections after treatment with AS+MQ or artemether plus lumefantrine (AL) [9, 22–24], which suggests that 86N could be a potential marker of MQ and lumefantrine resistance in vivo. However, no clear association between *pfmdr*1 184F mutation and MQ failure has been established, although this allele is widespread in Cambodia [25]. Western Cambodia has a higher prevalence of MQ resistance than does eastern Cambodia, and this is consistent with an increased frequency of multiple copies of *pfmdr*1 and the 184F mutation in the western part of the country, compared with the eastern part of the country [25, 26]. Although AS+MQ treatment policy for uncomplicated falciparum malaria applies for the whole of Cambodia, the difference in drug pressure between western and eastern Cambodia is well known. The available data indicate high efficacy (~100%) of AS+MQ in eastern Cambodia, compared with Pailin in western Cambodia, where a reduced efficacy (79%) of AS+MQ has been reported [6, 25].

Microsatellites are important markers to identify regions in the genome that have been under selection. During the process of selection, a beneficial mutation spreads through the population that results in a reduction of heterozygosity at both the selected locus and neutral flanking microsatellite loci (genetic hitchhiking) [27]. Under continuous selection, the mutation eventually gets fixed in the population, and sequence diversity is reduced around the selected locus (selective sweep). Evidence of selective sweeps around drug-resistant *pfcrt* and *dhfr*, *dhps* alleles due to CQ and SP drug pressure, respectively, have been described in several *P. falciparum* populations [28–32]. Microsatellite analysis around these alleles also revealed that CQ and SP resistance originated independently at only a few places and later spread to other parts of the world, which suggests the role of gene flow in the evolution of drug resistance [29, 30, 32–34]. Hence, it is important to investigate whether evolving MQ resistance has a similar genetic basis.

Previous studies have addressed the genetic basis of *pfmdr*1 gene amplification and found that the amplification involves a wide chromosomal region (up to 100 kb) and contains multiple copies of the *pfmdr*1 gene [11, 15, 35]. Recently, these findings were further

confirmed in *P. falciparum* isolates from the Thailand-Myanmar border, which showed that a 15–49 kb region of the chromosome 5 is amplified and contains 2–4 copies of *pfmdr*1 [36]. It is not known whether the amplification of the *pfmdr*1 gene in Cambodian parasites is similar.

Here, we have attempted to understand the nature of selective sweeps occurring around *pfmdr*1 caused by the spread of adaptive mutation and/or multiple copies of this gene in the *P. falciparum* population in Cambodia. This study also addresses geographical differences in the *pfmdr*1 haplotype pattern between eastern and western Cambodia.

## MATERIALS AND METHODS

### Sample collection, DNA isolation, *pfmdr*1 genotyping, and copy number estimation

Clinical isolates of *P. falciparum* were collected from patients with uncomplicated falciparum malaria from 4 sites across Cambodia: Pailin and Kampong Seila in western Cambodia and Memut and Rattanakiri in eastern Cambodia, as described earlier [25]. The location of the study sites and distribution of MQ resistance is shown in Figure 1. Epidemiologically, malaria incidence is much lower in western Cambodia than it is in eastern Cambodia. Eastern Cambodia is less developed, has a greater population of ethnic minorities, and has a very poor public health system. In eastern Cambodia, local transmission within villages is more common, and all age groups are affected. In western Cambodia, malaria is more predominant among adults, who are usually occupationally exposed in the jungles (ie, outside of their villages), than it is among other age groups.

This study was approved by the Institutional Review Boards of the Cambodia National Ethics Committee for Health Research, the US Naval Medical Research Unit No.2 (Jakarta, Indonesia), and the University of North Carolina at Chapel Hill (Chapel Hill, NC).Written informed consent was obtained from each participant before blood samples were collected. The patients were treated with AS+MQ in accordance with current national antimalarial drug policy.

DNA was extracted from filter paper blood spots using QIAamp Mini kit (Qiagen). The *pfmdr*1 copy number estimation and genotyping of *pfmdr*1 codons 86, 184, 1034, 1042, and 1246 was done previously using real-time polymerase chain reaction (PCR) [25]. These coded DNA samples were sent to the Centers for Disease Control and Prevention laboratory at Atlanta, Georgia, for further analysis. Of 158 DNA samples, 62 had multiple copies (≥2) of *pfmdr*1, whereas the remaining 96 samples had single copies of *pfmdr*1 [25]. We also performed direct sequencing of partial fragments of the *pfmdr*1 (for codons 86–184 and 1034–1246) on these samples to analyze any additional mutations. The *pfmdr*1 fragments were amplified in 2 separate PCR reactions. The first fragment (~799 base pairs [bp]) covered codons 86–184 was amplified using AL6875F (5′-CCGTTTAAATGTTTACCTGCAC-3′) and AL6876R (5′-TGGGGTATTGATTCGTTGCAC-3′) primers with the following PCR cycling parameters: initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The second fragment (~909 bp) that covered codons 1034–1246 was amplified using AL6794F (5′-TATGCATACTGTTATTAATTATGG-3′) and AL6795R (5′-TTCGATAAATTCATCTATAGCAG-3′) primers. The PCR cycling parameters for these primers were also the same as for the first fragment, except that an annealing temperature of 56°C was used. The PCR product was sequenced on an ABI 3130xl Genetic Analyzer using BigDye Terminator, version 3.1 (Applied Biosystems). The internal primers AL6877F (5′-GTATGTGCTGTATTATCAGGAG-3′) and AL6878R (5′-

AGCCTCTTCTATAATGGACATG-3′) were used to sequence the first fragment, whereas the second fragment was sequenced using AL6794F and AL6795R primers.

### Microsatellite allele scoring and statistical analysis

All 158 isolates were first assayed for 8 neutral microsatellite loci on chromosomes 2 and 3, unlinked to any gene under selection [37]. Of 158 samples, 65 had multiple alleles at ≥1 neutral loci and thus were excluded from the study. The remaining 93 isolates were further analyzed for 13 microsatellite loci (−99, −54, −29.5, −9.3, −4.2, and −3.3 kb upstream and 0, 0.16, 0.45, 3.6, 9.1, 23.3, 89 kb downstream) flanking the *pfmdr*1 gene on chromosome 5. Primer sequences and PCR cycling parameters for all 8 neutral loci and 13 *pfmdr*1 loci have been described elsewhere [31, 36, 37]. PCR products were separated on ABI 3130xl Genetic Analyzer and analyzed using Gene-Mapper software, version 3.7 (Applied Biosystems).

The genetic variation at each locus was measured as expected heterozygosity ($H_e$) and number of alleles per locus ($A$). $H_e$ was calculated using the formula $(H_e)=[n/(n-1)][1-\sum p_i^2]$, where $n$ is the number of samples genotyped for that locus and $p_i$ is the frequency of the $i$th allele. The Microsatellite ToolKit was used to compute $H_e$, $p_i$ as well as $A$ [38]. The sampling variance for $H_e$ was calculated as $2(n-1)/n^3\{2(n-2)[\sum(p_i^3-(\sum p_i^2)^2]\}$. The closest 8 loci (± 9 kb) around *pfmdr*1 (−9.3, −4.2, −3.3, 0 [within gene], 0.16, 0.45, 3.6 and 9.1 kb) were used for grouping isolates or parasites into haplotypes. The haplotypes were considered to be unique if they differed at even 1 of the 8 loci analyzed.

Significant associations between pairs of loci were determined on the basis of an exact test of linkage disequilibrium performed in Arlequin, version 3.01 [39]. To correct for multiple testing, Bonferroni correction was used. The genetic differentiations between groups (single copy vs multiple copies and wild-type [184Y] vs mutant [184F] alleles) were determined through Wright's fixation index ($F_{ST}$) using Arlequin, version 3.01 [39].

We used the same 8 loci (± 9 kb) to make a median-joining haplotypes network (Network, version 4.5.1.0.) to predict the genetic relationship among the haplotypes. Median joining networks are used for reconstructing the phylogeny of regions with reticulate evolution [40].

## RESULTS

### *pfmdr*1 Mutant alleles and copy number in Cambodia

We sequenced the *pfmdr*1 region covering codons 86–184 and 1034–1246 in Cambodian *P. falciparum* isolates. We grouped the isolates from all 4 sites on the basis of whether they harbored a single copy or multiple (≥2) copies of *pfmdr*1. Overall, the *pfmdr*1 sequencing results for 93 isolates (from patients with single-clone infections) revealed that only a minority (2.15%) contained mutations at codon 86 (86Y), whereas ~54% of the isolates harbored a mutation at codon 184 (184F). None of the isolates contained mutation at codon 1246. Mutations at codons 1034 and 1042 were only observed in conjunction with mutation at codon 184 (Table 1). Two major differences in the distribution of *pfmdr*1 alleles and copy number were observed between isolates from western and eastern Cambodia. First, we observed an unbiased distribution of 184F mutation in isolates from western Cambodia (Pailin and Kampong Seila), where both isolates with a single copy and isolates with multiple copies of *pfmdr*1 carried the 184F mutation. This was in striking contrast to the eastern Cambodian (Memut and Rattanakiri) population, where the 184F mutation was completely absent in isolates with multiple copies of *pfmdr*1. Second, the 86Y mutation and the recently described mutations 130K and 1109I [41] were observed exclusively in the

eastern population, albeit at low frequencies (3.5%, 3.5%, and 7.1%, respectively). However, both populations contained the double mutant 184F plus 1042D, although this was found exclusively in isolates that contained a single copy of *pfmdr*1.

### Genetic variation at microsatellite loci flanking *pfmdr*1 and selective sweep of 184F allele

We attempted to study the genetic hitchhiking of flanking *pfmdr*1 loci resulting from the spread of multiple copies of *pfmdr*1 and/or mutant 184F allele among these isolates. In this regard, we compared the level of heterozygosity ($H_e$) and number of alleles per locus (*A*) on the basis of single versus multiple copies of *pfmdr*1 and wild-type (184Y) versus mutant (184F) groups (Figures 2 and 3 and Table 2). Overall, fewer alleles and less genetic diversity were found in the loci surrounding *pfmdr*1 than were found in the neutral loci on chromosomes 2 and 3 (Table 2). However, there were no obvious differences in the heterozygosities for any of the 13 loci between samples with single copies and those with multiple copies of *pfmdr*1. The single-copy and multiple-copy groups had almost identical mean $H_e$ values (± standard deviation [SD]) of 0.75 ± 0.03 and 0.76 ± 0.04, respectively (Figure 2*A* and 2*B*). However, interpopulation analysis revealed markedly reduced $H_e$ at almost all of the loci in the western Cambodian population, compared with the eastern Cambodian population (Figure 2*C* and 2*D*).

Interestingly, evidence of genetic hitchhiking was seen that was due to selective sweep of 184F *pfmdr*1 allele in the population. There was an overall reduction of 28% in mean $H_e$ (± SD) observed for the isolates carrying the 184F allele (0.56 ± 0.05), compared with those harboring the wild-type 184Y allele (0.84 ± 0.02) (Figure 3*A* and 3*B* and Table 2). The difference in $H_e$ between wild-type (0.86) and mutant (0.53) alleles was greater (33% reduction) in the upstream region of *pfmdr*1. On the other hand, the difference in $H_e$ between wild-type (0.82) and mutant (0.60) alleles was relatively less (22% reduction) in the downstream region (Figure 3*A*). To investigate the role of 184F-driven selective sweep, we compared the heterozygosities around the 184Y and 184F alleles in isolates from eastern Cambodia. The results revealed a reduction in heterozygosity around 184F, compared with the wild-type allele, in the eastern Cambodian population (Figure 3*C* and 3*D*). The reduction around the 184F allele was greater in the isolates from western Cambodia (Figure 3*C* and 3*D*). We could not compare heterozygosities around the 184Y allele in western Cambodia, because there were only 4 isolates that harbored this allele.

### Linkage disequilibrium between loci flanking *pfmdr*1

The pattern of linkage disequilibrium was also deduced to explore the extent of 184F-induced selective sweeps operating in the population. The pair-wise linkage disequilibrium distribution between the closest (± 9 kb) *pfmdr*1 microsatellite loci is shown in Figure 4. Significant linkage disequilibrium was observed between loci close to *pfmdr*1 in the group with the mutant 184F allele, but no association was observed between loci in the group with the 184Y allele (Figure 4*A*). On the other hand, we found significant linkage disequilibrium between these loci in both groups with single or multiple copies of the *pfmdr*1 gene (Figure 4*B*).

### Genetic differentiation and relationships among *pfmdr*1 microsatellite haplotypes

The genetic differentiation between the 184Y (wild-type) and 184F (mutant) groups using the closest 8 microsatellite loci (± 9 kb) around the *pfmdr*1 gene was measured employing Wright's fixation index, $F_{ST}$. Significant $F_{ST}$ ($F_{ST}$, 0.03; $P < .001$) was observed between the wild-type (184Y) and mutant (184F) groups, as would be expected because of the decrease in genetic diversity within this subpopulation. Unsurprisingly, a low but insignificant level of genetic differentiation was observed between subpopulations with a single copy and those with multiple copies of *pfmdr*1 ($F_{ST}$, 0.003; $P > .05$).

The same 8 loci (± 9 kb) were also used for classifying the 93 isolates into 66 haplotypes (H1–H66) (Figure 5). When we looked at the distribution of haplotypes irrespective of *pfmdr*1 copy number by geography, we found fewer haplotypes in western Cambodia (19 haplotypes among 37 isolates) than in eastern Cambodia (51 haplotypes among 56 isolates), which suggests less genetic diversity in western Cambodia. Only 4 haplotypes (H1, H8, H9, and H12) were shared between eastern and western isolates, including isolates that bore a single copy and those that bore multiple copies of *pfmdr*1 (Figure 5). Overall, the subpopulation with multiple copies of *pfmdr*1 displayed a relatively smaller number of haplotypes (27) than did the subpopulation with single copies (45) (Figure 5). Only 6 haplotypes (H1, H2, H3, H5, H8, and H9) were shared by subpopulations with both single and multiple *pfmdr*1 copies, and all of them had the *pfmdr*1 184F mutation (Figure 5). We also found that wild-type and mutant *pfmdr*1 alleles did not share any microsatellite haplotypes. The haplotypes with the mutant 184F allele (on genetic backgrounds of both single and multiple *pfmdr*1 copies) were found to form 3 main independent clusters (Figure 5).

## DISCUSSION

Resistance to AS+MQ has developed on the Thailand–Cambodia border. This study provides comprehensive data on the distribution of *pfmdr*1 alleles and the pattern of selective sweeps in 4 sites in Cambodia with different levels of transmission and different drug resistance profiles. As has been shown elsewhere [25], our results from *pfmdr*1 sequencing also confirmed the high prevalence (~86%) of the 184F allele in western Cambodia (Table 1), where a significant level of MQ resistance has been reported. On the other hand, the prevalence of 184F allele in eastern Cambodia was low (~32%), which also correlates with the reduced level of MQ resistance in this region (Table 1). In western Cambodia, the *pfmdr*1 amplification was found to occur on both 184Y and 184F backgrounds, in contrast with eastern Cambodia, where all of the amplification was found on the 184Y background (Table 1). A possible explanation for this is that the *pfmdr*1 amplification is independent of the acquisition of the 184F mutation by the parasites. Although an increased copy number in the *pfmdr*1 gene has been associated with MQ resistance, the significance of the 184F mutation remains less well understood [9, 42]. A study on the Southeast Asian *P. falciparum* population showed that isolates that fall under category I (all wild-type *pfmdr*1 codons: 86N, 184Y, 1034S, and 1042N) and category III (mutation at only 184 codon: 86N, 184F, 1034S, and 1042N) exhibited increased resistance to MQ and AS [17]. Also, the isolates with amplified *pfmdr*1 fell into category I or III and were significantly more resistant to these drugs. A recent study from Cambodia demonstrated that isolates with single 184F mutation had a significantly elevated 50% inhibitory concentration for MQ [41]. Similar results were obtained in studies from Tanzania and Uganda, where treatment with another widely used ACT (AL) selected for reinfecting parasites with 86N, 184F, and 1246D *pfmdr*1 alleles [24, 43, 44]. The wild-type 86N allele has been found to be associated with increased 50% inhibitory concentrations to MQ and AS [9, 18, 42, 45].

We characterized microsatellites flanking (± 99 kb) the *pfmdr*1 gene to estimate the strength of genetic hitchhiking resulting from the spread of the 184F mutant allele and/or amplification events in *pfmdr*1. As evident from Figure 3, 184F mutant allele appears to be under strong selection with reduced variation spanning from 99 kb upstream to 88 kb downstream of the gene. Generally, downstream loci exhibited a lower reduction in $H_e$, except at the 0.45-kb locus, where we found a sharp reduction of 57%, compared with wild-type *pfmdr*1 (Table 2 and Figure 3). We could not find in the PlasmoDB any other potential gene under selection around this locus that could be responsible for such a sharp reduction in $H_e$. Present data seems to suggest that the 184F allele has undergone a selective sweep, but it

is not clear whether MQ or other drug pressure is the cause for this selection. Although a previous study by Nair et al [36] reported that a significant proportion (30%; $n = 326$) of the Thailand isolates harbor the 184F allele, the authors did not mention any genetic hitchhiking resulting from the spread of this allele.

Amplification of the *pfmdr*1 gene was found to occur on multiple haplotype backgrounds based on the typing of 13 microsatellite loci (Figure 5). Our observation indicating multiple origins for the development of strains with multiple copies of *pfmdr*1 in Cambodia is in agreement with previous observations in Thailand and other studies [35, 36]. Nair et al [36] have described a "soft selective sweep" in the isolates with multiple copies of *pfmdr*1 from the border of Thailand and Myanmar. They have shown a "limited" reduction (~42%; range, 21%–63%) in heterozygosities in the 16 loci surrounding (± 18 kb) *pfmdr*1 in isolates bearing multiple copies, compared with those carrying a single copy. Our results are also consistent with the soft selective sweep model, because it is evident that putatively advantageous alleles with multiple copies of *pfmdr*1 have entered the population recurrently (Figure 5). Thus, we may not have detected a decrease in heterozygosity simply because the strength of that pattern is affected by the number and neutral divergence among adaptive equivalent alleles [46]. The amplification of *pfmdr*1 has been suggested to be a frequent event, and an increase from a single-copy state to having 2 copies of *pfmdr*1 has been found to occur in every $10^8$ parasites [47]. The rate of occurrence of gene duplication is much higher than that of mutation within codons, which occurs at a much lower frequency ($1{:}10^{14}$) [48]. In the presence of MQ drug pressure, an increase in *pfmdr*1 copy number has been observed, whereas deamplification occurs under CQ pressure in laboratory isolates [12–14, 49].

We attempted to study the genetic relationships among haplotypes using Network to visualize whether the multiple *pfmdr*1 copy–bearing haplotype emerged from the single-copy haplotype (Figure 5). Indeed, the multiple *pfmdr*1 copy group was found to share some haplotypes with the single-copy group. Possible explanations for not finding the progenitor haplotype from which single or multiple *pfmdr*1 copies may have emerged in our sample set could be attributable to a recent selective sweep (ie, not enough time has passed to detect selective changes) or attributable to recurring *pfmdr*1 copy number changes that promote duplication events on multiple genetic backgrounds. The negligible $F_{ST}$ value and similarity between the amount of linkage disequilibrium between single and multiple *pfmdr*1 copy sub-populations suggests that the frequency of duplication events is independent of the genetic background. Other studies similarly suggest multiple and independent origins of *pfmdr*1 gene amplification [15, 35, 36].

In conclusion, this study provides evidence that rapid changes in *pfmdr*1 copy number are evolving on multiple genetic backgrounds. It remains to be seen whether the amplified *pfmdr*1 gene will spread in the population as rapidly as CQ-and SP-resistant genotypes. The 184F allele appears to be under selective pressure, but it remains to be established to what extent this mutation influences resistance to MQ. Moreover, it will be important to monitor whether the dissemination of the 184F could also assist in spreading strains with multiple copies of the *pfmdr*1 gene in the population. Therefore, continuing molecular surveillance using genetic markers may help track the spread of MQ resistant parasites and allow better containment of MQ resistance.

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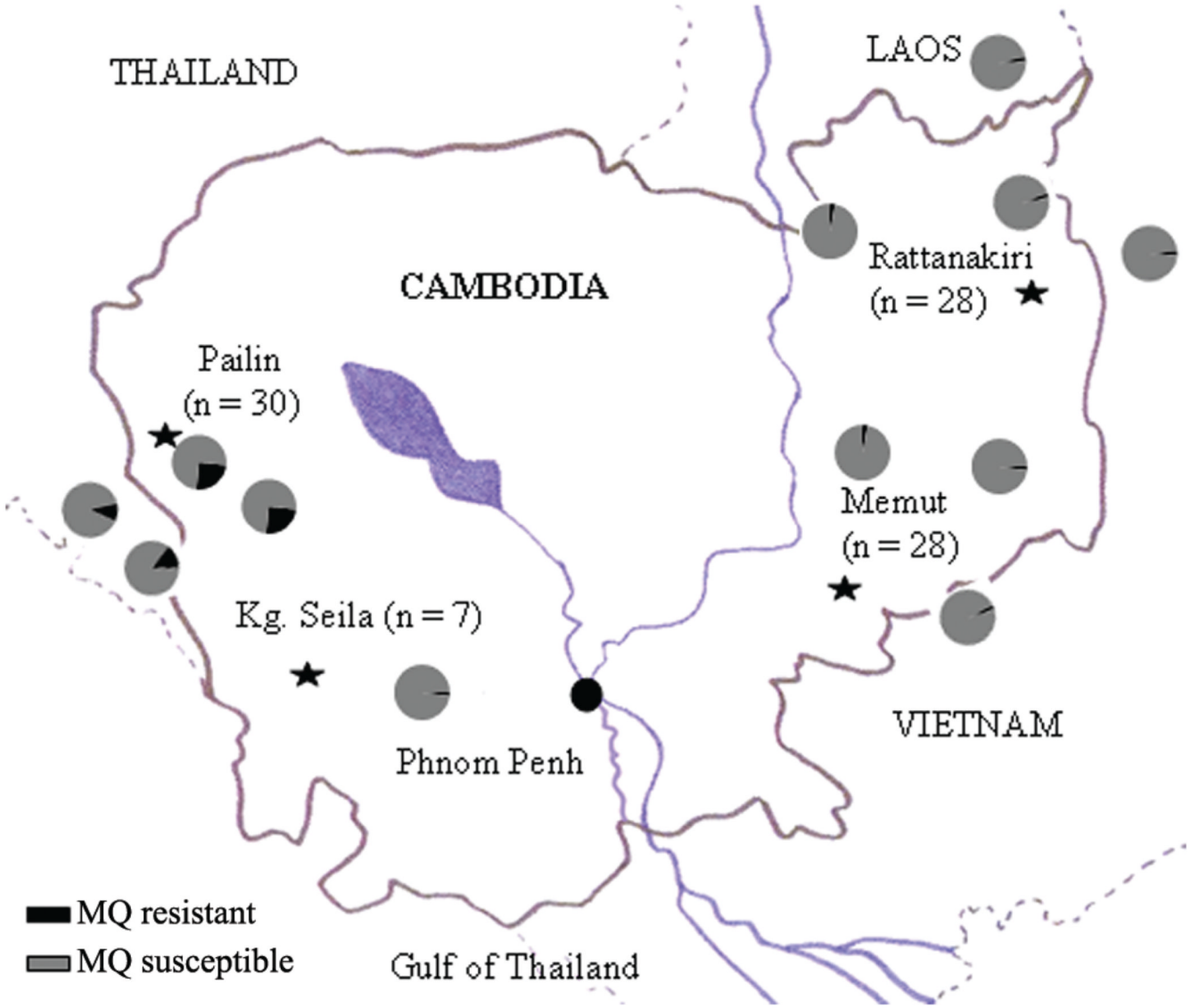


**Figure 1.**
A map of Cambodia showing the location of the 4 sites (Pailin, Kampong Seila, Memut, and Rattanakiri) from which the isolates used in this study were obtained. The proportion of mefloquine (MQ) resistance in Cambodia and bordering areas (Thailand to the west, Vietnam to the east, and Laos to the north) is indicated by pie charts.

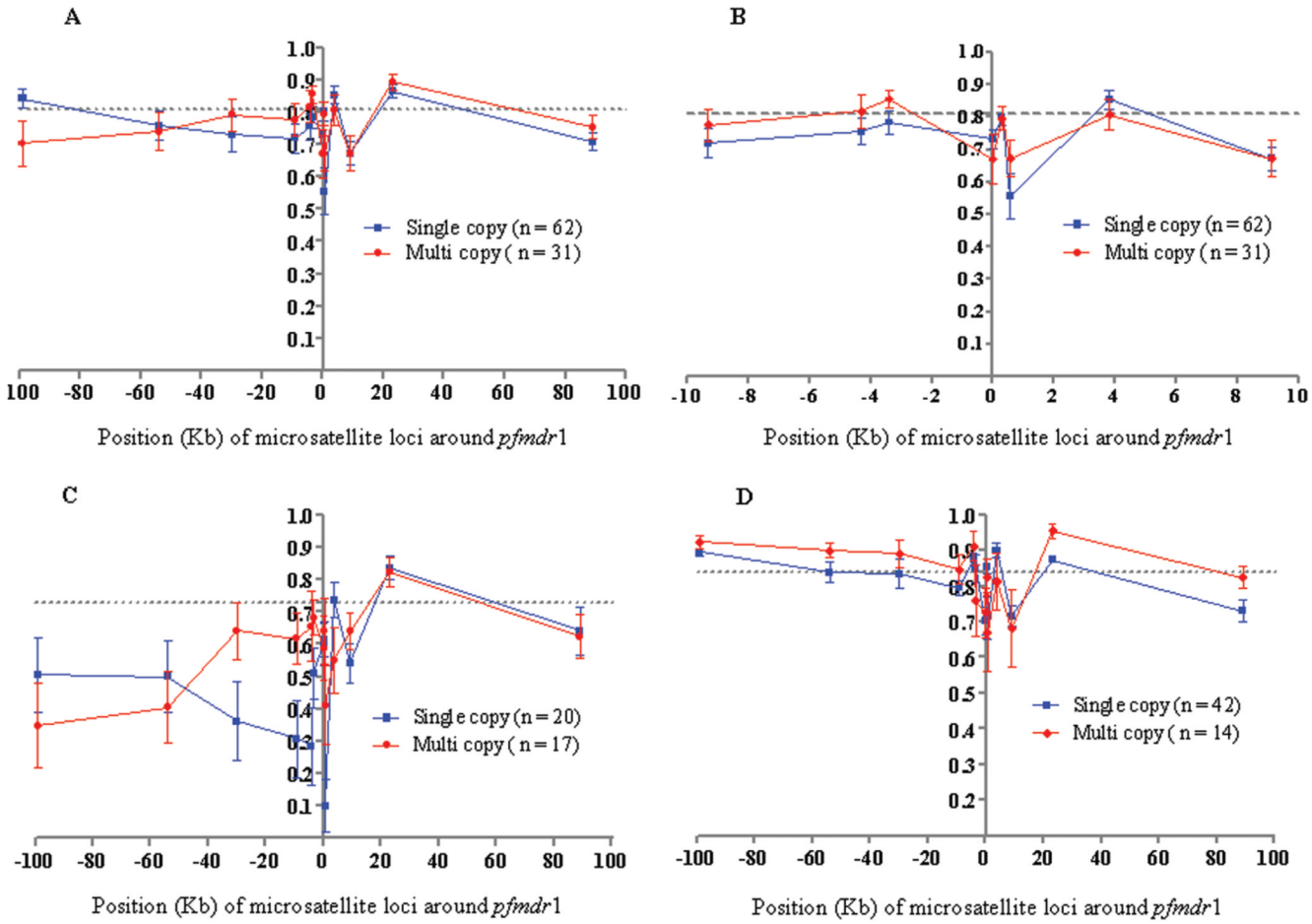


**Figure 2.**

The expected heterozygosity ($H_e$) at 13 microsatellite loci around *pfmdr*1. Comparison of $H_e$ between groups of isolates with a single copy or multiple copies of *pfmdr*1. The mean $H_e$ at neutral loci on chromosomes 2 and 3 is shown by a dotted line. The error bars indicate ± 1 standard deviation. *A*, isolates from both western and eastern Cambodia; *B*, detail showing the closest 8 microsatellite loci flanking (± 9 kb) *pfmdr*1 for isolates from western and eastern Cambodia; *C*, isolates from western Cambodia; *D*, isolates from eastern Cambodia.

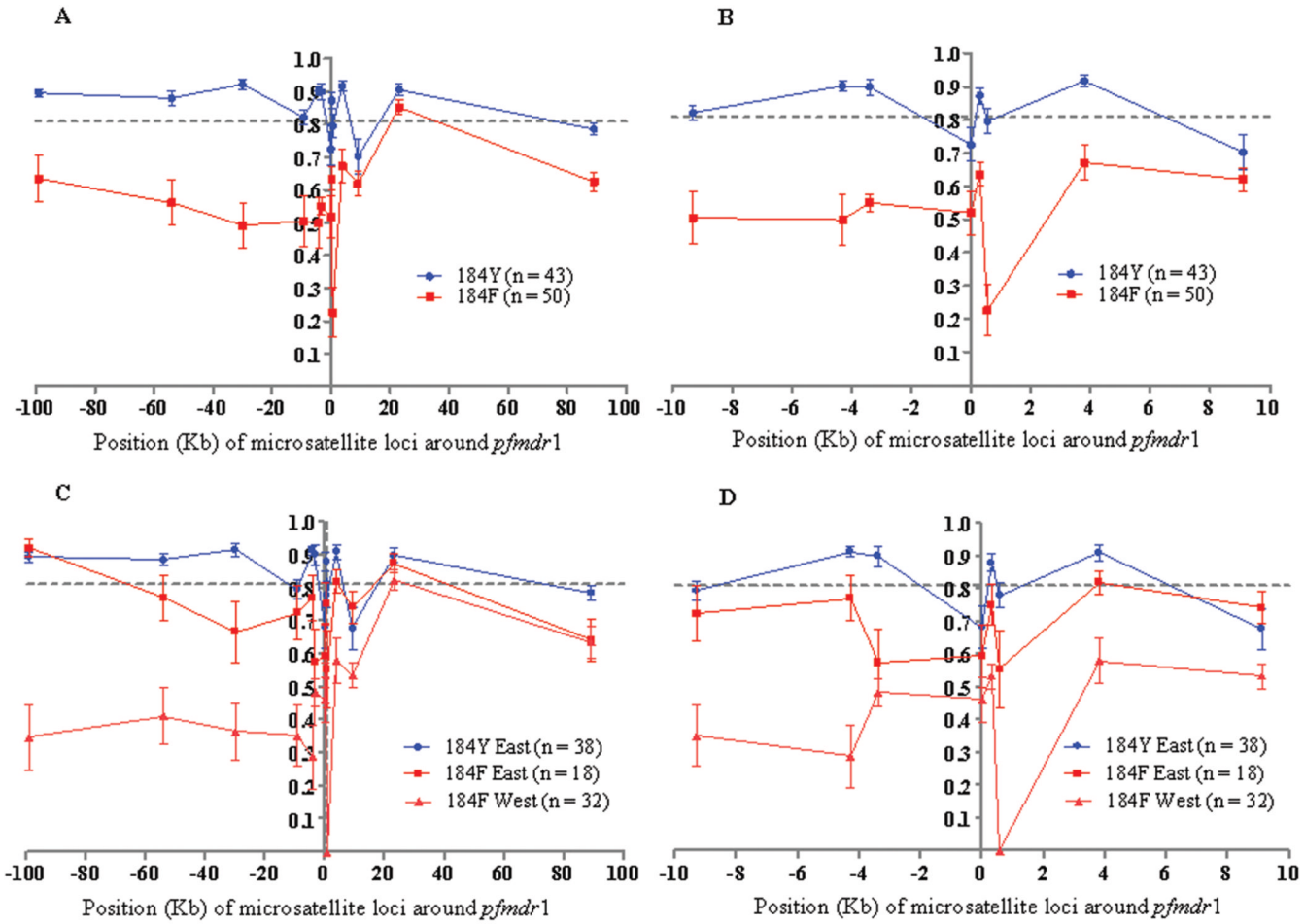


**Figure 3.**

The expected heterozygosity ($H_e$) at 13 loci around wild-type (184Y) and mutant (184F) *pfmdr*1. Comparison of $H_e$ between 184Y and 184F *pfmdr*1 groups of isolates from both western and eastern Cambodia *(A)* and the closest 8 microsatellite loci flanking (± 9 kb) *pfmdr*1 *(B)*, and comparison of 184F group of isolates from western Cambodia with 184F and 184Y groups of isolates from eastern Cambodia *(C)* and the closest 8 microsatellite loci flanking (± 9 kb) *pfmdr*1 *(D)*. The mean $H_e$ at neutral loci on chromosomes 2 and 3 is shown by a dotted line. The error bars indicate ± 1 standard deviation.

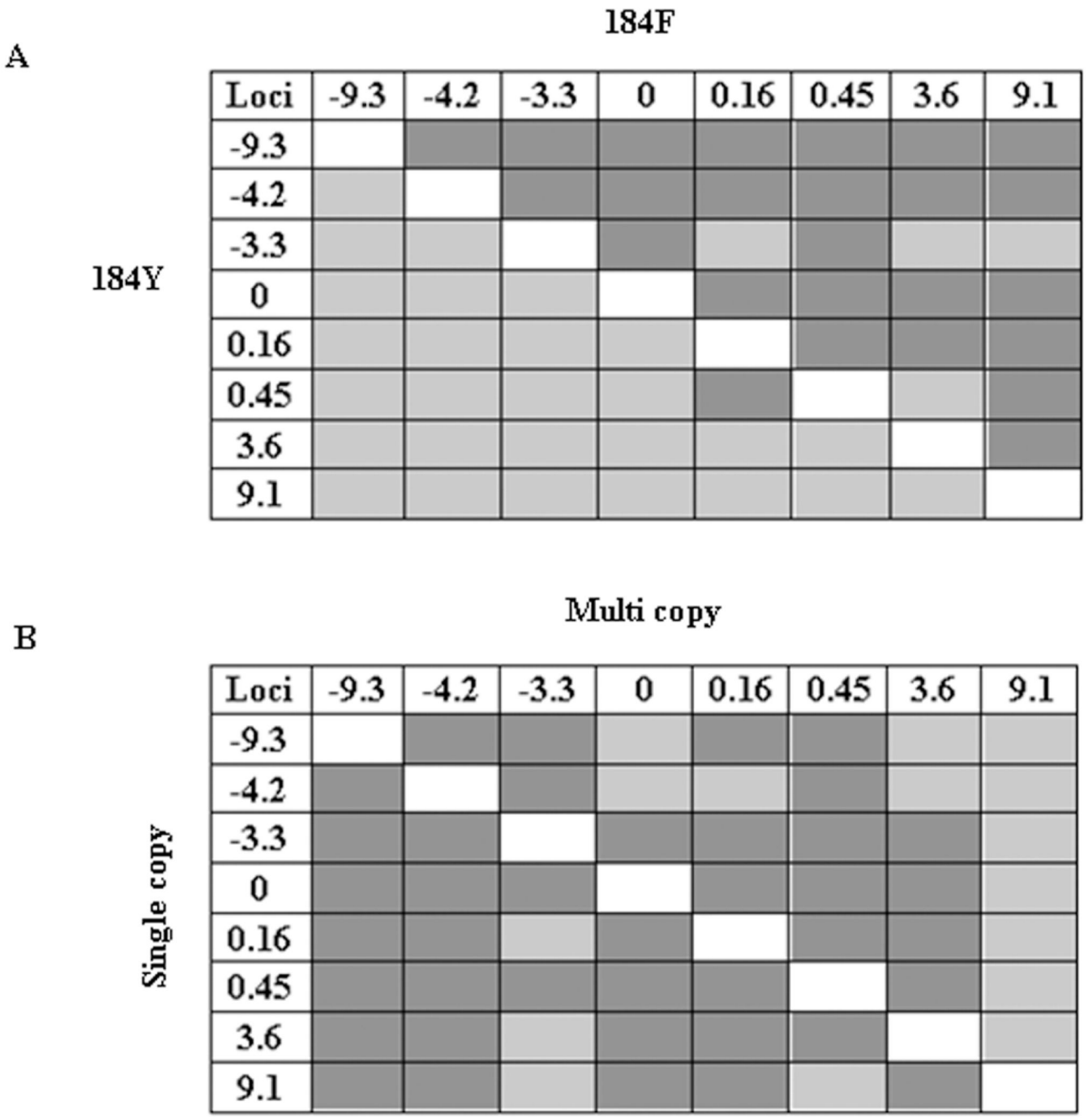


**Figure 4.**

Pair-wise linkage disequilibrium around *pfmdr*1 (± 9 kb). *A*, linkage disequilibrium between loci within the wild-type group (lower half of the diagonal) and mutant group (upper half of the diagonal). *B*, linkage disequilibrium between loci within the group of isolates with a single copy of *pfmdr*1 (lower half of the diagonal) and those with multiple copies of *pfmdr*1 (upper half of the diagonal). Each cell shows a comparison between polymorphic pairs of loci. $P < .006$ was considered to be significant after Bonferroni correction. Light gray cells represent *P* values that are not significant (>.006), and dark gray cells indicate statistically significant *P* values (<.006).

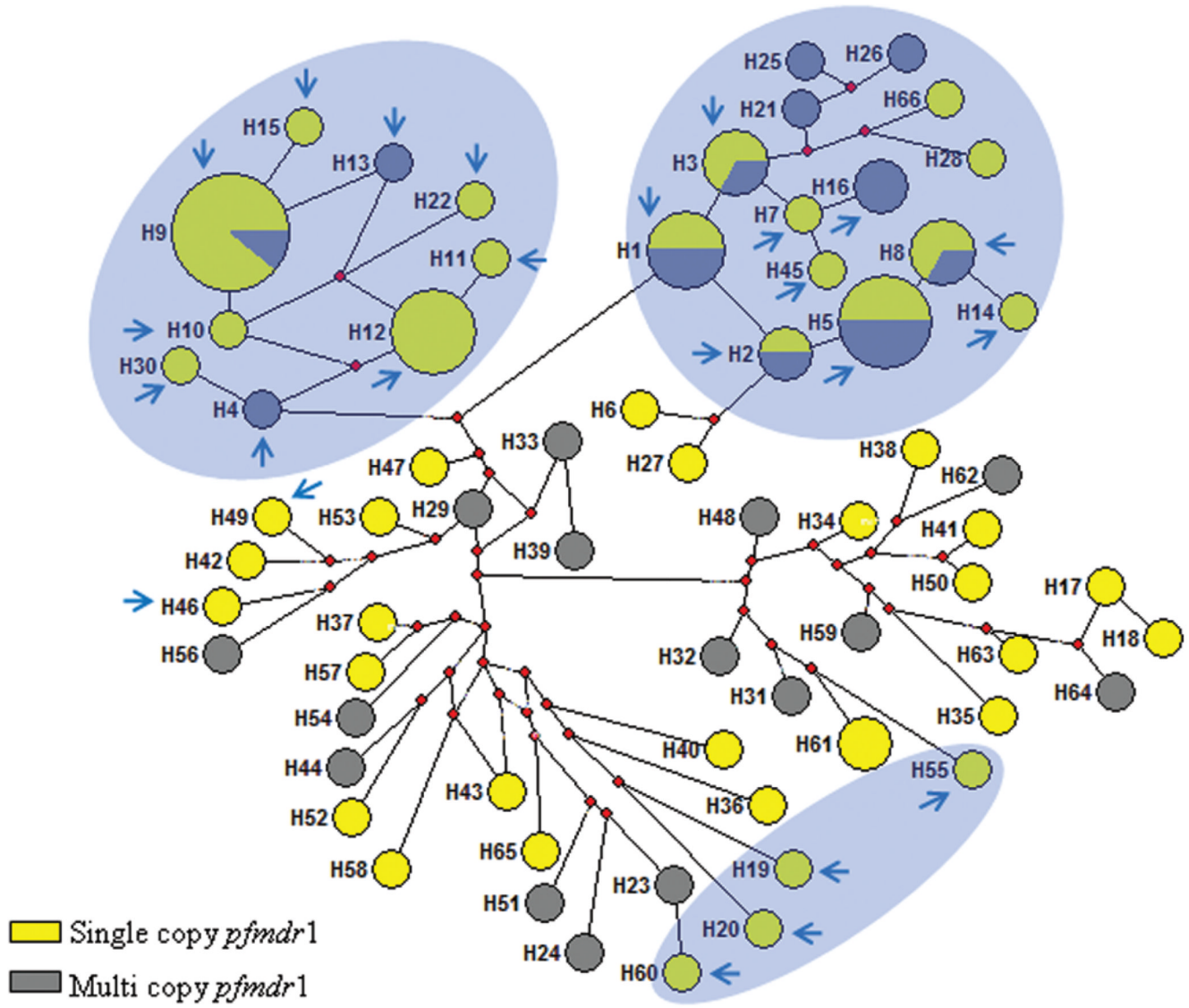


**Figure 5.**

Median-joining network showing genetic relationships among *pfmdr*1 haplotypes in Cambodia. The closest 8 loci (± 9 kb) were used for constructing the network. Each circle in this network represents a unique haplotype, with the size of the circle being proportional to the number of isolates harboring that haplotype. The circles shown in yellow and gray represent isolates carrying single and multiple copies of *pfmdr*1, respectively. The haplotype shared by both isolates with single and isolates with multiple *pfmdr*1 copies (proportion indicated in pie charts) are shown as a circle with both yellow and gray shading. The red dots are the median vectors that imply software-generated "hypothetical haplotypes," joining 2 haplotypes in the diagram from which single and/or multiple *pfmdr*1 copy–containing haplotypes may have originated. The isolates with 184F are marked by blue arrows enclosed in the shaded clusters.

**Table 1**

Distribution of Mutations at Codons 86, 130, 184, 1034, 1042, and 1109 in *pfmdr*1 Gene in Cambodian *Plasmodium falciparum* Isolates

| Study site, copy number | No. of isolates (*n* = 93) | Wild-type *pfmdr*1 | Single mutation | | | | Double mutation | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 86Y | 130K | 184F | 1109I | 184F plus 1034C | 184F plus 1042D | 130K plus 1109I |
| Western Cambodia | | | | | | | | | |
| Pailin | | | | | | | | | |
| SC | 16 | 1 | … | … | 12 | … | 1 | 2 | … |
| MC | 14 | 4 | … | … | 10 | … | … | … | … |
| Kampong Seila | | | | | | | | | |
| SC | 4 | … | … | … | 4 | … | … | … | … |
| MC | 3 | … | … | … | 3 | … | … | … | … |
| Eastern Cambodia | | | | | | | | | |
| Rattanakiri | | | | | | | | | |
| SC | 16 | 9 | … | … | 7 | … | … | … | … |
| MC | 12 | 8 | 1 | … | … | 3 | … | … | … |
| Memut | | | | | | | | | |
| SC | 26 | 12 | 1 | 1 | 8 | … | … | 3 | 1 |
| MC | 2 | 2 | … | … | … | … | … | … | … |
| Overall | | | | | | | | | |
| SC | 62 | 22 | 1 | 1 | 31 | … | 1 | 5 | 1 |
| MC | 31 | 14 | 1 | … | 13 | 3 | … | … | … |

**NOTE.** No singleton mutations were found at *pfmdr*1 codons 1034 and 1042. MC, isolates bearing multiple (≥2) copies of *pfmdr*1; SC, isolates bearing a single copy of *pfmdr*1.

**Table 2**

Genetic Characteristics of the 13 Microsatellite Loci around *pfmdr*1 on Chromosome 5 and 8 Neutral Microsatellite Loci (4 Each on Chromosomes 2 and 3) Based on *pfmdr*1 Copy Number and 184F Mutation

| | Based on copy number | | | | Based on Y184F | | | |
|---|---|---|---|---|---|---|---|---|
| | Single copy ($n$ = 62) | | Multiple copies ($n$ = 31) | | 184Y ($n$ = 43) | | 184F ($n$ = 50) | |
| **Loci** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** |
| *pfmdr*1 | | | | | | | | |
| −99 kb | 13 | 0.84 ± 0.03 | 8 | 0.70 ± 0.07 | 11 | 0.89 ± 0.01 | 10 | 0.63 ± 0.07 |
| −54 kb | 12 | 0.75 ± 0.04 | 7 | 0.74 ± 0.06 | 12 | 0.88 ± 0.02 | 7 | 0.56 ± 0.07 |
| −29.5 kb | 14 | 0.73 ± 0.05 | 9 | 0.79 ± 0.05 | 14 | 0.92 ± 0.01 | 7 | 0.49 ± 0.07 |
| −9.3 kb | 7 | 0.72 ± 0.04 | 7 | 0.77 ± 0.05 | 8 | 0.82 ± 0.02 | 6 | 0.50 ± 0.07 |
| −4.2 kb | 11 | 0.76 ± 0.04 | 11 | 0.81 ± 0.05 | 13 | 0.90 ± 0.01 | 7 | 0.49 ± 0.07 |
| −3.3 kb | 12 | 0.78 ± 0.03 | 10 | 0.85 ± 0.02 | 14 | 0.90 ± 0.02 | 4 | 0.55 ± 0.02 |
| 0 kb | 8 | 0.73 ± 0.02 | 8 | 0.67 ± 0.07 | 10 | 0.72 ± 0.05 | 5 | 0.51 ± 0.06 |
| 0.16 kb | 10 | 0.81 ± 0.02 | 7 | 0.79 ± 0.03 | 12 | 0.87 ± 0.02 | 5 | 0.63 ± 0.03 |
| 0.45 kb | 10 | 0.56 ± 0.07 | 6 | 0.67 ± 0.05 | 9 | 0.79 ± 0.03 | 5 | 0.22 ± 0.07 |
| 3.6 kb | 14 | 0.85 ± 0.02 | 9 | 0.80 ± 0.04 | 13 | 0.91 ± 0.01 | 7 | 0.67 ± 0.05 |
| 9.1 kb | 5 | 0.67 ± 0.03 | 6 | 0.67 ± 0.05 | 7 | 0.70 ± 0.05 | 5 | 0.61 ± 0.03 |
| 23.3 kb | 11 | 0.86 ± 0.02 | 11 | 0.89 ± 0.02 | 12 | 0.90 ± 0.01 | 10 | 0.85 ± 0.02 |
| 89 kb | 5 | 0.71 ± 0.02 | 5 | 0.75 ± 0.03 | 6 | 0.78 ± 0.01 | 4 | 0.62 ± 0.03 |
| Mean value | 10.15 | 0.75 ± 0.03 | 8 | 0.76 ± 0.04 | 10.84 | 0.84 ± 0.02 | 6.30 | 0.56 ± 0.05 |
| Chr 2[a] | | | | | | | | |
| C2M27 | 13 | 0.91 ± 0.02 | 12 | 0.91 ± 0.02 | 14 | 0.92 ± 0.01 | 12 | 0.85 ± 0.03 |
| C2M29 | 10 | 0.68 ± 0.06 | 9 | 0.77 ± 0.07 | 10 | 0.74 ± 0.06 | 11 | 0.71 ± 0.06 |
| C2M34 | 17 | 0.91 ± 0.02 | 17 | 0.95 ± 0.01 | 15 | 0.92 ± 0.01 | 17 | 0.91 ± 0.02 |
| C2M33 | 15 | 0.89 ± 0.02 | 11 | 0.89 ± 0.02 | 14 | 0.92 ± 0.01 | 14 | 0.88 ± 0.02 |
| Mean value | 13.75 | 0.85 ± 0.05 | 12.25 | 0.88 ± 0.03 | 13.25 | 0.87 ± 0.04 | 13.50 | 0.84 ± 0.04 |
| Chr 3[a] | | | | | | | | |
| C3M40 | 16 | 0.89 ± 0.02 | 14 | 0.93 ± 0.02 | 15 | 0.91 ± 0.02 | 14 | 0.87 ± 0.02 |

| | Based on copy number | | | | Based on Y184F | | | |
|---|---|---|---|---|---|---|---|---|
| | Single copy ($n$ = 62) | | Multiple copies ($n$ = 31) | | 184Y ($n$ = 43) | | 184F ($n$ = 50) | |
| **Loci** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** | ***A*** | ***$H_e$* ± SD** |
| C3M88 | 16 | 0.91 ± 0.01 | 11 | 0.92 ± 0.01 | 12 | 0.91 ± 0.01 | 13 | 0.91 ± 0.01 |
| C3M69 | 11 | 0.85 ± 0.02 | 8 | 0.82 ± 0.03 | 11 | 0.88 ± 0.02 | 10 | 0.82 ± 0.03 |
| C3M39 | 6 | 0.44 ± 0.07 | 3 | 0.31 ± 0.11 | 6 | 0.59 ± 0.07 | 3 | 0.19 ± 0.07 |
| Mean value | 12.25 | 0.77 ± 0.11 | 12.25 | 0.75 ± 0.14 | 11 | 0.82 ± 0.07 | 10 | 0.71 ± 0.16 |

**NOTE.** The isolates were considered as multiple copy isolates if they contained ≥2 copies of *pfmdr*1 gene as determined by real-time quantitative polymerase chain reaction. *A*, number of alleles per locus; $H_e$, expected heterozygosity; SD, standard deviation.

[a]The GenBank accession numbers for these neutral microsatellite loci on chromosomes 2 and 3 are G37889, G37891, G37995, G37886, G37979, G42719, G37915, and G37913.