Distinctive Origin and Spread Route of Pyrimethamine-Resistant Plasmodium falciparum in Southern China

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Southeast Asia (the Thailand-Cambodia border) has been considered the primal epicenter for most antimalarial drug resistance; however, numerous molecular epidemiological studies have successively reported multiple independent origins of sulfadoxine-pyrimethamine (SP) resistance-associated Plasmodium falciparum dhfr (pfdhfr) and pfdhps alleles in other areas. To better understand the origin and evolutionary pathway of the SP resistance in Southeast Asia, a total of 374 P. falciparum field isolates from the Yunnan-Burma border and Hainan Island in southern China have been collected for comprehensive investigations on the mutation patterns of the pfdhfr/pfdhps genes as well as their microsatellite haplotypes. By comparative analysis of single-nucleotide polymorphism (SNP) genotyping and flanking microsatellite haplotypes, we reveal a unique origin of pyrimethamine-resistant mutations in Pfdhfr gene in Hainan Island and an oriented spread route of the pyrimethamine resistance from the Thailand-Cambodia border into the Hainan area, which reflects the geographical traits and SP administration histories in the two geographically independent areas. Moreover, genetic linkages between the high-level SP resistance-conferring pfdhfr/pfdhps alleles have been established in the isolates from the Yunnan-Burma border, raising the concern of a genetic basis in adopting combination chemotherapies against falciparum malaria.

Since the prevalence of chloroquine resistance in malaria chemotherapy worldwide, the wide use of combination therapies such as sulfadoxine-pyrimethamine (SP), an affordable alternative to chloroquine, had led to increasing multidrug resistance in malarial parasites, which has hampered therapeutic efficacy in many areas (1, 2). Recently, resistance to artemisinin (ART) in Plasmodium falciparum has been reported in Southeast Asia (SEA), causing the current situation of first-line treatment in malaria control to deteriorate (3–5). As one of the partner drugs of artemisinin-based combination therapy (ACT), SP combination is still the only drug treatment recommended by WHO for intermittent preventive treatment (IPT) in those vulnerable populations because of its safety in pregnant women and infants and its long action. Therefore, the fundamental understanding of how SP resistance emerged and spread globally will definitely contribute to the development of novel intervention strategies against widespread multidrug resistance and prevention of potential drug resistances in malarial parasites.

Resistance to SP in P. falciparum parasites was established mainly by site mutations in the genes encoding dihydrofolate reductase (dhfr) at codons 50, 51, 59, 108, and 613 and dihydropteroate synthase (dhps) at codons 436, 437, 540, 581, and 613 (6–10). These key mutations are suggested to appear in a stepwise manner and to be able to act synergistically to enhance the level of SP resistance both in vivo and in vitro (10, 11). Molecular epidemiological studies such as site-mutation genotyping and haplotype analysis of microsatellites surrounding the target gene locus have provided crucial information in tracing the origin, evolutionary history, and spread route of drug resistance in various areas where malaria is endemic. In this way, a common ancestry (CNRNLS) of the triple mutant P. falciparum dhfr (pfdhfr) allele was discovered at the Thailand-Cambodia border, the epicenter of pyrimethamine resistance. This mutant allele evolved and was introduced into other regions in Southeast Asia and Africa through a similar spread pathway of chloroquine resistance (12–15). Nevertheless, multiple indigenous origins of some single, double, or triple pfdhfr alleles were also reported in Southeast Asia, Africa, South America, and Papua New Guinea (16–19). To date, the evolution and spread of resistant lineages are traced by the pfdhfr rather than the pfdhps gene, but there are still several surveys that showed that unlike the pfdhfr gene, various sulfadoxine-resistant pfdhps alleles originated independently in each of those areas, including Thailand, Cambodia, Kenya, Cameroon, and Venezuela (17, 18, 20–22).

In Asia, resistance to SP was first found on the Thailand-Cambodia border in 1960s and quickly spread to neighboring countries in Southeast Asia (SEA) (23, 24). In the 1970s and 1980s, there existed a wide range of SP resistance in this area, where Fansidar was widely used. Consequently, the efficacy of SP decreased sharply and the cure rates were very low in SEA regions, especially on the Thailand-Burma (42%) and Thailand-Cambodia (32%) borders (2). As one of the regions in Southeast Asia where malaria is endemic, the southern areas of China, including the Yunnan-Burma border and Hainan Province (a geographically isolated island located in the South China Sea), have also experienced the use of SP combination therapy and thereby have encountered the occurrence and spread of SP resistance. How-
ever, the SP therapy programs in the two regions were not identi-
cal, as one was introduced as an antimalarial prophylactic remedy
in Yunnan from the middle of 1960s until the early 1990s, whereas
in Hainan Island, pyrimethamine was first introduced in 1959 and
then combined with sulfadoxine in several villages from 1967 to
1972. Due to the distinct histories of drug administration, geo-
graphical environments, and population migration in these two
areas, the field isolates of *P. falciparum* collected from Yunnan and
Hainan are of great interest for characterization of the mutation
patterns of the *pfdhfr/pfdhps* genes and related flanking microsat-
ellite loci and thereby tracking the origin and evolution of SP
resistance-associated alleles. To date, no such comprehensive inves-
tigation of SP drug resistance in the two areas has been performed,
though a few small-scale genotyping studies on the *pfdhfr* or
*pfdhps* genes have been described (25).

In the present study, we have addressed this issue by profiling
the SP resistance-associated *pfdhfr/pfdhps* alleles, as well as the
microsatellite haplotypes flanking the *pfdhfr* gene, in *P. falciparum*
isolates from Yunnan and Hainan Provinces and further evaluat-
ing the relationship between the two populations. Our results
present evidence of the different selection on *pfdhfr* and *pfdhps*
alleles in the two geographically independent areas and reveal an
independent origin of the *pfdhfr* ANCNI and ANRNI alleles in
Hainan Island. Moreover, we show significant genetic linkages
between the high-level SP-resistant *pfdhfr/pfdhps* alleles in the
population of the Yunnan-Burma border, suggesting that multi-
ple mutations in both *pfdhfr* and *pfdhps* play an critical role in
establishing the genetic linkage across chromosomal boundaries
which reciprocally stabilize the existing multiple mutations in the
population even after use of the drugs has been ceased for longer
than 20 years.

**MATERIALS AND METHODS**

**Study sites and sample collection.** The malaria patients involved in this
study were all local residents in the Yunnan-Burma border area and
Hainan Island with a primary diagnosis of falciparum malaria. After
obtaining written informed consent and ethical approval, we collected 374
*P. falciparum* clinical isolates from symptomatic malaria patients seeking
care at the local Center for Disease Control and Prevention (CDC) in
Yunnan and Hainan from 2003 to 2008. Six sampling sites were included
in this study, three located on the China-Burma border (Yunnan Prov-
ince) and the others on the west coast of the island (Hainan Province).
The Yunnan isolates (*n* = 230) were mainly from venous blood, while the
Hainan isolates (*n* = 119) were mostly from finger prick blood (adsorbed
onto Whatman filter paper). The study was approved by the Ethical Re-
view Board of Second Military Medical University, China.

**DNA isolation and genotyping methods.** The parasite DNA was ex-
tacted from 200 μl venous blood or finger prick blood spot by using the
QIaAmp DNA Blood Minikit according to the manufacturer’s recom-
mandations (Qiagen, Germany). All these samples were genotyped for
*pfdhfr* mutations at codons 16, 51, 59, 108, and 164 and *pfdhps* mutations
at codons 436, 437, 540, 581, 613, 640, and 645 by pyrosequencing. The
sequences of primers used for *pfdhfr* and *pfdhps* genotyping were de-
scribed by Zhou et al. (26). Each DNA sample was tested by nested PCR.
The primary amplification of *pfdhfr* was done with the following param-
eters: 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 65°C for
60 s; and 65°C for 5 min. The second amplification of *pfdhps* was done with
the following parameters: 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for
30 s, and 65°C for 60 s; and 65°C for 5 min. The 650-bp product of *pfdhfr*
and 750-bp product of *pfdhps* were subjected to sequencing on the
ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA,
USA). Samples with multiple peaks at any genotyped single-nucleotide
polymorphism (SNP) codon (mixed genotype) were excluded from SNP
analysis and microsatellite genotyping.

**Microsatellite analysis.** To assess the selective sweeps of *pfdhfr*
resistance determinants, we investigated polymorphic microsatellite repeats
(TA) within a 60-kb flanking region of the *pfdhfr* gene. Seven loci located
on chromosome 4 linked to *pfdhfr* were chosen, i.e., 50 kb, 30 kb, 3.87 kb,
and 0.1 kb upstream and 1.48 kb, 5.87 kb, and 54 kb downstream. Nested
PCR was performed using fluorescence end-labeled primers; the se-
quences of primers and the cycling parameters were previously described
by Nair et al. (27). As a reference of the baseline heterozygosity of all these
isolates, 13 loci on the other chromosomes which were regarded as puta-
tively neutral microsatellite markers were integrated into this study (28).
The amplified products were then detected by electrophoresis on an ABI
377 sequencer and analyzed with GeneScan software v3.7 (Applied Bio-
systems, Foster City, CA, USA). Samples with two or more peaks at the
same locus were treated as exceptions.

**Statistical analysis.** The Pearson correlation between *pfdhfr* and
*pfdhps* resistance alleles which represented the hierarchy among different
genes was calculated with Cluster version 3.0 and Tview (http://rana.
lbl.gov/EisenSoftware.htm). The results were shown in terms of a heat
map indicating the hierarchical information, where *pfdhfr* was shown in
columns and *pfdhps* in rows. Each box represented the frequency of the
isolate carrying a certain *pfdhfr/pfdhps* allele (a *χ*² test had been done in
advance; *P* < 0.01). Linkage disequilibrium (LD) analysis between pairs of
*pfdhfr/pfdhps* point mutations was performed with TASSL software. Sam-
bles with multiple infections detected at any SNP site were excluded from
the LD analysis. Significant LD between the loci should meet the require-
ments of *D*’ > 0.8, *r*’ > 0.2, and *P* < 0.01 simultaneously.

To measure the genetic diversity, we evaluated the expected heterozy-
gosity (*H*<sub>e</sub>) at all *pfdhfr* and neutral microsatellite loci by using Genalex
software, version 6. *H*<sub>e</sub> was calculated by using the formula *H*<sub>e</sub> = [n/(n − 1)] *π*<sub>e</sub> / [1 − Σ *p*<sub>i</sub>]<sup>2</sup> (29), where *n* is the number of
infections sampled and *p*<sub>i</sub> is the frequency of the *i*<sup>th</sup> allele. The sampling variance of *H*<sub>e</sub> was calculated
according to the formula with a slight modification of the standard dip-
loid variance, *(n − 1/n)*<sup>2</sup> *π*<sub>e</sub>[(2 − 3 *Σ *p*<sub>i</sub>)<sup>2</sup> / [2(1 − Σ *p*<sub>i</sub>)]]<sup>2</sup> (29). Different mean
*H*<sub>e</sub> values were compared by using the Mann-Whitney *U* test in SPSS
(version 19.0). A *P* value of <0.05 was considered statistically significant.

To track genetic lineages of *pfdhfr* alleles, we constructed a median-join-
tree based on 7-locus microsatellite haplotypes through NETWORK
version 4.5.1.0. In addition, for determining the number of origins pre-
cisely, the haplotypes of 4 loci at positions 3.87 kb and 0.1 kb upstream
and 1.48 kb and 5.87 kb downstream within 10 kb were selected for this anal-
ysis.

**RESULTS**

**Identification of infection complexity in field isolates.** The iso-
lates of *Plasmodium falciparum* were collected from the Yunnan-
Burma border and Hainan Island, two geographically indepen-
dent regions in southern China (Fig. 1A). Each region included
three sampling sites: Tengchong City (*n* = 92), Dehong City (*n* =
78), and Lazan County (*n* = 60) on the Yunnan-Burma border
and Dongfang County (*n* = 50), Ledong County (*n* = 37),
and Sanya City (*n* = 32) on Hainan Island (see Table S1 in the supple-
mental material). Among them, 25 isolates (6.68%) were excluded from
this study because of multiple infections detected by SNP
 assay (data not shown). The rest of the 349 samples were subse-
quently subjected to pyrosequencing. Of them, 100 were found to
contain multiple alleles at one or more neutral loci through micro-
satellite analysis (13 loci). Therefore, a total of 249 isolates were
finally recruited in the microsatellite haplotype analysis around the
*pfdhfr* gene locus on chromosome 4.

**Regional distribution of *pfdhfr* and *pfdhps* resistance alleles in
the two populations.** By specific amplification and sequencing of the
*pfdhfr/pfdhps* alleles, we found a total of 7 *pfdhfr* and 14
pfdrhps haplotypes in this study, most of which have been described in previous reports. There were two novel pfdrhps mutants (SGDAA and FGEHA) with extremely low frequencies in Tengchong City in Yunnan. In addition, two newly reported triple mutants, ANGAA and SGNGA, were also found in this site (20, 30), indicating a relatively high rate of transmission rate P. falciparum parasites in this area.

Approximately, 92.61% of the Yunnan isolates harbored pfdrhfr triple (AIRNI and ANRNL), quadruple (AIRNL), and quintuple (VIRNL) mutants. There was only one isolate with the wild-type pfdrhfr allele (ANCSI (0.43%), and no single mutants were found in this region. In contrast, the Hainan isolates exhibited predominantly single (ANCNI, 19.33%) and double (ANRNI, 67.23%) mutants, whereas both high-level drug resistance-related mutants (AIRNI/ANRNL/VIRNL) and the wild-type allele (ANCSI) were rare (5.88% and 7.56%, respectively). Similar to the case for the pfdrhfr alleles, high-level resistant pfdrhps mutants, including two predominant triple mutants, AGFAA and SGEHA, and other mutants such as AGEGA, FGEGA, AGEA, AGNAA, and SGNGA, were observed in 90.43% of the Yunnan-derived isolates. The frequencies of pfdrhps double mutants (SGEAA/SGDAA/AGKAA/SGKGA), single mutants (SGKAA), and the wild type (SAKAA) were 6.97%, 0.43%, and 2.17%, respectively. For the P. falciparum isolates collected from Hainan Island, however, the pfdrhps alleles showed a distinct distribution pattern where the wild type (SAKAA) was rarely observed (82.35%) compared to the high-level drug resistance alleles (5.89% for triple mutants and 2.52% for double mutants) (Fig. 1B; see Table S1 in the supplemental material).

With respect to individual sampling sites, further analysis showed similar distribution patterns of pfdrhfr alleles in Lazan, Tengchong, and Dehong in Yunnan, where the quadruple mutant AIRNL and the triple mutant ANRNL were the major alleles in all the three sites. In Hainan Island, however, it is notable that Dongfang City exhibited a varied status of the pfdrhfr mutants, which were composed mainly of ANCNI, ANRNI, and wild-type ANCSI alleles, while only the double mutant ANCNI allele was prevalent in the other sites (Ledong and Sanya) (Fig. 2A). For the pfdrhps gene, no significant difference in the mutation profiles was observed among the three sites in either Yunnan or Hainan (Fig. 2B).

**Genetic linkage between sites in pfdrhfr and pfdrhps.** The similar SP resistance status observed in the sites in Yunnan indicated a potential genetic linkage between the pfdrhfr and pfdrhps gene loci, which appeared and was fixed during the multidrug selections, as was discovered in P. vivax recently (31). In order to address this key point for P. falciparum isolates, we rearranged all these alleles determined in this study into a cross table to determine the linkages between pfdrhfr and pfdrhps alleles (Fig. 3A). Strong linkage was established in two major groups, AGFHA/AGNAA/ANRNI/AIRNL and SGEAA/SGDAA/ANRNL/AIRNL (pfdhfr correlation, >5), which are linked to high-level SP-resistant mutants, and SAANRNI/ANCNI/ANCSI and SGKAA-ANCNI (pfdhps correlation, >5), which represent low-level SP resistance alleles. For the two genes on different chromosomes in stabilizing and fixing the existed multidrug resistance within these parasite populations even after the removal of the SP drugs in these areas for a long time. Thus, we further analyzed the pfdrhfr/pfdrhps alleles from Yunnan (n = 230) and Hainan (n = 119) via the same strategy. The results showed that most of the Yunnan isolates belonged to the aforementioned high-level SP-resistant groups and that the Hainan isolates fell in the SP-sensitive groups (Fig. 3A, Yunnan and Hainan). Several other pfdrhfr-pfdrhps pairs showed weaker linkages without statistical significance.

The significant linkages between pfdrhfr and pfdrhps mutants strongly supported the role of linkage disequilibrium (LD) between sites of the two genes on different chromosomes in stabilizing and fixing the existed multidrug resistance within these parasite populations even after the removal of the SP drugs in these areas for a long time. Thus, we further analyzed the LD of SNP mutations among them. Using TASSEL software, we showed a significant LD between pfdrhfr codons 437 and pfdrhfr codon 51 (D’ = 0.91, r2 = 0.25, P < 0.001) or pfdrhfr codon 164 (D’ = 0.85, r2 = 0.36, P < 0.001) in this population (Fig. 3B). It is notable that, unlike the previous finding by McCollum et al. (22), no significant association was observed between pfdrhfr codon 437 and the key pfdrhfr mutation site 59 or 108 (P > 0.01). In addition, for the alleles within the pfdrhfr or pfdrhps gene, significant LDs were observed between pfdrhfr codons 59 and 108 (D’ = 1, r2 = 0.28, P < 0.001) and pfdrhps codons 437 and 436 (D’ > 0.8, r2 > 0.2, P < 0.001) or 540 (D’ = 1, r2 = 0.64, P < 0.001), suggesting that the
synchronous mutations on adjacent SNP sites might increase the level of SP resistance synergistically.

**Selective sweeps of *pfdhfr* drug resistance alleles.** The distinct regional distribution pattern of *pfdhfr* alleles in Hainan suggests a more complex origin and evolutionary pathway of pyrimethamine resistance on this island. To clarify this issue, we examined the genetic diversity and selective sweeps around *pfdhfr* alleles in 349 field isolates by microsatellite analysis of 7 loci around each of five major *pfdhfr* mutations and the wild-type allele. Due to PCR amplification failure or multiple infections on some microsatellite loci, 249 isolates were finally subjected for this analysis. We first determined the heterozygosity ($H_e$) of all these alleles on each of the 4 loci, i.e., 5.87 kb and 0.1 kb upstream and 1.48 kb and 3.84 kb downstream from the *pfdhfr* gene within a range of 10 kb. The mean $H_e$ of the wild-type allele (ANCSI) was greater ($H_e = 0.605 \pm 0.013$) than those of alleles with single (ANQNI, $H_e = 0.321 \pm 0.068; P = 0.010$), double (ANRNI, $H_e = 0.290 \pm 0.014; P = 0.013$), triple (AIRNN, $H_e = 0.030 \pm 0.017 [P < 0.001]$), and ANRNL, $H_e = 0.121 \pm 0.019 [P < 0.001]$), or quadruple ($H_e = 0.103 \pm 0.006; P < 0.001$) mutations. This observation is compatible with the previously established model of positive directional selection; i.e., a progressive decline in $H_e$ correlates with an increase in the number of favorable mutations of the *pfdhfr* gene (32). For three other loci, 57 kb and 30 kb upstream and 54 kb downstream from the *pfdhfr* gene, however, no significant differences in $H_e$ were observed between the wild-type and other mutant alleles, probably because the diversity of these loci was not influenced by drug selection since they located far from the *pfdhfr* locus (Fig. 4A). In addition, we also measured 10 neutral loci on chromosomes 1, 3, 5, 6, 7, 9, and 10 as references. The mean $H_e$ of these loci ($H_e = 0.751 \pm 0.062$) was significantly greater than that of the 4 loci flanking the mutant *pfdhfr* gene locus ($P < 0.001$) but similar to that of the wild type ($P = 0.103$).

Next, we further compared the overall diversity of the *pfdhfr* locus between the two regions in southern China. Figure 4B shows a significant lower mean $H_e$ in Yunnan (0.383 \pm 0.003) than in Hainan (0.603 \pm 0.014) ($P = 0.016$), suggesting a stronger effect of drug selection in the Yunnan-Burma area. Moreover, a site-based analysis revealed a distinct profile of the genetic diversity of the *pfdhfr* locus in Dongfang isolates ($H_e = 0.568 \pm 0.008$), whereas the other two sites in Hainan showed a pattern similar to that in Yunnan ($H_e_{\text{Ledong}} = 0.359 \pm 0.034; H_e_{\text{Sanya}} = 0.464 \pm 0.008$).
This is interesting since it indicates an independent origin or evolutionary pathway of pfdhfr mutant alleles in the Dongfang region. A further analysis of the evolutionary pathway of the resistance-conferring mutations in the pfdhfr and pfdhps genes did not show an apparent difference between Hainan (Dongfang) and Yunnan (see Fig. S1 in the supplemental material), which points to a unique origin of the pfdhfr drug resistance mutations in the Dongfang region.

**Microsatellite haplotype and genetic relationship of dhfr alleles.** To classify the origin of pfdhfr mutant alleles in Yunnan and Hainan isolates, we performed analysis of microsatellite haplotypes with the 4 loci within 10 kb flanking the pfdhfr locus. In total, there were 31 various microsatellite haplotypes, H1 to H31, in the recruited 249 isolates, and according to the relationship among them, they were able to be classified into two major groups corresponding to H1 and H19 (Fig. 5). The haplotype H1, displaying a microsatellite combination of 193-175-201-110 bp on 4 loci, was described previously as the Southeast Asia (SEA) haplotype (19, 28). The H19 haplotype, 199-155-201-104, however, had not been reported yet. Accordingly, this novel microsatellite haplotype of the pfdhfr locus was designated the HN haplotype here. The derivatives of the two haplotypes were called SEA variant and HN variant haplotypes, respectively, and that containing mixed microsatellite types was designated the SEA-HN hybrid haplotype. Figure 6 lists the categories of all microsatellite haplotypes in the Yunnan and Hainan isolates analyzed in this study. It shows that SEA haplotype derivatives were mainly detected in 96% of the Yunnan isolates and 47.2% of Hainan isolates, corresponding to the triple or quadruple mutant pfdhfr alleles. Meanwhile, approximately 30.6% of the Hainan isolates presented the HN haplotype and its variants, most of which were from Dongfang samples with single or double mutations of the pfdhfr gene.

To further address this point, we also drew a median-joining network diagram tree of these microsatellite haplotypes based on the polymorphism on those loci flanking the pfdhfr locus. Two major independent groups were constructed (Fig. 6) and represent the high-level (YN/SEA type) and low-level (HN type I) drug resistance alleles. It should be noted that a few pfdhfr double mutants with SEA haplotypes from Hainan isolates, mainly from the Ledong site, also migrated into the Yunnan group (HN type II), suggesting a common ancestor of the pfdhfr mutant alleles in Ledong and Yunnan. Taken together, this clearly showed that in Yunnan, the pyrimethamine resistance of *P. falciparum* parasites could be traced to an ancestor originating in the well-character-
ized Southeast Asia isolates. However, at least two origins of \textit{pfdhfr} mutations existed in the geographically isolated Hainan Island under multidrug selections. The Dongfang isolates represented a novel source of the pyrimethamine resistance in \textit{P. falciparum} field isolates.

**DISCUSSION**

Hainan and Yunnan Provinces are the two representative high-risk regions of endemicity of falciparum malaria in southern China. In the past 2 decades, resistance to pyrimethamine-sulfadoxine has appeared in a stepwise fashion since the widespread use of SP combination chemotherapy in these areas. In this study, samples of field isolates were collected from 2003 to 2008, when SP were replaced by artemisinin-based combination therapy for a long period. The genotyping analysis of drug resistance-conferring \textit{pfdhfr} and \textit{pfdhps} alleles revealed a more severe situation of SP resistance in the isolates from Yunnan area. Compared to the pyrimethamine resistance, it is notable that there is a delay in the emergence and development of sulfadoxine resistance alleles in both areas. This observation supports the presumption that the \textit{pfdhps} resistance takes place only after a substantial fraction of the population has been selected for pyrimethamine resistance (22, 33, 34), and it likely reaffirms the previous clinical finding that mutations in the \textit{pfdhfr} gene plays a major role in the failure of SP treatment against falciparum malaria (35, 36). Though the SP drug selection was assumed to act independently and differently on \textit{dhfr} and \textit{dhps} loci (37, 38), the asymmetry in the selection pattern suggests a potential genetic linkage between the two loci across chromosomal boundaries during the coselection of the drug combination (22, 31). In this study, LD analysis of the \textit{pfdhfr} and \textit{pfdhps} alleles discovered strong linkages of some SNPs between the major high-level resistance alleles in the population. In another, parallel study on \textit{P. vivax} isolates from Yunnan, we have shown that the high-resistance mutations in positions 57, 61, and 117 of \textit{pvdhfr} (chromosome 5) and position 383 of \textit{pvdhps} (chromosome 14) are genetically linked (31). Little is known about when and how these genetic linkages of certain SNPs in the \textit{dhfr} and \textit{dhps} genes took place across chromosomal boundaries, but it has been proved that such genetic relationships tend to induce the emergence and development of multiple drug resistance mutations and stabilize them within a parasite population after use of the drugs has been ceased for as long as 20 years. Therefore, this should be taken into account before the adoption of a combined chemotherapy against malaria.

**FIG 4** Selective sweep around \textit{dhfr} alleles in different regions. (A) \(H_e\) comparison of the wild type and five mutant groups (single, double, triple, quadruple, and quintuple mutations) on loci 57 kb, 30 kb, 5.87 kb, and 0.1 kb upstream and 1.48 kb, 3.84 kb, and 54 kb downstream around the \textit{pfdhfr} locus. The dotted line crossing the y axis indicates the mean \(H_e\) at 10 neutral microsatellite markers on other chromosomes. (B) Selection patterns of \textit{pfdhfr} alleles in Yunnan (black line) and Hainan (red line) are showed in the upper panel. A detailed description of the selection patterns in three sampling sites of Hainan is shown in the lower panel. The error bars indicate standard deviations (SD).
In China, Yunnan and Hainan Provinces are two geographically independent areas that both suffer from a prevalence of drug-resistant *P. falciparum* as well as *P. vivax*, though the drug selection pressure forced by SP combination chemotherapy has been ceased for approximately 20 years. The nonidentical distribution patterns of *pfdhfr* and *pfdhps* alleles in the parasite populations from the two areas partially reflect their history of drug administration. Unlike the case for the *pfdhps* alleles, it is intriguing to observe heterogeneity of prevalent patterns of the *pfdhfr* alleles within the sites in Hainan Island, which points to a unique evolution pathway of pyrimethamine resistance gene in the Dongfang site. Consistent with this finding, the systematic microsatellite-based analysis clearly distinguishes the origin and evolution pathway of Dongfang isolates from those of the other isolates. Particularly, we found a novel “HN” microsatellite haplotype in Dongfang City, which included nearly one-third of the Hainan isolates, whereas the other sites in Hainan and all the sites in the Yunnan area shared a common ancestor, the SEA haplotype, with Southeast Asia isolates. The observation that the HN haplotype was absent in all of the Yunnan isolates suggests that it is still limited to this geographically isolated island, though the representative SEA type from the SEA epicenter, the Thailand-Cambodia border, has invaded the Dongfang site in Hainan, resulting in full or hybrid types.
This phenomenon is further supported by the map of genetic relationships among \textit{pf}\textit{dhfr} alleles in southern China, where nearly all the Dongfang isolates belong to the HN type I cluster and other Hainan isolates fall into the YN/SEA subgroup. These data suggest a spread route of the SEA-type resistant \textit{pf}\textit{dhfr} alleles from Southeast Asian countries to China-Burma border and then to Hainan Island. Currently, how the unique HN-type pyrimethamine resistance in the Dongfang region originated and evolved and how the SEA type spread from the SEA epicenter to this isolated island are still not clear, but factors including the genetic characteristics of parasites and host, transmission of the local mosquito vector, and population migration among various areas where malaria is endemic might be involved in this process.

In conclusion, the results presented here show that (i) the geographically different distributions of drug-resistant \textit{pf}\textit{dhfr} and \textit{pf}\textit{dhps} alleles are fixed in southern China, which reflects their distinct histories of antimalaria chemotherapy, (ii) genetic linkage exists between certain \textit{pf}\textit{dhfr} and \textit{pf}\textit{dhps} alleles, and (iii) unlike in the Yunnan area where the common origin (SEA type) is prevalent, multiple origins of pyrimethamine resistance, including the novel HN type I, are present in Hainan (Dongfang), though the evolutionary pathway is similar to that in Yunnan. Taking the findings together, we have provided here a better understanding of SP resistance in the area in Asia where primary drug-resistant malaria is endemic.

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