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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 artimisinin (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 164 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 (CNRNL) 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-

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ever, the SP therapy programs in the two regions were not identical, 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, geographical 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 microsatellite loci and thereby tracking the origin and evolution of SP resistance-associated alleles. To date, no such comparative investigation 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 evaluating 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 multiple 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 Province) 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 Review Board of Second Military Medical University, China.

DNA isolation and genotyping methods. The parasite DNA was extracted from 200 μl venous blood or finger prick blood spot by using the QIAamp DNA Blood Minikit according to the manufacturer's recommendations (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 described by Zhou et al. (26). Each DNA sample was tested by nested PCRs. The primary amplification of *pfdhfr* 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 second amplification of *pfdhps* was done with the following parameters: 95°C for 3 min; 35 cycles of 95°C for 30 s, 52°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 sequences 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 putatively 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 Biosystems, 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 Treeview (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. Samples with multiple infections detected at any SNP site were excluded from the LD analysis. Significant LD between the loci should meet the requirements of $D' > 0.8$, $r^2 > 0.2$, and $P < 0.01$ simultaneously.

To measure the genetic diversity, we evaluated the expected heterozygosity (H_e) at all *pfdhfr* and neutral microsatellite loci by using Genalex software, version 6. H_e was calculated by using the formula $H_e = [n/(n-1)](1 - \sum p_i^2)$ (29), where n is the number of infections sampled and p_i is the frequency of the ith allele. The sampling variance of H_e was calculated according to the formula with a slight modification of the standard diploid variance, $2(n-1)/n^3\{2(n-2)[\sum p_i^3 - (\sum p_i^2)^2]\}$ (29). Different mean H_e 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-joining tree based on 7-locus microsatellite haplotypes through NETWORK version 4.5.1.0. In addition, for determining the number of origins precisely, 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 analysis.

RESULTS

Identification of infection complexity in field isolates. The isolates of *Plasmodium falciparum* were collected from the Yunnan-Burma border and Hainan Island, two geographically independent 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 supplemental 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 subsequently subjected to pyrosequencing. Of them, 100 were found to contain multiple alleles at one or more neutral loci through microsatellite 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

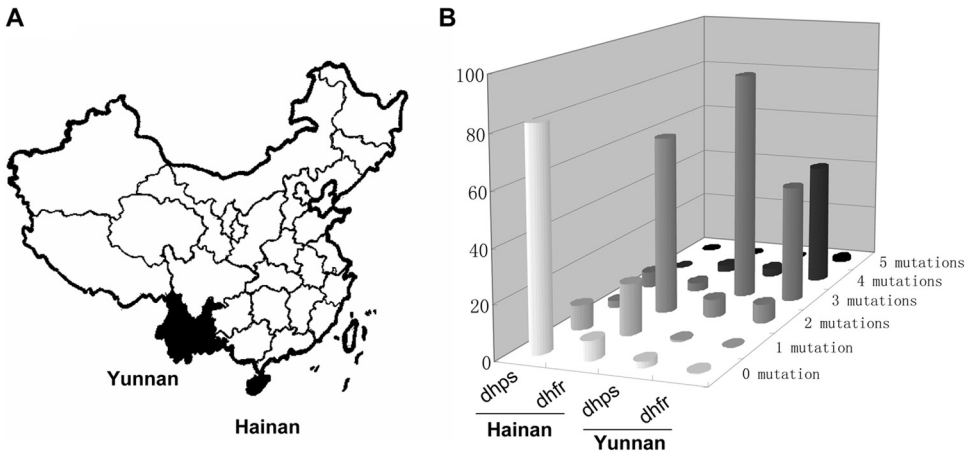

FIG 1 Distribution patterns of drug resistance-associated *pfdhfr* and *pfdhps* mutations in *Plasmodium falciparum* isolates from the Hainan and Yunnan areas. (A) The black parts of the map represent the two high-risk regions of endemicity of falciparum malaria in southern China (Yunnan and Hainan Provinces). (B) The number of site mutations represents various SNP haplotypes as listed in Table S1 in the supplemental material.

pfdhps haplotypes in this study, most of which have been described in previous reports. There were two novel *pfdhps* mutants (SGDAA and FGEGA) with extremely low frequencies in Tengchong City in Yunnan. In addition, two newly reported triple mutants, AGNAA 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 *pfdhfr* triple (AIRNI and ANRNL), quadruple (AIRNL), and quintuple (VIRNL) mutants. There was only one isolate with the wild-type *pfdhfr* 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 *pfdhfr* alleles, high-level resistant *pfdhps* mutants, including two predominant triple mutants, AGEAA and SGEGA, and other mutants such as AGEGA, FGEGA, AGEAT, AGNAA, and SGNGA, were observed in 90.43% of the Yunnan-derived isolates. The frequencies of *pfdhps* 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 *pfdhps* alleles showed a distinct distribution pattern where the wild type (SAKAA) was highly predominant (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 *pfdhfr* 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 *pfdhfr* mutants, which were composed mainly of ANCNI, ANRNI, and wild-type ANCSI alleles, while only the double mutant ANRNI allele was prevalent in the other sites (Ledong and Sanya) (Fig. 2A). For the *pfdhps* 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 *pfdhfr* and *pfdhps*. The similar SP resistance status observed in the sites in Yunnan indicated a potential genetic linkage between the *pfdhfr* and *pfdhps* 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 *pfdhfr* and *pfdhps* alleles (Fig. 3A). Strong linkage was established in two major groups, AGEAA-AIRNL/ANRNL/AIRNI and SGEGA-AIRNL/ANRNL/AIRNI (*dhps-dhfr* correlation, >5), which are linked to high-level SP-resistant mutants, and SAKAA-ANRNI/ANCNI/ANCSI and SGKAA-ANCNI (*dhps-dhfr* correlation, >5), which represent low-level SP resistance or wild type (χ^2 test, $P < 0.01$) (Fig. 3A, Southern China). Considering the geographical differences among these isolates, we further analyzed the *pfdhfr*/*pfdhps* 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 *pfdhfr-pfdhps* pairs showed weaker linkages without statistical significance.

The significant linkages between *pfdhfr* and *pfdhps* 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 *pfdhps* codon 437 and *pfdhfr* codon 51 ($D' = 0.91$, $r^2 = 0.25$, $P < 0.001$) or *pfdhfr* codon 164 ($D' = 0.85$, $r^2 = 0.36$, $P < 0.001$) in this population (Fig. 3B). It is notable that, unlike the previous finding by McCollum et al. (22), no significant association was observed between *pfdhps* codon 437 and the key *pfdhfr* mutation site 59 or 108 ($P > 0.01$). In addition, for the alleles within the *pfdhfr* or *pfdhps* gene, significant LDs were observed between *pfdhfr* codons 59 and 108 ($D' = 1$, $r^2 = 0.28$, $P < 0.001$) and *pfdhps* codons 437 and 436 ($D' > 0.8$, $r^2 > 0.2$, $P < 0.001$) or 540 ($D' = 1$, $r^2 = 0.64$, $P < 0.001$), suggesting that the

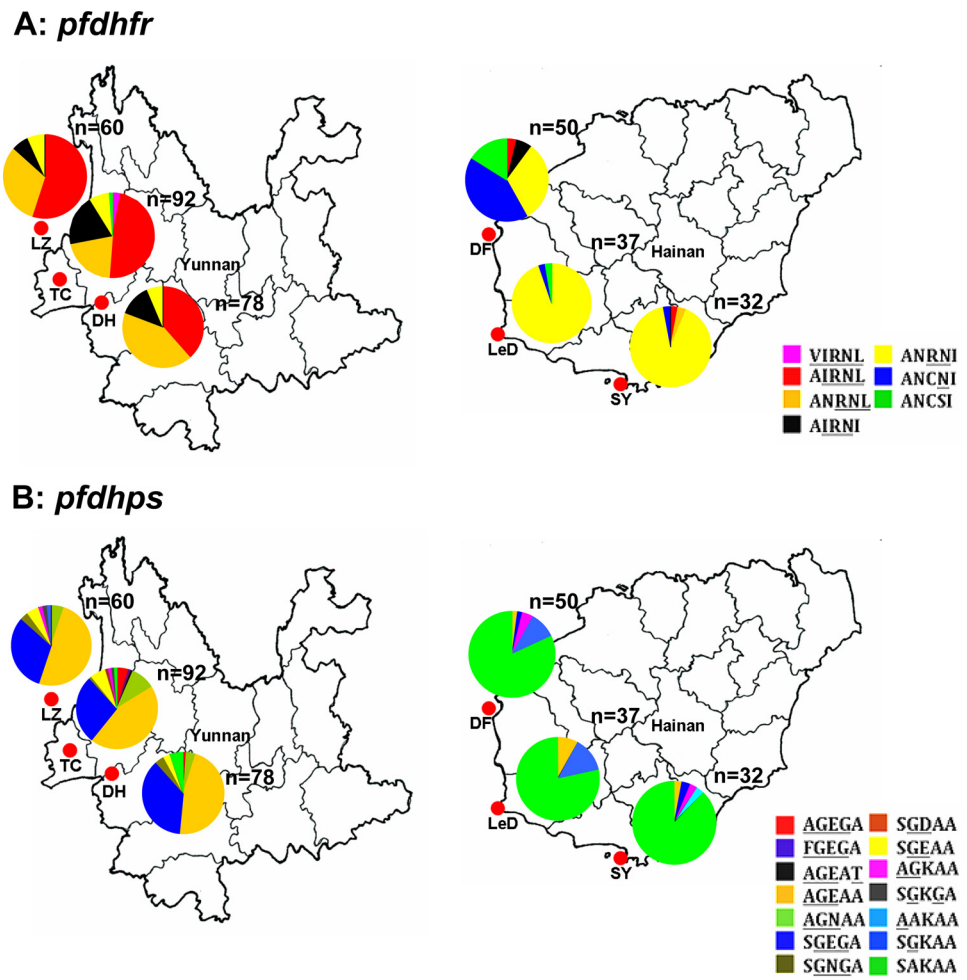

FIG 2 Local distributions of *pfdhfr* and *pfdhps* resistance alleles in Yunnan and Hainan. (A) Pie charts of the six sampling sites on the China-Burma border and western coast of Hainan Island illustrate the proportions of all 7 *pfdhfr* alleles. (B) Proportions of all 14 *pfdhps* alleles in the 6 sampling sites. The various *pfdhfr* or *pfdhps* alleles are shown in different colors. LZ, Lazan ($n = 60$); TC, Tengchong ($n = 92$); DH, Dehong ($n = 78$); DF, Dongfang ($n = 50$); LeD, Ledong ($n = 37$); SY, Sanya ($n = 32$).

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 wide-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 (ANCNI, $H_e = 0.321 \pm 0.068$; $P = 0.010$), double (ANRNI, $H_e = 0.290 \pm 0.014$; $P = 0.013$), triple (AIRNI, $H_e = 0.030 \pm 0.017$ [$P < 0.001$] 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 ± 0.003) than in Hainan (0.603 ± 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 ($H_{eLedong} = 0.359 \pm 0.034$; $H_{eSanya} = 0.464 \pm$

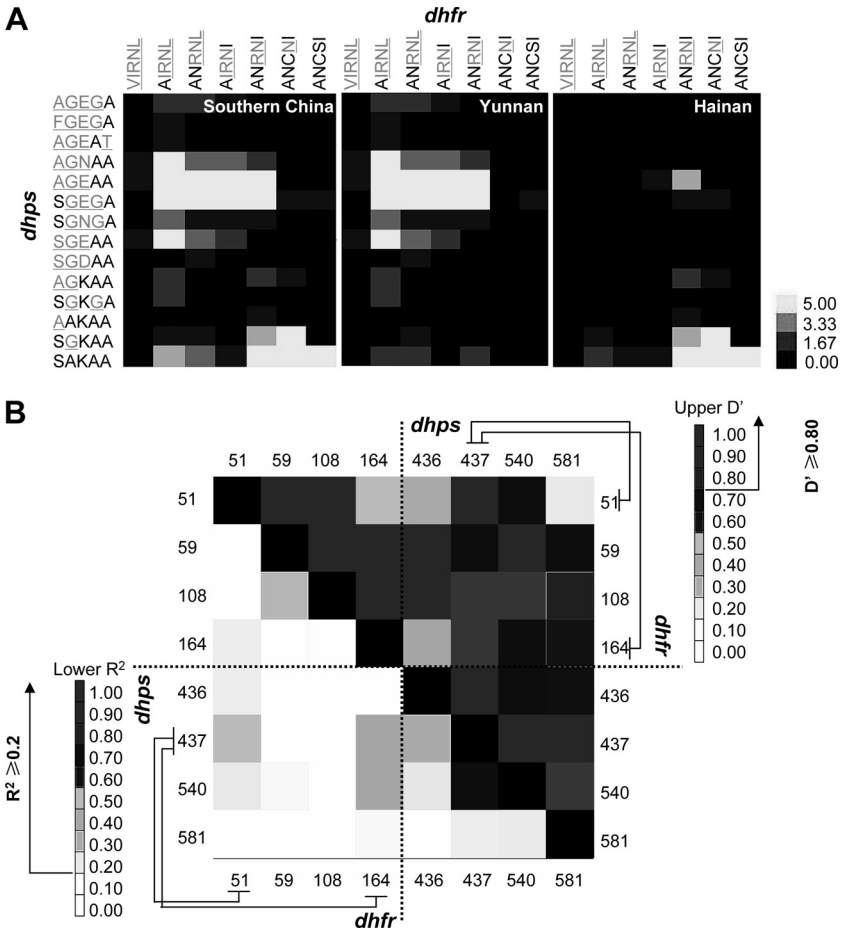

FIG 3 Genetic linkage between *pfdhfr* and *pfdhps* alleles. (A) Linkage clustering. The level of linkage was measured by the Pearson correlation and is displayed in different colors (a χ^2 test had been performed in advance, $P < 0.01$). The black, gray-yellow, and yellow grids represent zero, weakly positive, and strongly positive correlations, respectively. The numbers in the bar chart refer to the values of Pearson correlation grades. (B) LD analysis. The numbers on the *x* and *y* axes indicate SNP sites in *pfdhfr* and *pfdhps*. The upper right shows *D′* values for all the SNP pairs, while the lower left represents r^2. The grids in different colors indicate the LD level between each pairs. The arrows show the baseline in determining the significant LDs.

0.107) ($P = 0.021$). 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* gene. 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-

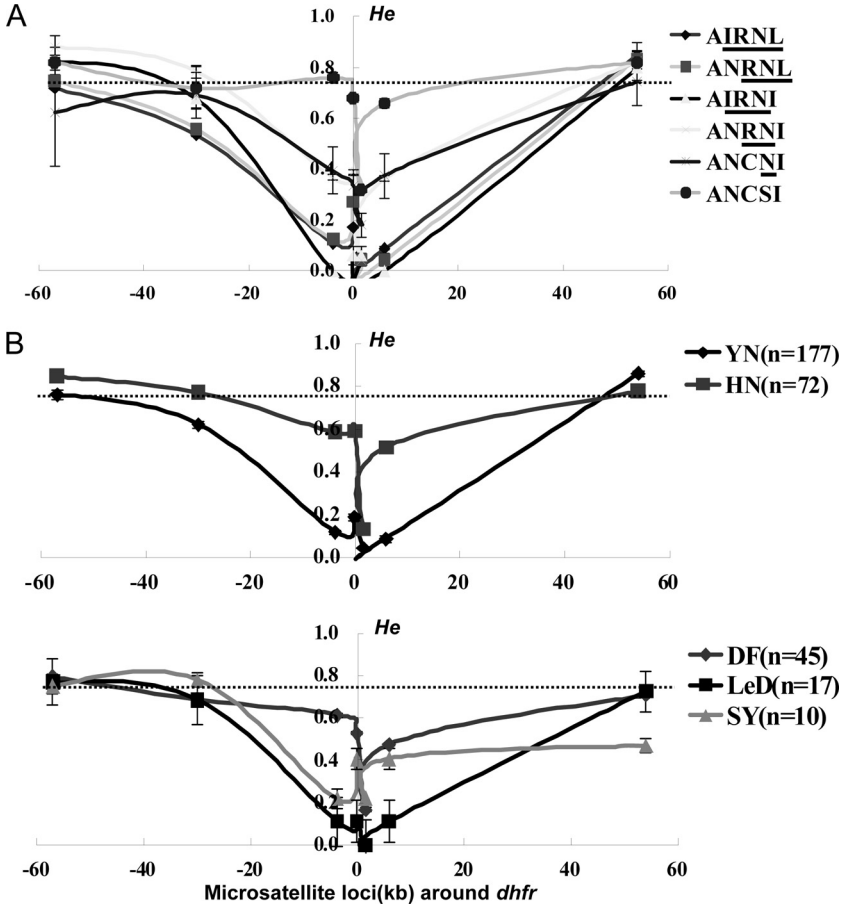

FIG 4 Selective sweep around *dhfr* alleles in different regions. (A) H_e comparison of the wild type and five mutant groups (single, double, triple, quadruple, and quintuple mutations) on loci 57 kb, 30 kb, 5.87 kb, and 0.1 kb upstream and 1.48 kb, 3.84 kb, and 54 kb downstream around the *pfdhfr* locus. The dotted line crossing the *y* axis indicates the mean H_e at 10 neutral microsatellite markers on other chromosomes. (B) Selection patterns of *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).

ized Southeast Asia isolates. However, at least two origins of *pfdhfr* mutations existed in the geographically isolated Hainan Island under multidrug selections. The Dongfang isolates represented a novel source of the pyrimethamine resistance in *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 *pfdhfr* and *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 *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 *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 *dhfr* and *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 *pfdhfr* and *pfdhps* alleles discovered strong linkages of some SNPs between the major high-level resistance alleles in the population. In another, parallel study on *P. vivax* isolates from Yunnan, we have shown that the high-resistance mutations in positions 57, 61, and 117 of *pvdhfr* (chromosome 5) and position 383 of *pvdhps* (chromosome 14) are genetically linked (31). Little is known about when and how these genetic linkages of certain SNPs in the *dhfr* and *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.

H	Allele	Bp length of microsatellite markers at four loci				Region						Haplotypes
		-3.87	-0.1	1.48	5.87	LZ	DH	TC	LeD	DF	SY	
H1	VIRNL	193	175	201	110			2				SEA
H2	VIRNL	193	175	201	108			1				SEA Variant
H1	AIRNL	193	175	201	110	29	9	31		1		SEA
H3	AIRNL	195	175	201	110			3				SEA Variant
H4	AIRNL	193	165	201	110	1		2				SEA Variant
H5	AIRNL	193	175	201	112	1	1					SEA Variant
H6	AIRNL	193	175	207	110			1				SEA Variant
H7	AIRNL	193	175	201	130			1				SEA Variant
H8	AIRNL	193	163	201	110	1						SEA Variant
H9	AIRNL	193	167	201	110			1				SEA Variant
H10	AIRNL	193	169	201	110						1	SEA Variant
H11	AIRNL	187	155	201	110			1				—
H12	AIRNL	187	175	201	110					1		SEA Variant
H13	AIRNL	193	175	199	110			1				SEA Variant
H14	AIRNL	193	179	201	110		1					SEA Variant
H15	AIRNL	193	175	201	98						1	SEA Variant
H1	ANRNL	193	175	201	110	17	9	11				SEA
H9	ANRNL	193	167	201	110		1	3				SEA Variant
H16	ANRNL	199	167	201	110	1		1				—
H17	ANRNL	193	175	201	102			1				SEA Variant
H13	ANRNL	193	175	199	110			1				SEA Variant
H18	ANRNL	193	155	201	110		2					SEA Variant
H1	AIRNI	193	175	201	110	4	9	14		3		SEA
H13	AIRNI	193	175	199	110			1				SEA Variant
H1	ANRNI	193	175	201	110	1	3	6	16	3	3	SEA
H19	ANRNI	199	155	201	104					6	1	HN
H20	ANRNI	195	175	201	102	2						—
H21	ANRNI	193	169	201	104					1		—
H18	ANRNI	193	155	201	110					1		SEA-HN hybrid
H22	ANRNI	193	155	201	104					1		SEA-HN hybrid
H23	ANRNI	199	155	201	110					1		SEA-HN hybrid
H13	ANRNI	193	175	199	110						1	SEA Variant
H3	ANRNI	195	175	201	110					1		SEA Variant
H9	ANRNI	193	167	201	110			1				SEA Variant
H19	ANCNI	199	155	201	104					13		HN
H1	ANCNI	193	175	201	110					1	1	SEA
H11	ANCNI	187	155	201	110					1		—
H23	ANCNI	199	155	201	110					1		SEA-HN hybrid
H13	ANCNI	193	175	199	110					1		SEA Variant
H24	ANCNI	199	155	199	104					1		HN Variant
H21	ANCNI	193	169	201	104					1		—
H25	ANCSI	187	155	199	110					2		—
H11	ANCSI	187	155	201	110			1		1		—
H21	ANCSI	193	169	201	104					1	1	—
H26	ANCSI	207	155	201	112			1				—
H27	ANCSI	197	195	201	104					1		—
H28	ANCSI	199	195	201	104					1		HN Variant
H29	ANCSI	193	175	201	104					1		SEA-HN hybrid
H30	ANCSI	205	175	201	98						1	—
H31	ANCSI	205	185	201	98				1			—
						57	35	85	17	45	10	249

FIG 5 Microsatellite haplotype profiles (H1 to H31) on four loci flanking *dhfr* in isolates from southern China. Each box represents the microsatellite type at codons 16, 51, 59, 108, and 164 of each *pfdhfr* allele in the six sampling sites. Identical colors (green and blue) represent proposed common lineages. Blue grids represent the SEA lineage, whereas the HN lineage is shown in green. The yellow grids under the column "Haplotypes" indicate a hybrid of the SEA and HN haplotypes based on microsatellite characteristics at loci −3.87, −0.1, and 5.87. The numbers under the column "Region" indicate the frequency of each haplotype in various regions. LZ, Lazan; TC, Tengchong; DH, Dehong; DF, Dongfang; LeD, Ledong; SY, Sanya.

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 SEA epicenter, the Thailand-Cambodia border, has invaded the Dongfang site in Hainan, resulting in full or hybrid types.

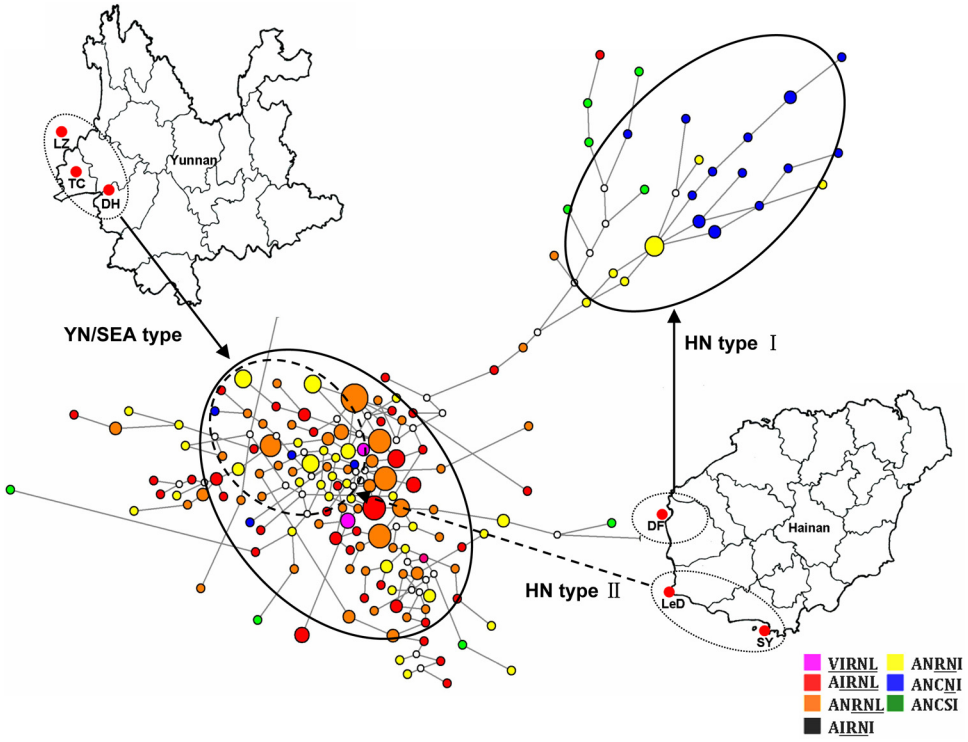

FIG 6 Genetic relationships among *pfdhfr* alleles in southern China. Based on 7-loci microsatellite polymorphism around *pfdhfr*, all these isolates ($n = 249$) were classified into 174 haplotypes. The sizes of the circles are proportional to the number of isolates showing particular 7-loci microsatellite haplotypes. Six types of *pfdhfr* alleles are showed in different colors. White dots connecting haplotypes within the network are hypothetical median vectors generated by the software. Two major independent networks, designated YN/SEA types and HN type I, are formed simultaneously in the population (circles with solid line). The HN type II circle (dashed line) represents partial Hainan isolates with double mutants belong to the YN/SEA type.

This phenomenon is further supported by the map of genetic relationships among *pfdhfr* 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 *pfdhfr* 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 *pfdhfr* and *pfdhps* alleles are fixed in southern China, which reflects their distinct histories of antimalaria chemotherapy, (ii) genetic linkage exists between certain *pfdhfr* and *pfdhps* 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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Y.Z., Q.Z., and W.P. conceived and designed the experiments. Y.Z. and H.Y. performed the experiments. Y.Z., H.Y., Q.Z., and W.P. analyzed the data. G.W. and Y.H. contributed reagents, materials, and analysis tools. Y.Z., Q.Z., and W.P. wrote the paper.

REFERENCES

1. **Talisuna AO, Bloland P, D'Alessandro U.** 2004. History, dynamics, and public health importance of malaria parasite resistance. Clin. Microbiol. Rev. **17:**235–254. http://dx.doi.org/10.1128/CMR.17.1.235-254.2004.
2. **Pinichpongse S, Doberstyn EB, Cullen JR, Yisunsri L, Thongsombun Y, Thimasarn K.** 1982. An evaluation of five regimens for the outpatient therapy of falciparum malaria in Thailand 1980-81. Bull. World Health Organ. **60:**907–912.
3. **Denis MB, Tsuyuoka R, Poravuth Y, Narann TS, Seila S, Lim C, Incardona S, Lim P, Sem R, Socheat D, Christophel EM, Ringwald P.** 2006. Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. Trop. Med. Int. Health **11:**1360–1366. http://dx.doi.org/10.1111/j.1365-3156.2006.01690.x.
4. **Denis MB, Tsuyuoka R, Lim P, Lindegardh N, Yi P, Top SN, Socheat D, Fandeur T, Annerberg A, Christophel EM, Ringwald P.** 2006. Efficacy of artemether-lumefantrine for the treatment of uncomplicated fal-

ciparum malaria in northwest Cambodia. Trop. Med. Int. Health **11:** 1800–1807. http://dx.doi.org/10.1111/j.1365-3156.2006.01739.x.
5. **Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM.** 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N. Engl. J. Med. **359:**2619–2620. http://dx.doi.org/10.1056/NEJMc0805011.
6. **Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ.** 1988. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U. S. A. **85:**9109–9113. http://dx.doi.org/10.1073/pnas.85.23.9109.
7. **Cortese JF, Plowe CV.** 1998. Antifolate resistance due to new and known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. Mol. Biochem. Parasitol. **94:**205–214. http://dx.doi.org/10.1016/S0166-6851(98)00075-9.
8. **Triglia T, Menting JG, Wilson C, Cowman AF.** 1997. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U. S. A. **94:** 13944–13949. http://dx.doi.org/10.1073/pnas.94.25.13944.
9. **Wang P, Read M, Sims PF, Hyde JE.** 1997. Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. Mol. Microbiol. **23:**979–986. http://dx.doi.org/10.1046/j.1365-2958.1997.2821646.x.
10. **Plowe CV, Kublin JG, Doumbo OK.** 1998. *P. falciparum* dihydrofolate reductase and dihydropteroate synthase mutations: epidemiology and role in clinical resistance to antifolates. Drug Resist. Updates **1:**389–396. http://dx.doi.org/10.1016/S1368-7646(98)80014-9.
11. **Gregson A, Plowe CV.** 2005. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol. Rev. **57:**117–145. http://dx.doi.org/10.1124/pr.57.1.4.
12. **Bjorkman A, Phillips-Howard PA.** 1990. The epidemiology of drug-resistant malaria. Trans. R. Soc. Trop. Med. Hyg. **84:**177–180. http://dx.doi.org/10.1016/0035-9203(90)90246-B.
13. **Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, Chandramohan D, Sharp B.** 2003. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet **361:**1174–1181. http://dx.doi.org/10.1016/S0140-6736(03)12951-0.
14. **Maïga O, Djimdé AA, Hubert V, Renard E, Aubouy A, Kironde F, Nsimba B, Koram K, Doumbo OK, Le Bras J, Clain J.** 2007. A shared Asian origin of the triple-mutant *dhfr* allele in *Plasmodium falciparum* from sites across Africa. J. Infect. Dis. **196:**165–172. http://dx.doi.org/10.1086/518512.
15. **Mita T, Tanabe K, Takahashi N, Culleton R, Ndounga M, Dzodzomenyo M, Akhwale WS, Kaneko A, Kobayakawa T.** 2009. Indigenous evolution of *Plasmodium falciparum* pyrimethamine resistance multiple times in Africa. J. Antimicrob. Chemother. **63:**252–255. http://dx.doi.org/10.1093/jac/dkn482.
16. **Bacon DJ, McCollum AM, Griffing SM, Salas C, Soberon V, Santolalla M, Haley R, Tsukayama P, Lucas C, Escalante AA, Udhayakumar V.** 2009. Dynamics of malaria drug resistance patterns in the Amazon basin region following changes in Peruvian national treatment policy for uncomplicated malaria. Antimicrob. Agents Chemother. **53:**2042–2051. http://dx.doi.org/10.1128/AAC.01677-08.
17. **Cortese JF, Caraballo A, Contreras CE, Plowe CV.** 2002. Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. J. Infect. Dis. **186:**999–1006. http://dx.doi.org/10.1086/342946.
18. **McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA.** 2007. Common origin and fixation of *Plasmodium falciparum dhfr* and *dhps* mutations associated with sulfadoxine-pyrimethamine resistance in a lowtransmission area in South America. Antimicrob. Agents Chemother. **51:**2085–2091. http://dx.doi.org/10.1128/AAC.01228-06.
19. **Mita T, Tanabe K, Takahashi N, Tsukahara T, Eto H, Dysoley L, Ohmae H, Kita K, Krudsood S, Looareesuwan S, Kaneko A, Björkman A, Kobayakawa T.** 2007. Independent evolution of pyrimethamine resistance in *Plasmodium falciparum* isolates in Melanesia. Antimicrob. Agents Chemother. **51:**1071–1077. http://dx.doi.org/10.1128/AAC.01186-06.
20. **Vinayak S, Alam MT, Mixson-Hayden T, McCollum AM, Sem R, Shah NK, Lim P, Muth S, Rogers WO, Fandeur T, Barnwell JW, Escalante AA, Wongsrichanalai C, Ariey F, Meshnick SR, Udhayakumar V.** 2010. Origin and evolution of sulfadoxine resistant *Plasmodium falciparum*. PLoS Pathog. **6:**e1000830. http://dx.doi.org/10.1371/journal.ppat.1000830.
21. **Pearce RJ, Pota H, Evehe MS, Bâ el-H, Mombo-Ngoma G, Malisa AL, Ord R, Inojosa W, Matondo A, Diallo DA, Mbacham W, van den Broek IV, Swarthout TD, Getachew A, Dejene S, Grobusch MP, Njie F, Dunyo S, Kweku M, Owusu-Agyei S, Chandramohan D, Bonnet M, Guthmann JP, Clarke S, Barnes KI, Streat E, Katokele ST, Uusiku P, Agboghoroma CO, Elegba OY, Cissé B, A-Elbasit IE, Giha HA, Kachur SP, Lynch C, Rwakimari JB, Chanda P, Hawela M, Sharp B, Naidoo I, Roper C.** 2009. Multiple origins and regional dispersal of resistant *dhps* in African *Plasmodium falciparum* malaria. PLoS Med. **6:**e1000055. http://dx.doi.org/10.1371/journal.pmed.1000055.
22. **McCollum AM, Basco LK, Tahar R, Udhayakumar V, Escalante AA.** 2008. Hitchhiking and selective sweeps of *Plasmodium falciparum* sulfadoxine and pyrimethamine resistance alleles in a population from central Africa. Antimicrob. Agents Chemother. **52:**4089–4097. http://dx.doi.org/10.1128/AAC.00623-08.
23. **Hurwitz ES, Johnson D, Campbell CC.** 1981. Resistance of *Plasmodium falciparum* malaria to sulfadoxine-pyrimethamine ('Fansidar') in a refugee camp in Thailand. Lancet **i:**1068–1070.
24. **Verdrager J.** 1986. Epidemiology of the emergence and spread of drug-resistant, falciparum malaria in South-East Asia and Australasia. J. Trop. Med. Hyg. **89:**277–289.
25. **Huang F, Tang L, Yang H, Zhou S, Liu H, Li J, Guo S.** 2012. Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* in Yunnan Province, China. Malar. J. **11:**243. http://dx.doi.org/10.1186/1475-2875-11-243.
26. **Zhou Z, Poe AC, Limor J, Grady KK, Goldman I, McCollum AM, Escalante AA, Barnwell JW, Udhayakumar V.** 2006. Pyrosequencing, a high-throughput method for detecting single nucleotide polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum*. J. Clin. Microbiol. **44:**3900–3910. http://dx.doi.org/10.1128/JCM.01209-06.
27. **Nair S, Williams JT, Brockman A, Paiphun L, Mayxay M, Newton PN, Guthmann JP, Smithuis FM, Hien TT, White NJ, Nosten F, Anderson TJ.** 2003. A selective sweep driven by pyrimethamine treatment in southeast Asian malaria parasites. Mol. Biol. Evol. **20:**1526–1536. http://dx.doi.org/10.1093/molbev/msg162.
28. **Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP.** 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol. Biol. Evol. **17:**1467–1482. http://dx.doi.org/10.1093/oxfordjournals.molbev.a026247.
29. **Nei M.** 1987. Molecular evolutionary genetics. Columbia University Press, New York, NY.
30. **Alam MT, Vinayak S, Congpuong K, Wongsrichanalai C, Satimai W, Slutsker L, Escalante AA, Barnwell JW, Udhayakumar V.** 2011. Tracking origins and spread of sulfadoxine-resistant *Plasmodium falciparum dhps* alleles in Thailand. Antimicrob. Agents Chemother. **55:**155–164. http://dx.doi.org/10.1128/AAC.00691-10.
31. **Ding S, Ye R, Zhang D, Sun X, Zhou H, McCutchan TF, Pan W.** 2013. Anti-folate combination therapies and their effect on the development of drug resistance in *Plasmodium vivax*. Sci. Rep. **3:**1008. http://dx.doi.org/10.1038/srep01008.
32. **Pearce R, Malisa A, Kachur SP, Barnes K, Sharp B, Roper C.** 2005. Reduced variation around drug-resistant *dhfr* alleles in African *Plasmodium falciparum*. Mol. Biol. Evol. **22:**1834–1844. http://dx.doi.org/10.1093/molbev/msi177.
33. **Bonizzoni M, Afrane Y, Baliraine FN, Amenya DA, Githeko AK, Yan G.** 2009. Genetic structure of *Plasmodium falciparum* populations between lowland and highland sites and antimalarial drug resistance in western Kenya. Infect. Genet. Evol. **9:**806–812. http://dx.doi.org/10.1016/j.meegid.2009.04.015.
34. **Nzila AM, Nduati E, Mberu EK, Hopkins Sibley C, Monks SA, Winstanley PA, Watkins WM.** 2000. Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate pyrimethamine/sulfadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum*. J. Infect. Dis. **181:**2023–2028. http://dx.doi.org/10.1086/315520.
35. **Gatton ML, Martin LB, Cheng Q.** 2004. Evolution of resistance to sulfadoxine-pyrimethamine in *Plasmodium falciparum*. Antimicrob. Agents Chemother. **48:**2116–2123. http://dx.doi.org/10.1128/AAC.48.6.2116-2123.2004.
36. **Mockenhaupt FP, Teun Bousema J, Eggelte TA, Schreiber J, Ehrhardt S, Wassilew N, Otchwemah RN, Sauerwein RW, Bienzle U.** 2005. *Plasmodium falciparum dhfr* but not *dhps* mutations associated with sul-

phadoxine-pyrimethamine treatment failure and gametocyte carriage in northern Ghana. Trop. Med. Int. Health **10:**901–908. http://dx.doi.org/10.1111/j.1365-3156.2005.01471.x.

37. **Nzila AM, Mberu EK, Sulo J, Dayo H, Winstanley PA, Sibley CH, Watkins WM.** 2000. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. Antimicrob. Agents Chemother. **44:**991–996. http://dx.doi.org/10.1128/AAC.44.4.991-996.2000.
38. **Sibley CH, Hyde JE, Sims PF, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Nzila AM.** 2001. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? Trends Parasitol. **17:**582–588. http://dx.doi.org/10.1016/S1471-4922(01)02085-2.