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# Rapid selection of *dhfr* mutant allele in *Plasmodium falciparum* isolates after the introduction of sulfadoxine/pyrimethamine in combination with 4-aminoquinolines in Papua New Guinea

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**Abstract**

To overcome the declining efficacy of the 4-aminoquinolines in Papua New Guinea, sulfadoxine/pyrimethamine (SP) was combined with the 4-aminoquinolines as the first line treatment for falciparum malaria since 2000. To assess how this change had affected SP resistant gene polymorphisms, we determined allele frequencies of *dhfr* and *dhps* in 113 *Plasmodium falciparum* isolates from Wewak, East Sepik of Papua New Guinea in 2002 and 2003. In *dhfr*, double mutant (ACNRNVI) was the predominant allele with a prevalence of 91%. We found a significant decrease of wild *dhfr* allele prevalence (7%) compared with that reported in the adjacent area of East Sepik called the Wosera region (57%), before the drug policy changed in 1990–1993. Between 2002 and 2003, the prevalence of this allele decreased from 15% to 3% ($P = 0.02$). Two distinct microsatellite haplotypes flanking *dhfr* were found in isolates with *dhfr* double mutant, suggesting the selection of preexisting SP resistant parasites rather than a frequent occurrence of *dhfr* mutations. The *dhfr*/*dhps* quartet mutations (ACNRNVI in *dhfr* and SGEAA in *dhps*) were identified in six of the isolates (8%) from 2003. This genotype, which is associated with in vivo resistance to SP, has not been reported before in Papua New Guinea. These findings suggest that isolates resistant to SP were rapidly selected despite the use of the SP combination therapy, probably because of their preexisting high level of resistance to the 4-aminoquinoline partner drug.


*Keywords:* *Plasmodium falciparum*; Chloroquine; Sulfadoxine/pyrimethamine; Resistance; *Dhfr*; *Dhps*; Combination therapy; Microsatellite

## 1. Introduction

The spread of drug resistant *Plasmodium falciparum* (*P. falciparum*) is a major public health problem in the malaria endemic regions. Sulfadoxine/pyrimethamine (SP) inhibits two enzymes in the folate synthesis pathway of *P. falciparum*, i.e., dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). The amino acid change at position 108 from S to N in the *dhfr* usually represents an initial mutation with low in vitro resistance (Peterson et al., 1988). Additional mutation(s) at positions 16, 50, 51, 59, 140, and 164 will then lead to higher resistance. Similarly, in *dhps*, amino acid change at position 437 from A to G represents an initial mutation to sulfadoxine whereas additional mutation(s) at positions 436, 437, 540, 581 and 613 elevated levels of in vitro sulfadoxine resistance (Triglia and Cowman, 1994; Triglia et al., 1998). Surveillance of *dhfr* and *dhps* mutations is considered an inexpensive and reliable tool for the assessment of SP resistant levels in the malaria endemic regions.

Because of the widespread development of chloroquine resistance, Kenya (Ogutu et al., 2000), Malawi (Bloland et al., 1993), and South Africa (Bredenkamp et al., 2001) switched

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their first-line antimalarial drugs for falciparum infection from chloroquine to SP. However, SP resistance was reported soon after its use as the monotherapy in these countries (Mberu et al., 2000; Sibley et al., 2001; Bwijo et al., 2003; Roper et al., 2003). In particular, in the province of KwaZulu-Natal in South Africa where SP was introduced as the first-line therapy in 1988, SP treatment failed and reached an unacceptable level (70%) by 2000. The first-line treatment was soon changed to the coartemether (artemether + lumefantrine) (Bredenkamp et al., 2001).

In the case of tuberculosis and AIDS, combination therapy delays the selection of drug resistant pathogens. For falciparum malaria, artesunate and mefloquine became the first widely used drug combination in Southeast Asia where the resistance to both drugs was prevented (Wilairatana et al., 1998; Nosten et al., 2000).

In Papua New Guinea, oral administration of 4-aminoquinoline (chloroquine or amodiaquine) was used for many years as the standard treatment for uncomplicated malaria. However, chloroquine resistant *P. falciparum* has become highly prevalent in many endemic regions in Papua New Guinea after its first emergence in 1976 (Han and Grimmond, 1976). In 2000, the combination of either one of the 4-aminoquinoline with SP was officially introduced as the first-line treatment for falciparum malaria. SP has therefore been used almost exclusively as a partner drug. Although the addition of chloroquine to SP offered lesser therapeutic advantage when compared with SP alone (Checchi et al., 2004; Talisuna et al., 2004a), it was expected that the introduction of the combination therapy could delay the activity of SP resistance (Rieckmann and Cheng, 2002).

We investigated whether the combination therapy (4-aminoquinoline + SP) will protect against the spread of resistance to SP in place where 4-aminoquinoline resistances are preexisting by determining the frequencies of *dhfr* and *dhps* haplotypes in *P. falciparum* isolates collected from Wewak, East Sepik in 2002 and 2003. We also investigated the microsatellite markers flanking the *dhfr* locus to assess the evolutionary origins of *dhfr* mutant alleles.

## 2. Materials and methods

### 2.1. Study site and patients

The study included patients attending Town and Wiryi clinics in Wewak, East Sepik Province (Fig. 1) in November 2002 and 2003, respectively. In this province, the average temperature is 27 (min 24; max 30) with an annual rainfall of about 2000 mm. Malaria is hyper-endemic with minor monthly variation, transmitted mainly by *Anopheles farauti* and *An. koliensis*. *P. falciparum* is the predominant parasite species in the province. Together with the previously reported sporozoite rate of Anopheles vectors (0.7%) in East Sepik (Benet et al., 2004), the annual entomological inoculation rate in this area was estimated to be 51 infective bites per person per year. Although the combination of either one of the 4-aminoquinoline with SP was officially adopted as the first-line treatment for falciparum malaria since 2000 in Papua New Guinea, this policy amendment was not widely implemented in our study site till 2001. Antifolate drug is exclusively used for the malaria infection. Patients of all age groups with clinical symptoms of uncomplicated malaria, e.g. fever, diarrhea, and vomiting were microscopically examined for the presence of parasites using thick and thin smears stained with Giemsa. *P. falciparum* infected patients who met the following criteria were recruited for this study: (1) asexual parasitemia from 1000 to 80,000 $\mu L^{-1}$, (2) no intake of antimalarials in the preceding 4 weeks, and (3) informed consent from patient or parent. The malaria-infected patients were treated with the combination of either one of the

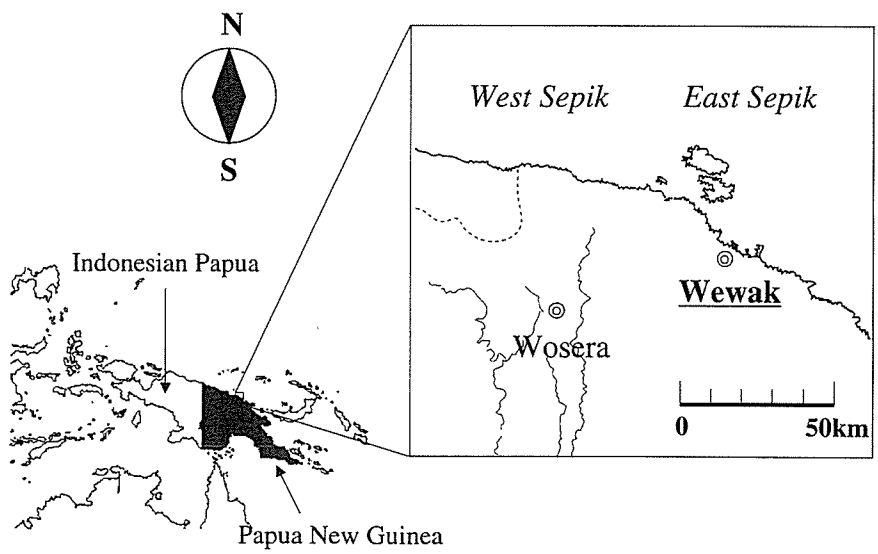


Fig. 1. Location of Wewak region in Papua New Guinea.

4-aminoquinoline and SP according to the official treatment policy.

### 2.2. Dhfr and dhps alleles

A finger prick blood sample (75 μL) was collected on chromatography filter paper ET31CHR (Whatman Limited, Kent, UK) before treatment. *P. falciparum* DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) with some modifications as described elsewhere (Sakihama et al., 2001). *Dhfr* and *dhps* were amplified by polymerase chain reaction (PCR) and amplified products were subjected to direct sequencing using primer pairs with BigDye terminator v1.1 cycle sequencing kit in ABI 377 DNA Sequencer (Applied Biosystems, Warrington, Cheshire, UK) as described by Reeder et al. (1996).

### 2.3. Microsatellite haplotyping

Nucleotide length variations (determined by the number of TA repeats) of microsatellite markers were determined to infer the origin of these alleles. We measured three microsatellite markers located at 0.1 kb and 3.87 kb upstream and 1.48 kb downstream of the *dhfr* locus as described by Nair et al. (2003). In brief, semi-nested PCR was performed using fluorescent end-labeled primers. Size variations of the amplified products were determined by electrophoresis on an ABI 377 and analyzed with Genescan software (Applied Biosystems). In the event of two or more polymorphisms being detected, we considered these isolates as mixed infections.

### 2.4. Statistical analysis

We calculated expected heterozygosity ($h$) at each microsatellite locus as $h = [n/(n-1)]\ [1 - \sum pi^2]$, where $n$ is the number of infections sampled and $pi$ is the frequency of the $i$th allele. We estimated the variance of h using a Taylor's series expansion. Fisher's exact probability test was used to assess statistical associations of allele frequencies between 2002 and 2003. $P < 0.05$ was considered statistically significant.

## 3. Result

### 3.1. Dhfr and dhps alleles

A total of 266 symptomatic patients were screened for eligibility in 2002 and 2003, and 173 were found to have *P. falciparum* mono-infections. Out of the total infected patients, 113 patients satisfied the inclusion criteria and were recruited for the study. *Dhfr* and *dhps* sequences were successfully determined in all the 113 blood samples (Table 1). Two of the seven loci in *dhfr* were found to be polymorphic corresponding to amino acid positions 59 and 108. The combination of the two mutations (ACNRNVI) was the predominant allele yielding prevalences of 83% and 96% for 2002 and 2003, respectively. The prevalences of wild *dhfr* alleles was reduced from 15% (2002) to 3% (2003) ($P = 0.02$). These prevalences of the wild allele were considerably lower than the prevalence (59%) reported in 1990–1993 in the Wosera region, an area adjacent to East Sepik (Reeder et al., 1996).

Table 1
Combinations of *dhfr* and *dhps* alleles in *Plasmodium falciparum* isolates from Wewak, Papua New Guinea in 2002 and 2003

| *Dhfr/dhps* haplotype | No. of samples | |
|---|---|---|
| *Dhfr* + *dhps* | 2002 ($n = 40$) | 2003 ($n = 73$) |
| ACNCSVI[a] + SAKAA[b] | 6 (15.0%) | 2 (2.7%) |
| ACNC*N*VI + SAKAA | 1 (2.5%) | 1 (1.4%) |
| ACNRNVI + SAKAA | 33 (82.5%) | 63 (86.3%) |
| ACNRNVI + *C*AKAA | 0 | 1 (1.4%) |
| ACNRNVI + S*G*E*A*A | 0 | 6 (8.2%) |

[a] Amino acid residues at positions 16, 51, 59, 108, 140, and 164 in *dhfr*.
[b] Amino acid residues at positions 436, 437, 540, 581, and 613 in *dhps*.

Three mutations were detected within the *dhps* gene; at amino acid positions 436, 437 and 540; but 100% ($n = 40$) and 90% ($n = 66$) of the isolates still harbored the wild allele derived in 2002 and 2003, respectively (Table 1). All mutations were detected in seven isolates derived in 2003. Six of them harbored double mutations at positions 436 and 437 (S*G*E*A*A) and all these six isolates also had the double mutant (ACNRNVI) allele in *dhfr*. These isolates therefore had *dhfr/dhps* quartet mutations (ACNRNVI + S*G*E*A*A).

### 3.2. Microsatellite polymorphisms around the dhfr locus

Associations between *dhfr* alleles and microsatellite markers flanking *dhfr* are shown in Table 2. Isolates harboring mixed microsatellite polymorphisms at any positions ($n = 14$) were excluded from this analysis. We found significant differences of h between parasites harboring wild and mutant *dhfr* (ACNRNVI) alleles in microsatellite markers located at 0.1 kb (0.75 in wild versus 0.39 in mutant, $P < 0.001$) and 3.87 kb (0.86 versus 0.41 in mutant, $P < 0.0005$) upstream of the *dhfr* locus.

Table 2
*Dhfr* alleles and microsatellite haplotypes around *dhfr* in 99 *P. falciparum* isolates from Wewak, Papua New Guinea in 2002 and 2003

| *Dhfr* allele | Microsatellite haplotype[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 156/192/203 | 156/194/203 | 156/198/203 | 156/202/205 | 156/204/205 | 172/194/203 | 173/206/203 | 176/192/203 | 176/194/203 | 178/194/203 |
| ACNCSVI | 1 | 1 | 1 | – | – | 2 | 1 | 2 | – | – |
| ACNCNVI | – | – | – | – | – | – | – | – | – | 2 |
| ACNRNVI | | 2 | | 64 | 1 | | | | 20 | 2 |

[a] Size (bp) of microsatellite markers (−0.1 kb/−3.87 kb/+1.48 kb flanking *dhfr*).

Six microsatellite haplotypes were observed in eight isolates harboring wild *dhfr* allele. In contrast, among the 89 isolates harboring ACNRNVI allele, the microsatellite polymorphisms flanking *dhfr* were considerably restricted. Only two distinct haplotypes were predominant with the respective prevalences of 72% (156/202/205 haplotype) and 22% (176/194/203 haplotype). Among the remaining three haplotypes, two (156/194/203; $n = 2$ and 156/204/205; $n = 1$) were similar to the 156/202/205 haplotype, and one (178/194/203; $n = 2$) was similar to the 176/194/203 haplotype, suggesting that the three minor microsatellite haplotypes evolved from the two distinct major haplotypes (156/202/205 and 176/194/203) in the event of recombination.

Among the six isolates harboring the "*dhfr/dhps* quartet mutations", five isolates shared a same microsatellite haplotype but one is different, suggesting that the origins of the *dhfr/dhps* quartet mutant was not single in Papua New Guinea.

## 4. Discussion

In 2000, Papua New Guinea officially introduced the combination of SP with 4-aminoquinoline as a new treatment policy for uncomplicated malaria. Previously, SP had only been used in combination with quinine for severe malaria or after a first-line 4-aminoquinoline therapy has failed.

In the present study, a significant decrease of the *dhfr* wild allele was observed from 2002 to 2003 in the Wewak region, East Sepik province, 2–3 years after the change of the treatment policy. In addition, our observed *dhfr* wild allele prevalences (15% in 2002 and 3% in 2003) were considerably lower than that observed in the adjacent area of East Sepik province called the Wosera region (57%, 1990–1993) (Reeder et al., 1996).

There is a possibility that the observed pattern of increased SP resistance in 2003 results from the isolates come from a unique or from a little number of clusters of related malaria cases. However, the population of our study site in East Sepik is large, estimated to be several thousands of people. Records of patient's habitation demonstrated that malaria positive cases were fairly distributed over the 61 areas and the rate of related malaria cases were considerably low (5%). Thus, the possibility that the *P. falciparum* isolates in our study were mutually dependent would be excluded.

Our results suggest that reduced sensitivity to SP has rapidly developed in Papua New Guinea after the introduction of SP, even when combined with 4-aminoquinoline. In Uganda, rapid selection of *dhfr* mutations at position 59 and 108 was similarly reported after the introduction of SP in combination with previously used chloroquine (Sendagire et al., 2005).

The fact that the decrease of the *dhfr* wild allele was observed 2 and 3 years after the adoption of the new drug policy in 2000 is interesting. We could probably explain this phenomenon as follows: first, although the drug policy was officially changed in 2000, this policy amendment was not fully implemented till 2001 in our study site. Second, some patients tended to use self-medication (commonly 4-aminoquinoline alone) when they had a fever because of the difficult accessibility to the clinics. Third, the isolates harboring a mutant ACNRNVI allele has shown low to moderate levels of in vitro resistance to pyrimethamine (Peterson et al., 1988), which appears to be associated with a slower selection of this allele in the presence of SP.

The effect of SP as a monotherapy has previously been reported from several endemic regions. In Killifi, Kenya, significant decrease of in vitro $IC_{50}$ values to pyrimethamine was observed in *P. falciparum* field isolates several years after the introduction of SP monotherapy (Mberu et al., 2000). In KwaZulu-Natal province, South Africa, the prevalences of the resistant allele, *dhfr* triple mutant (ACIRNVI), increased from 22% to 38% in 7 and 11 years after SP monotherapy became a first-line treatment (Roper et al., 2003). Our data suggests that a combination therapy in Papua New Guinea did not prevent the development of SP resistance. We believe that the result was mainly because of the already compromised efficacy of this 4-aminoquinoline partner drug. In the study area, in vitro resistance of *P. falciparum* isolates to chloroquine is high (82%), with 93% prevalence of *dhrf* K76T mutation (Mita et al., in press). The in vitro resistance to amodiaquine is similarly high (100%) (data not shown).

The microsatellite variation flanking *dhfr* was restricted in isolates harboring *dhfr* double mutant from Papua New Guinea, producing two distinct microsatellite haplotypes. Reduced microsatellite diversity around *dhfr* indicates strong selection of resistant isolates in the presence of SP (Pearce et al., 2005). The increased prevalence of the *dhfr* double mutant would thus be a result of the selection of this allele rather than frequent mutations in the parasite populations in the presence of pyrimethamine drug. This is consistent with the previous findings in South America (Cortese et al., 2002), Southeast Asia (Nair et al., 2003), and South Africa (Roper et al., 2003).

We also obtained evidence for the emergence of a mutant *dhps* SGEAA allele in 2003, which has not been reported before in Papua New Guinea. In a previous study from Madang and Maprik Provinces in 2000 and 2001, all the 187 isolates harbored wild *dhps* allele (Casey et al., 2004). High prevalences of the mutant *dhps* allele have been reported in other geographic areas like in Thailand (78%) (Ngo et al., 2003) and Vietnam (100%) (Biswas et al., 2000) where a high level of SP resistance has been documented. Also in South Africa, the escalation of SP resistance in endemic regions has been reported to occur concomitantly with the emergence of *dhps* SGEAA allele (Roper et al., 2003). In our study, all six isolates with *dhps* SGEAA mutant allele also harbored *dhfr* ACNRNVI allele, i.e., *dhfr/dhps* 'quartet' mutant haplotype. The *dhfr/dhps* 'quartet' mutant isolates have been associated with high rates of RII/RIII resistance to SP, e.g. 83% in Indonesian Papua as compared to 0% in *dhfr/dhps* 'double' mutant (ACNRNVI + - SAKAA) (Nagesha et al., 2001). Similarly, in Uganda, two mutations at positions 59 and 108 in *dhfr* combined with one at position 540 in *dhps* significantly increased the risk for SP parasitological failure (Talisuna et al., 2004b).

In conclusion, we observed a significant decrease in the prevalence of wild *dhfr* allele in Papua New Guinea with the introduction of SP combined with the 4-aminoquinoline in 2000. We also found the higher resistant genotype in 2003, *dhfr/*

*dhps* 'quartet' mutant, which has not been reported in Papua New Guinea before the drug policy change. Thus, we argue that the combination therapy introduced to prevent the selection of *dhfr/dhps* resistant alleles in 2000 was not as effective as the underlying drug policy had anticipated.

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# Independent Evolution of Pyrimethamine Resistance in *Plasmodium falciparum* Isolates in Melanesia[▽]

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**Pyrimethamine resistance in *Plasmodium falciparum* has previously been shown to have emerged once in Southeast Asia, from where it spread to Africa. Pyrimethamine resistance in this parasite is known to be conferred by mutations in the gene encoding dihydrofolate reductase (*dhfr*). We have analyzed polymorphisms in *dhfr* as well as microsatellite haplotypes flanking this gene in a total of 285 isolates from different regions of Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Nearly all isolates (92%) in Melanesia were shown to carry a *dhfr* double mutation (CNRNI [underlining indicates the mutation]) at positions 50, 51, 59, 108, and 164, whereas 98% of Southeast Asian isolates were either triple (CIRNI) or quadruple (CIRNL) mutants. Microsatellite analysis revealed two distinct lineages of *dhfr* double mutants in Melanesia. One lineage had the same microsatellite haplotype as that previously reported for Southeast Asia and Africa, suggesting the spread of this allele to Melanesia from Southeast Asia. The other lineage had a unique, previously undescribed microsatellite haplotype, indicative of the de novo emergence of pyrimethamine resistance in Melanesia.**

Malaria is a major cause of morbidity and mortality in large areas of the tropical world. The antifolate drug sulfadoxine-pyrimethamine (SP) has been widely used to treat uncomplicated malaria, mainly as a monotherapy, but also in combination with other antimalarial drugs in most regions of endemicity for malaria in the world.

Pyrimethamine and sulfadoxine inhibit two separate enzymes in the folate synthesis pathway of *Plasmodium falciparum*: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. Point mutations at amino acid positions 16, 50, 51, 59, 108, and 164 in the DHFR gene (*dhfr*) are the major causes of resistance to pyrimethamine (3, 17, 18, 24). The mutation at position 108 (Ser→Asn) appears to be an initial prerequisite for a significant (10-fold) increase in in vitro resistance (24). Additional mutations at other amino acid positions within the gene are associated with stepwise increases in resistance. Isolates harboring four mutations at positions 51, 59, 108, and 164 (CIRNL at positions 50, 51, 59, 108, and 164 [mutations are indicated by underlining]) show the highest pyrimethamine resistance so far described.

Various *dhfr* alleles have been observed in regions of endemicity (30). A *dhfr* triple mutant (CIRNI) represents the most common type in Africa and Southeast Asia, while the *dhfr* quadruple mutant (CIRNL) is observed predominantly in Thailand and some other regions in Southeast Asia where SP resistance is very high (1, 12, 30). Two distinct triple *dhfr* mutant genotypes (RICNI and CICNL) are prevalent in South America (2, 18). A five-amino-acid insertion after position 30, termed the Bolivia repeat, is also exclusive to South America, suggesting two unique and different evolutionary origins of pyrimethamine resistance in South America (2).

The migration of drug-resistant alleles can be traced by the analysis of microsatellite markers closely linked to the gene conferring resistance. Microsatellite analysis flanking *pfcrt* has revealed that chloroquine resistance evolved independently in at least four different regions: Southeast Asia, two regions in South America, and New Guinea (31). Meanwhile, all *dhfr* triple (CIRNI) and quadruple (CIRNL) mutants from Southeast countries displayed the same or nearly identical microsatellite haplotypes flanking *dhfr* (12). Strikingly, pyrimethamine-resistant isolates in Africa also harbored microsatellite haplotypes identical to those found in Southeast Asia (21), suggesting a single origin of pyrimethamine resistance in Southeast Asia, which subsequently spread to Africa. However, whether the Melanesian *dhfr* mutants originated in Southeast Asia or arose independently remains unclear.

In the present study, we determined *dhfr* and microsatellite haplotypes flanking the gene in *P. falciparum* isolates from Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Our

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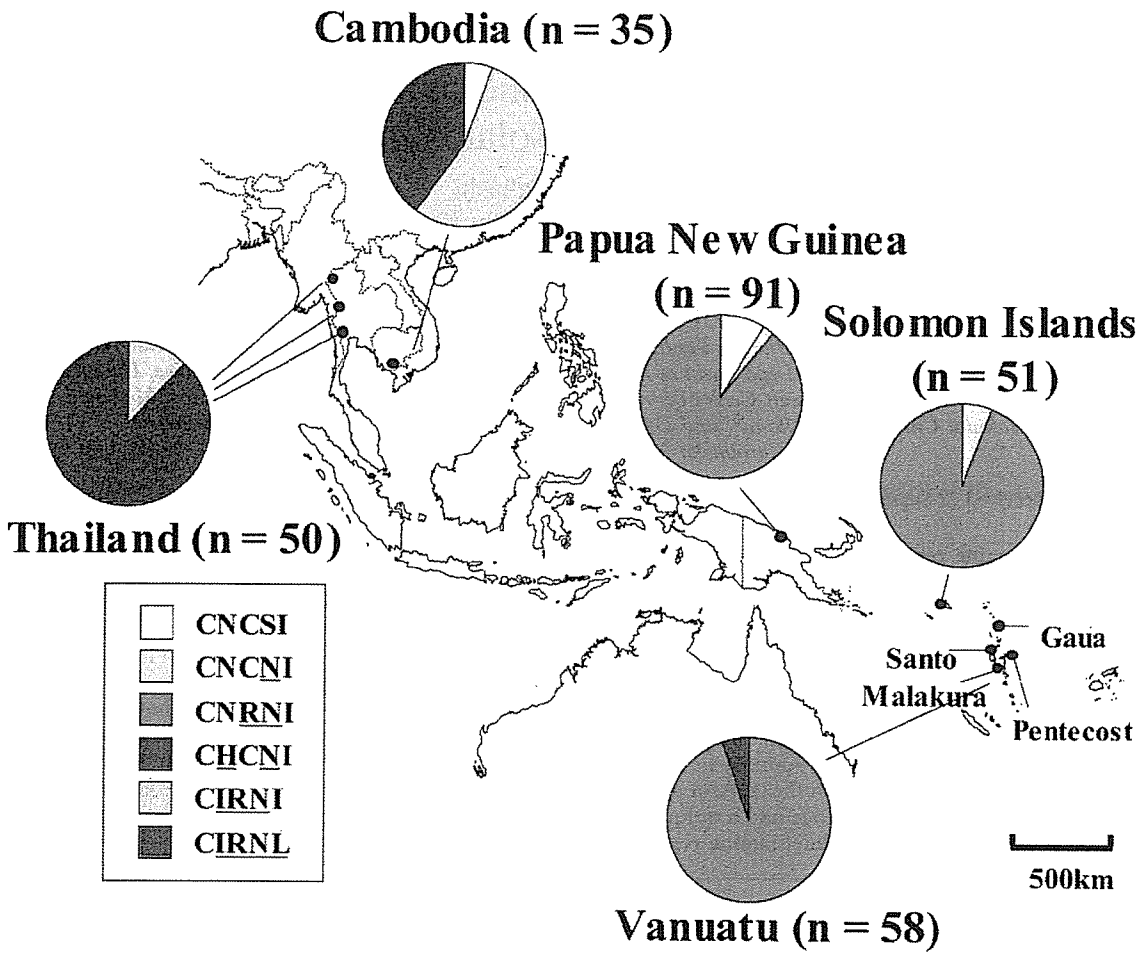


FIG. 1. Frequency of *dhfr* genotypes in *P. falciparum* isolates from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand.

results show two major lineages of pyrimethamine resistance in Melanesia. One has apparently originated in Melanesia, while the other originated in Southeast Asia and spread to Africa and Melanesia. This is the first unambiguous demonstration of the unique evolution of *P. falciparum* pyrimethamine resistance in Melanesia.

## MATERIALS AND METHODS

**Study site and patients.** Blood samples were obtained from *P. falciparum*-infected patients living in five countries where malaria is endemic: (i) Papua New Guinea, where isolates were from finger-prick blood samples taken during in vitro studies at a town clinic in Wewak, East Sepik Province, in November of both 2002 and 2003 (9, 10); (ii) Solomon Islands, where isolates from venous-blood samples were taken as part of a cross-sectional survey of three villages located in the northwestern part of Guadalcanal in 1995 and 1996 (23); (iii) Vanuatu, where isolates were from finger-prick blood samples obtained during a cross-sectional survey of rural villages and primary schools from February to March 1996 to 1998 in four islands, Gaua, Santo, Pentecost, and Malakula (22); (iv) Cambodia, where isolates were obtained from finger-prick blood samples taken during a cross-sectional survey of rural villages in Chumkiri, Kampot Province, in December 2004; (v) Thailand, where isolates were obtained from pretreatment venous blood samples taken during in vitro studies at a town clinic located at the western border of Tak, Kanchanaburi and Ratchaburi provinces, from 2001 to 2002.

**DNA preparation.** Finger-prick blood (75 μl) was spotted onto chromatography filter paper ET31CHR (Whatman Limited, Kent, United Kingdom). Venous blood was transferred into heparin-containing test tubes. Parasite DNA was purified using a QIAamp DNA blood mini kit (QIAGEN, Germany) according to the manufacturer's instructions with some modifications (22).

***dhfr* genotyping.** *dhfr* was amplified by PCR, and amplified products were directly sequenced with a BigDye Terminator 1.1 cycle sequencing kit in the ABI 377 DNA sequencer (GE Healthcare UK Ltd., Buckinghamshire, England) as previously reported (10, 19).

**Microsatellite haplotyping.** In order to determine the evolutionary history of pyrimethamine-resistant alleles of *dhfr*, we measured variation in the number of TA repeats at six microsatellite loci closely linked to the gene. These were located on chromosome 4, 0.1, 3.87, and 4.49 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr*. In some cases, in order to estimate the limit of genetic hitchhiking, which is defined as a valley of reduced variation around *dhfr*, an additional six-microsatellite markers were analyzed at 7.55, 29.61, 57.68, and 363.33 kb upstream and 30.31 and 299.72 kb downstream of *dhfr*. Polymorphisms in these microsatellite markers were determined as previously described (12). Briefly, seminested PCR was performed using fluorescent end-labeled primers. Size variations in the amplified products were determined by electrophoresis on an ABI 377 sequencer and analyzed with GeneScan software (GE Healthcare UK Ltd.). Samples with two or more peaks at the same locus in the electropherogram were considered to be mixed infections and were excluded from further analysis.

Polymorphism between microsatellite markers is measured as variation in nucleotide length derived from various numbers of TA repeats. Microsatellite haplotypes harboring an association of bp 200-194-176-106-203-111 at microsatellite positions 4.49, 3.87, and 0.1 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr* were designated "SEA" haplotypes, and those harboring an association of bp 220-202-156-100-205-111 were designated "Melanesia" haplotypes. Microsatellite haplotypes showing slight differences at one or two microsatellite markers from the SEA haplotype, e.g., at bp 204-, 200-176-106-203-111

TABLE 1. Microsatellite polymorphisms in 15 *P. falciparum* isolates with wild-type *dhfr* or single-mutant *dhfr*

| Isolate | Country[a] | Size (bp) of microsatellite marker at indicated position (kb) | | | | | |
|---|---|---|---|---|---|---|---|
| | | −4.49 | −3.87 | −0.1 | +0.52 | +1.48 | +5.87 |
| CNCSI | Cambodia | 198 | 206 | 156 | 94 | 203 | 105 |
| ($n$ = 10) | Cambodia | 198 | 206 | 156 | 94 | 203 | 105 |
| | PNG | 202 | 196 | 156 | 94 | 203 | 121 |
| | PNG | 214 | 198 | 156 | 94 | 203 | 123 |
| | PNG | 202 | 192 | 156 | 96 | 203 | 115 |
| | PNG | 204 | 194 | 172 | 96 | 203 | 103 |
| | PNG | 204 | 194 | 172 | 92 | 203 | 103 |
| | PNG | 204 | 206 | 172 | 100 | 203 | 111 |
| | PNG | 202 | 192 | 176 | 96 | 203 | 115 |
| | PNG | 202 | 192 | 176 | 96 | 203 | 115 |
| CNCNI | Solomon | 210 | 194 | 172 | 96 | 203 | 113 |
| ($n$ = 5) | Solomon | 204 | 208 | 176 | 94 | 203 | 120 |
| | Solomon | 204 | 208 | 176 | 94 | 203 | 120 |
| | PNG | 210 | 194 | 178 | 102 | 203 | 113 |
| | PNG | 210 | 194 | 178 | 102 | 203 | 113 |

[a] PNG, Papua New Guinea; Solomon, Solomon Islands.

(underlining indicates the differences), were considered SEA variation haplotypes. Similarly, haplotypes displaying minor variation at one or two markers from the Melanesia haplotype, e.g., bp 220-202-156-100-205-113, were considered Melanesia variation haplotypes. Isolates showing mixed *dhfr* genotypes and/or microsatellite haplotypes were excluded from analysis.

**Statistical analysis.** We calculated the expected heterozygosity ($h$) at each microsatellite locus as $h = [n/(n-1)][1 - \Sigma p_i^2]$, where $n$ is the number of infections sampled and $p_i^2$ is the frequency of the $i^{th}$ allele. The sampling variance of $h$ was calculated according to the following formula (23), a slight modification of the standard diploid variance (13), $[2/n(n-1)]\{2(n-2)[\Sigma pi^3 - (\Sigma pi^2)^2] + \Sigma pi^2 - (\Sigma pi^2)^2\}$. A $P$ value of <0.05 was considered statistically significant.

**Nucleotide sequence accession number.** The complete sequence of the allele identified has been submitted to the DDBJ and assigned accession number AB271908.

## RESULTS

***dhfr* genotypes.** Among a total of 314 samples, 29 (9%) had multiple *dhfr* alleles and/or were of mixed microsatellite haplotypes and so were excluded from this analysis. *dhfr* allele types and flanking microsatellite haplotypes were thus determined for 285 isolates (91 from Papua New Guinea, 51 from the Solomon Islands, 58 from Vanuatu, 35 from Cambodia, and 50 from Thailand). The frequencies of *dhfr* genotypes differed considerably between Southeast Asia and Melanesia (Fig. 1). In Southeast Asia, nearly all parasites (98%) carried either triple (CIRNI at positions 50, 51, 59, 108, and 164) or quadruple (CIRNL) mutations at *dhfr*. In Cambodia, triple and quadruple mutants were near equally prevalent. In Thailand, 85% of isolates were quadruple mutants. In Melanesia, nearly all isolates (92%) harbored a *dhfr* double mutation (CNRNI). The pyrimethamine-sensitive, wild-type allele (CNCSI) was found in only Papua New Guinea and at relatively low prevalence (8%). Neither the triple (CIRNI) nor the quadruple (CIRNL) mutant was found in Melanesia. A unique CHCNI allele was observed in three isolates from Gaua Island, Vanuatu.

**Polymorphism in microsatellite markers flanking *dhfr*.** The polymorphisms in six microsatellite markers flanking *dhfr* (−4.49 to 5.87 kb) from wild-type ($n$ = 10) or single-mutant ($n$ = 5) isolates are shown in Table 1. For those parasites carrying pyrimethamine-sensitive, wild-type alleles of *dhfr*, microsatellite markers were highly polymorphic. In contrast, *dhfr* double-mutant isolates ($n$ = 184) showed remarkably little diversity at all loci (Fig. 2). Similarly, nearly all triple ($n$ = 25) and quadruple ($n$ = 58) mutants displayed limited microsatellite polymorphism at each locus (Fig. 2). The expected heterozygosity ($h$) at each microsatellite marker is given in Table 2. In isolates carrying wild-type or single-mutant *dhfr* alleles, $h$ was high (0.60 to 0.89) at all six loci located between 4.49 kb upstream and 5.87 kb down-

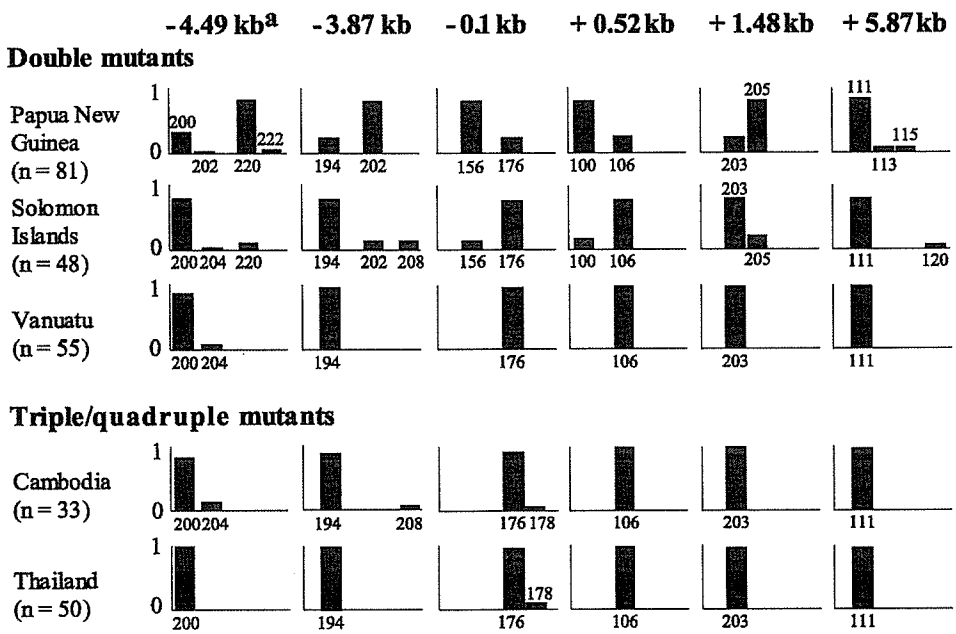


FIG. 2. Repeat length variations of six microsatellite markers flanking *dhfr* in *P. falciparum* isolates with *dhfr* double (CNRNI) and triple (CIRNI)/quadruple (CIRNL) mutants. $x$ axes, size (bp) of microsatellite markers. $y$ axes, frequency of microsatellite alleles. [a], location of microsatellite marker (distance from *dhfr*).

TABLE 2. Expected heterozygosity of microsatellite markers in *P. falciparum* isolates

| Isolate | No. of isolates | *h* of microsatellite marker at indicated position (kb) | | | | | | No. of haplotypes |
|---|---|---|---|---|---|---|---|---|
| | | −4.49 | −3.87 | −0.1 | +0.52 | +1.48 | +5.87 | |
| CNCSI | 10 | 0.78 | 0.84 | 0.69 | 0.71 | 0 | 0.89 | 8 |
| CNCNI | 5 | 0.60 | 0.60 | 0.80 | 0.80 | 0 | 0.60 | 5 |
| CNRNI | 184 | 0.54 | 0.49 | 0.48 | 0.49 | 0.48 | 0.17 | 2[a] |
| CIRNI | 25 | 0.28 | 0.22 | 0.08 | 0 | 0 | 0 | 1[a] |
| CIRNL | 58 | 0 | 0 | 0.10 | 0 | 0 | 0 | 1[a] |

[a] Number of major haplotypes.

stream of *dhfr*, except at the monomorphic +1.48-kb locus. In contrast, those isolates carrying the triple or quadruple mutations at *dhfr* had very low *h* values (0 to 0.28) at all microsatellite loci, indicating limited diversity in those isolates. Isolates carrying double mutations at *dhfr* had intermediate values of *h* (0.48 to 0.54) lying somewhere between those of the wild-type/single mutants and triple/quadruple mutants.

**Microsatellite haplotypes.** Different microsatellite haplotypes were found in isolates carrying wild-type *dhfr* and in those carrying single mutations; 8 haplotypes were found in 10 wild-type *dhfr* isolates, and 3 haplotypes were found in 5 single mutants (Table 1). In contrast, only two distinct microsatellite haplotypes (SEA/SEA variation and Melanesia/Melanesia variation) were observed in a total of 184 *dhfr* double-mutant isolates (Fig. 3). Identical or very similar haplotypes (SEA/SEA variation) were found in all *dhfr* triple or quadruple mutation-carrying isolates ($n$ = 83), suggesting that *dhfr* triple and quadruple mutants evolved directly from the *dhfr* double mutant.

In Southeast Asia, only SEA/SEA variation haplotypes were observed. These haplotypes were also predominant in Melanesian countries, except Papua New Guinea, where 78% of isolates were of the Melanesia/Melanesia variation haplotypes. In Vanuatu, all isolates showed SEA/SEA variation haplotypes. One isolate carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111) was observed in the Solomon Islands.

**Genetic hitchhiking in *dhfr* double-mutant parasites from Papua New Guinea.** These results suggest that the *dhfr* double mutants present today in Melanesia emerged independently in Southeast Asia and Melanesia. To determine the history of these two lineages, we measured the extent of genetic hitchhiking, which is determined by the distance of reduced microsatellite variation around *dhfr*. For this purpose, the variance

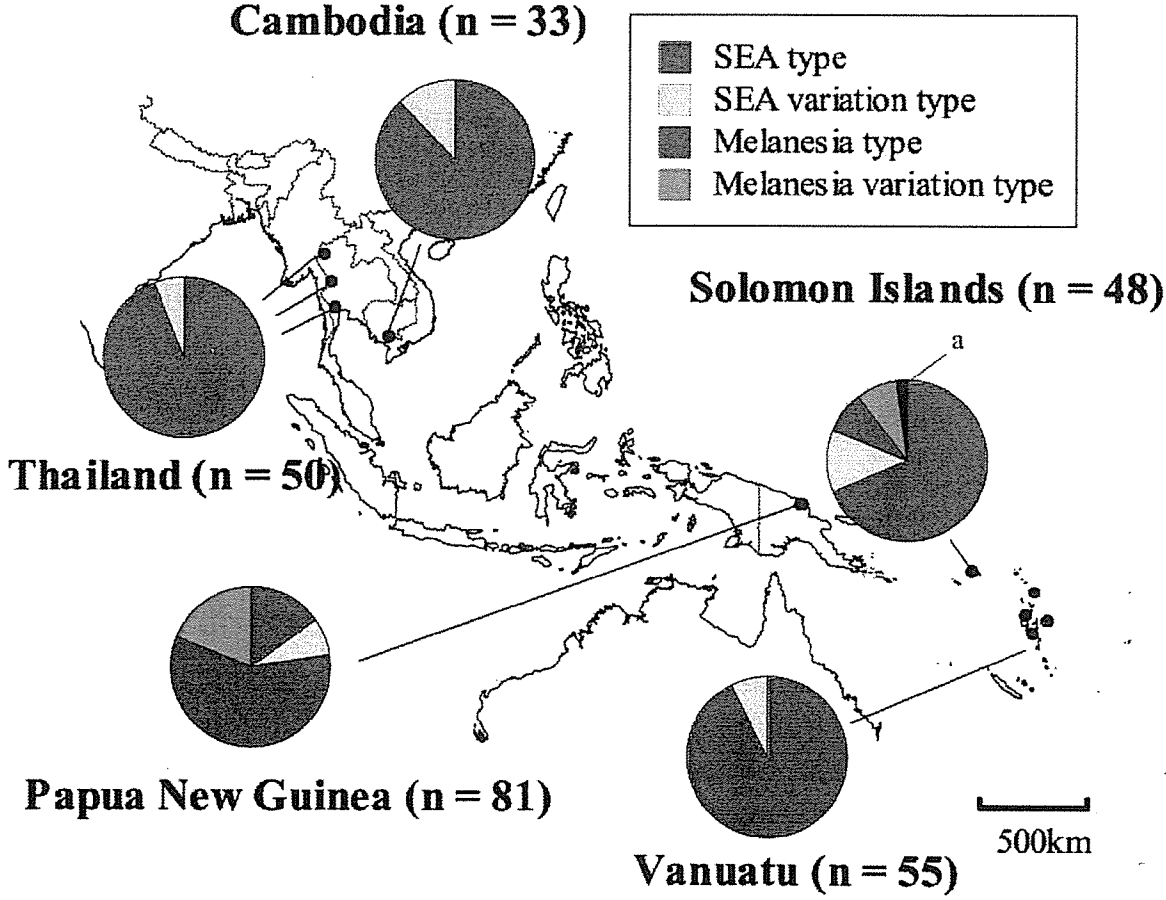


FIG. 3. Frequency of microsatellite haplotype flanking *dhfr* in *P. falciparum* isolates with *dhfr* double, triple, or quadruple mutants from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand. [a], isolate ($n$ = 1) carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111).

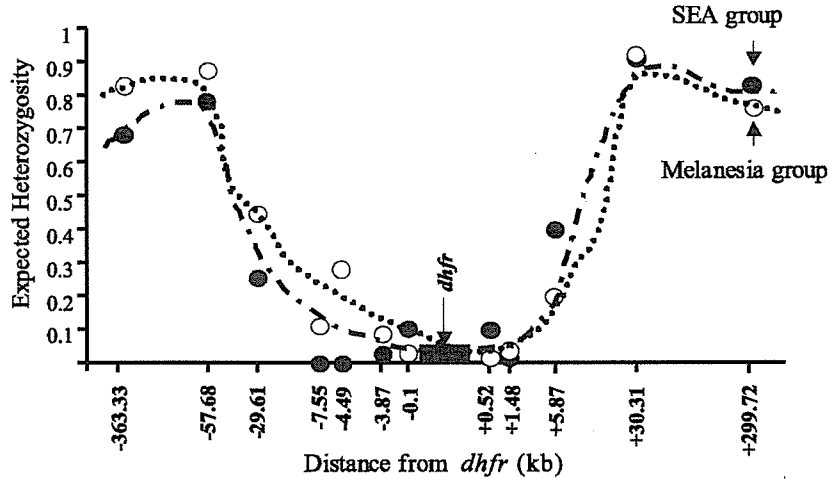


FIG. 4. Reduced microsatellite polymorphism near *dhfr* in *P. falciparum* isolates with CNRNI genotype from Papua New Guinea.

of *h* at 12 microsatellite markers spanning 363.33 kb upstream to 299.72 kb downstream of *dhfr* was measured for Papua New Guinean isolates with a *dhfr* double mutant displaying a SEA/SEA variation haplotype ($n$ = 17) and those displaying the Melanesia/Melanesia variation haplotype ($n$ = 64) (Fig. 4). The patterns of genetic hitchhiking in both haplotypes were similar within a distance of 58 kb upstream and 30 kb downstream of *dhfr*. These results suggest that these two lineages, both carrying the same point mutations (CNRNI), appeared coincidentally in Papua New Guinea.

## DISCUSSION

This study clearly shows that pyrimethamine-resistant *P. falciparum* evolved independently in Melanesia. It has previously been shown that a single lineage of pyrimethamine-resistant parasites arose in Southeast Asia, and subsequently spread to Africa (21). Pyrimethamine-resistant parasites from South America, which show *dhfr* genotypes different from those of other geographic areas, independently evolved in two foci within South America (2). Thus, there are at least four distinct independent origins of *dhfr* resistance presently identified. This is similar to the situation with chloroquine resistance, which has also been reported to have arisen independently a total of four times, once in Southeast Asia, twice in South America, and once in Melanesia (31).

A recent study has reported multiple origins of *dhfr* resistance within Kenya (6). However, care must be taken when basing conclusions about the origins of drug resistance on microsatellite variation from areas of high endemicity, such as Kenya. Two factors are likely to affect microsatellite polymorphism in areas of intense transmission. First, new microsatellite haplotypes are easily generated by meiotic recombination because of a high recombination rate and high prevalence of mixed-haplotype infections. Second, interallelic recombination within a microsatellite may generate new microsatellite alleles by an unequal crossing-over mechanism. Indeed, in a study by Roper et al. (21), nonuniform microsatellite haplotypes were noticeable around *dhfr* in pyrimethamine-resistant African isolates. These factors may be less important in areas of low transmission, such as Southeast Asia, and so do not compromise the conclusions of the present study.

Genetic hitchhiking reduces the expected heterozygosity of microsatellite markers around a selected gene, resulting in a valley of reduced variation. However, this association is easily broken down by recombination, resulting in a narrowing of the selection valley as the number of generations increases. In this study, we compared the selection valleys around the *dhfr* gene in two *dhfr* double mutants carrying SEA and Melanesia microsatellite haplotypes from Papua New Guinea. In both haplotypes, the microsatellite patterns within the valley were very similar from a distance of 58 kb upstream to 30 kb downstream of *dhfr*. The size of a selection valley is determined by several different parameters: the strength of the selection pressure on the mutant allele, the frequency of recombination, the transmission intensity, and the number of parasite generations since the emergence of the selected allele (16). In this analysis, these four parameters could be considered equal because all isolates were sampled from the same area. Thus, these results indicate that two ancestors of the *dhfr* double mutant in Papua New Guinea emerged coincidentally: one came from Southeast Asia, and the other arose independently within Melanesia. Although the appearance of the two resistant lineages emerged nearly simultaneously, we consider that the Melanesian-resistant type might have appeared slightly earlier than the influx of the SEA-resistant type. This is because if the SEA-resistant type migrated to Papua New Guinea earlier, it would have swept away microsatellite polymorphisms linked to the wild *dhfr*. Therefore, the possibility that a novel *dhfr*-resistant type having distinct microsatellite haplotypes appeared soon after the sweep in Papua New Guinea seems very unlikely.

The way drugs are used within regions of endemicity affects the generation and selection of resistant alleles. SP was widely used in Thailand and Cambodia during the 1970s and 1980s as the first-line treatment for uncomplicated malaria. In Melanesian countries, SP was introduced as a first-line treatment during the mid-1990s. Up until this time, pyrimethamine monotherapy was infrequently used for the treatment of malaria and other infections. It is difficult, therefore, to explain the nearly simultaneous emergence of pyrimethamine-resistant parasites

in this area. It is, however, possible to attribute the emergence and spread of the triple and quadruple *dhfr* mutants to the widespread use of SP in Southeast Asia during the 1970s and 1980s.

We consider that weak and persistent pyrimethamine pressure by medicated salt projects, a form of mass drug administration, may explain the first selection of pyrimethamine-resistant parasites (15, 25). In the late 1950s and early 1960s, medicated salt projects were carried out in four different endemic regions: Indonesian Papua, the Thailand-Cambodia border, Ghana, and Iran (15, 25). In Southeast Asia and Melanesia, this project was carried out from 1959 to 1962 and from 1960 to 1961, respectively. Pyrimethamine resistance in *P. falciparum* was reported within 3 months of the start of the project (1960) in Indonesian Papua (7). At the Thailand-Cambodia border, resistance also developed quickly and pyrimethamine was replaced by chloroquine beginning in 1961. A large number of people treated with subcurative doses of antimalarial drugs present ideal conditions for the emergence of drug resistance (4, 27, 28). This, combined with the long half-life of pyrimethamine (116 h) (29), would have facilitated the emergence of drug resistance within the areas covered by the medicated salt project: Indonesian Papua and the Thailand-Cambodia border.

The *dhfr* double mutant (CNRNI), which confers moderate resistance to pyrimethamine, is widely distributed in Africa, West Asia, and Melanesia. However, whether this mutant is regularly selected de novo or whether it has spread from a limited number of foci of emergence is not known. In the present study, only two distinct microsatellite haplotypes (SEA and Melanesia haplotypes) were observed in a total of 184 *P. falciparum* isolates harboring the *dhfr* double mutant from Papua New Guinea, Vanuatu, and the Solomon Islands, suggesting that the generation of two mutations at positions 59 and 108 in *dhfr* is not frequent. In laboratory isolates, key point mutations in *dhfr* have occurred at frequencies as high as $2.5 \times 10^{-9}$ per parasite replication, which predicts the generation of one mutant parasite in every malaria patient, assuming the number of parasites to be $10^{10}$ to $10^{12}$ in every infection (14). Consistently, the expected heterozygosities at microsatellite markers around *dhfr* were comparable between the wild-type and single *dhfr* mutant parasites. Thus, the initial mutation at position 108 in *dhfr* may occur relatively frequently (12, 20), but the generation and selection of an additional mutation at position 59 appear to be considerably less frequent. Mutations that render pathogens resistant to drug treatment are often associated with a loss of fitness (8, 11, 26). Resistant mutants may themselves develop compensatory mutations, which could then allow them to grow and survive in competition with wild-type forms (5, 26). The discrepancy between the frequent generation of the mutation at position 108 in *dhfr* and the rare occurrence of the *dhfr* double mutant as observed in this study may thus be reconciled by the requirement of complex compensatory mutations in a locus other than *dhfr* for restoring parasite fitness in natural populations.

In the present study, our samples of Papua New Guinea and Thailand were from individuals with clinical malaria, while samples from other sites were from cross-sectional studies of asymptomatic individuals. Symptomatic patients usually have higher parasite densities than do asymptomatic individuals. Thus, we cannot exclude the possibility that prevalences of microsatellite haplotypes may differ between isolates that cause disease and those that do not produce symptoms. However, we do not consider it very likely because there was no significant difference in the frequency distribution of genotypes of an antigen gene (*msp1*) between clinical patients and asymptomatic individuals in Melanesia (23).

In conclusion, this study provides strong evidence for the unique and independent origin of pyrimethamine resistance in Melanesia. The *dhfr* mutant, perhaps emerging from West Papua, has the same double mutations found in other geographic areas but distinct microsatellite haplotypes flanking the gene. Our results also show that the generation of double mutants with mutations at positions 59 and 108 of *dhfr* is a rare event, and this double mutation may be a first rate-determining step for the stable persistence of pyrimethamine resistance in *P. falciparum*.

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# A comparative study of the genetic diversity of the 42 kDa fragment of the merozoite surface protein 1 in *Plasmodium falciparum* and *P. vivax*

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**Abstract**

We investigated the genetic diversity of the 42 kDa fragment of the merozoite surface protein 1 (MSP-1) antigen in *Plasmodium falciparum* and *P. vivax*, as well as in non-human primate malarial parasites. This fragment undergoes a proteolytic cleavage generating two fragments of 19 kDa (MSP-$1_{19}$) and 33 kDa (MSP-$1_{33}$) that are critical in erythrocyte invasion. We found that overall the MSP-$1_{33}$ fragment exhibits greater genetic diversity than the MSP-$1_{19}$ regardless of the species. We have found evidence for positive natural selection only in the human malaria parasites by comparing the rate of non-synonymous versus synonymous substitutions. In addition, we found clear differences between the two major human malaria parasites. In the case of *P. falciparum*, positive natural selection is acting on the MSP-$1_{19}$ region while the MSP-$1_{33}$ is neutral or under purifying selection. The opposite pattern was observed in *P. vivax*. Our results suggest different roles of this antigen in the host–parasite immune interaction in each of the major human malarial parasites.


*Keywords:* Malaria; Merozoite; MSP-1; MSP-1 19 kDa; Genetic diversity; Vaccine; *Plasmodium*

## 1. Introduction

The malaria burden is particularly high in sub-Saharan Africa where *Plasmodium falciparum* is predominant. However, malaria "out of Africa" is characterized by the presence of *P. vivax*, the second most important malaria parasite in terms of its morbidity. Although there are clear biological and genetic differences between these two parasites (Coatney et al., 1971), they overlap in their geographic distribution and there is increasing evidence for their interaction (Snounou and White, 2004).

Among the antigens currently under consideration in malaria vaccine formulations, one of the most promising candidates is the major merozoite surface protein 1 (MSP-1) (Good et al., 2004). The MSP-1 antigen is expressed as a large protein of 190–200 kDa on the parasite surface (Holder and Freeman, 1982). This precursor undergoes two steps of proteolytic cleavage during the merozoite maturation. First, it is cleaved into four major fragments of 83, 30, 38 and 42 kDa (further referred to as MSP-$1_{83}$, MSP-$1_{30}$, MSP-$1_{38}$, and MSP-$1_{42}$) then, before erythrocyte invasion the MSP-$1_{42}$ fragment undergoes a second cleavage resulting in the generation of 33 and 19 kDa (MSP-$1_{33}$ and MSP-$1_{19}$) fragments where the latter remain on the merozoite surface during invasion.

*Plasmodium* spp. MSP-1 exhibits extensive genetic polymorphism (Tanabe et al., 1987; Putaporntip et al., 2002) that appears to be maintained by positive natural selection in *P. falciparum* (Hughes, 1992; Escalante et al., 1998; Conway et al.,

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2000) and *P. vivax* (Putaporntip et al., 2006). Similar observations have been made about other malarial vaccine antigens (see Escalante et al., 2004) on which the host immune system is considered the driving selective force that allows for the accumulation and frequent switch of suitable mutations in the parasite population. Under this scenario, mutations are maintained longer in the parasite population than expected if genetic drift were the sole process acting on the genetic polymorphism.

The conclusion that positive selection maintains the genetic diversity of genes encoding malarial antigens is supported, among others lines of evidence, by the observation in *P. falciparum* that non-synonymous nucleotide substitutions (those that change the amino acid) are more common than synonymous substitutions (mutations that do not change the amino acid) (Hughes and Hughes, 1995; Escalante et al., 1998, 2004). Since natural selection acts on phenotypic differences, an excess of non-synonymous substitutions over synonymous is considered evidence that natural selection is favoring the maintenance of genetic polymorphism.

In the case of *Plasmodium* spp. MSP-1, most of the genetic diversity analyses have subdivided the gene into blocks (segments) based on their level of genetic diversity but not using any other biological criteria (Tanabe et al., 1987; Putaporntip et al., 2002, 2006); however, few studies have been done considering the proteolytic fragments as functional units (Escalante et al., 1998).

The MSP-$1_{42}$ and MSP-$1_{19}$ fragments have received special attention in *P. falciparum* as part of vaccine formulations given that they are relatively conserved and antibodies against these fragments inhibit the parasite invasion into the red blood cells (Yang et al., 1999; Stanisic et al., 2004). In addition, the critical role of the MSP-$1_{19}$ fragment in the erythrocyte invasion is conserved even among distantly related species (O'Donnell et al., 2000).

An important characteristic of *P. vivax* is that it invades reticulocytes, a process that is mediated by specific proteins such as the reticulocyte binding proteins and Duffy receptor (Galinski et al., 1992; Chitnis and Miller, 1994). However, MSP-1 in *P. vivax* also appears to play an important role in this process (Rodríguez et al., 2002; Espinosa et al., 2003; Han et al., 2004; Sachdeva et al., 2004). Indeed, peptides with high specific binding activity (HSBA) to reticulocytes have been found in the MSP133 (Espinosa et al., 2003; Rodríguez et al., 2002).

This investigation aims to compare the genetic diversity of the MSP-$1_{42}$ in *Plasmodium* spp. focusing on *P. falciparum* and *P. vivax*. We have analyzed 120 sequences of the MSP-$1_{42}$ of *P. falciparum* and 75 sequences of the homologous region in *P. vivax*, and we have explored the genetic diversity of the MSP-$1_{33}$ and MSP-$1_{19}$ fragments. In the case of *P. vivax*, we further explored its genetic diversity by comparing it with the homologous regions in primate malarial parasites that are closely related to *P. vivax* (Escalante et al., 2005). Although we find evidence that positive natural selection is acting on the observed polymorphism in MSP-$1_{42}$, it operates differently in each of the two major human malarial parasites. We conclude that inferences made about *P. falciparum* MSP-1 cannot simply be "translated" into *P. vivax*.

## 2. Materials and methods

The gene encoding the 42 kDa fragment of MSP-1 or MSP-$1_{42}$ was amplified by polymerase chain reaction (PCR). The primers forward-GAA TGA TAT TCC TAA GAA GTT AGA GG and reverse-GAT AGA TTA TTT AAT AAG AAA AAA GAA CTT GGC CAA GAC AAA ATG C were used to amplify the partial *P. falciparum* 3′ sequences. The PCR conditions for amplifying the MSP-$1_{42}$ from *P. falciparum* were: a partial denaturation at 94 °C for 1 min and 30 cycles with 1 min at 94 °C, 1 min at 50 °C and 3 min extension at 72 °C. A final extension of 3 min was added in the last cycle. The primers forward-GAC CAA GTA ACA ACG GGA G and reverse-CAA AGA GTG GCT CAG AAC C were used for *P. vivax*, *P. cynomolgi*, *P. inui*, and *P. knowlesi*. In the case of *P. fragile*, we used the forward primer GAC CAA GTA ACA ACG GG. The PCR conditions for amplifying the MSP-$1_{42}$ from *P. vivax* and non-human primate malarias were: a partial denaturation at 94 °C for 3 min and 35 cycles with 1 min at 94 °C, 45′ at 50–58 °C and 2 min extension at 72 °C, a final extension of 10 min was added in the last cycle.

The amplified product was purified, cloned using the pGEM-TEasy Vector System I from Promega (USA), and sequenced. Both strands were sequenced from at least two clones. The alignment was performed using ClustalW Version 1.7 with manual editing using the alignment reported by Miller et al. (1993) in the case of *P. falciparum* and those reported by Putaporntip et al. (2002, 2006) in the case of *P. vivax* and related species.

In the case of *P. falciparum*, we sequenced the MSP-1 42 kDa in 34 isolates from Asembo Bay, western Kenya in this investigation. In addition, a total of 20 isolates (5 from India, 9 from Venezuela, and 6 from Thailand) were sequenced for the 3′ end. We used in our investigation prior published sequences (Chang et al., 1988; Qari et al., 1998; Jongwutiwes et al., 1992, 1993; Tanabe et al., 2004) and unpublished sequences under the accession numbers U20726–U20733 and U20653–U20656. A total of 120 MSP-$1_{42}$ sequences were considered in our analyses. In addition, we included 55 sequences of the MSP-$1_{19}$ reported in the literature (Kaneko et al., 1997; Kumar et al., 2005) and unpublished sequences under the accession numbers AF29507–AF29537 in order to obtain a complete picture of the MSP-$1_{19}$ alleles that have been reported.

In the case of *P. vivax*, we report five sequences from laboratory isolates (Rio Meta, Sumatra I, Indonesia I, Mauritania I, and Vietnam II) and used the sequences reported in literature (Putaporntip et al., 2000, 2002) for a total of 75 sequences. In addition, we analyzed 10 sequences from different isolates of *P. cynomolgi* (the sequence AY869723 from the GenBank together with new sequences from the strains B strain, Berok, Cambodian, Ceylonensis, Gombok, Mulligan, PT1, PT2, and RO), 15 sequences from isolates of *P. inui* (Celebes I and II, Hackeri, Hawking, Leaf Monkey I and II, Leucosphyrus, Mulligan, N-34, OS, Perak, Perlis, Philippine, Taiwan I and II), a sequence of *P. knowlesi* (Hackery strain), *P. hylobati* (parasite from gibbons), and *P. fragile* (Nilgiri strain). Information about the biology of these species and the origin of

the isolates can be found elsewhere (Coatney et al., 1971). All the primate malaria strains were provided by the Centers for Disease Control and Prevention. The sequences reported in this study are deposited in the GenBank with the accession numbers DQ907617–DQ907702.

### 2.1. Statistical analysis

We estimate genetic polymorphism by using the parameter $\pi$, which estimates the average number of substitutions between any two sequences. The average number of synonymous (Ds) and non-synonymous substitutions (Dn) between a pair of sequences was investigated to explore the effect of natural selection. The average numbers of synonymous and non-synonymous substitutions are estimated using two methods: Nei and Gojobori's method (1986) with the Jukes and Cantor correction, and the Li's method (1993) as implemented in the MEGA program (Kumar et al., 2001). We estimated the difference between Ds and Dn, its standard deviation was calculated using bootstrap with 1000 pseudo-replications for Ds and Dn, as well as a two tail Z-test on the difference between Ds and Dn (Nei and Kumar, 2000). The null hypothesis is that Ds = Dn; thus, we assumed as null hypothesis that the observed polymorphism was neutral.

The Tajima's $D$ statistic and $F^*$ from Fu and Li were estimated for testing the hypothesis that the allele frequency spectrum is compatible with the neutral model (Tajima, 1989; Fu and Li, 1993). Under the neutral model, Tajima's $D$ and $F^*$ are approximately equal to zero, thus any deviation from zero would indicate a departure from neutrality in the allele frequency spectrum.

Evidence for recombination was assessed by using the Rm parameter that estimates the minimum number of recombination events in the history of the sample. Rm is obtained using the four-gamete test (Hudson and Kaplan, 1985) and, as the name of the parameter indicates, it is a conservative estimate of the number of recombination events.

In the case of *P. vivax* and related non-human primate malarial parasites, the gene genealogy of the MSP-$1_{42}$ alleles was determined by using the Neighbor–Joining (Saitou and Nei, 1987) method with the Tamura-Nei model. The reliability of the nodes in the NJ tree was assessed by the bootstrap method with 1000 pseudo-replications. The genealogy was estimated using the MEGA program (Kumar et al., 2001). The assumption of neutrality was also tested in *P. vivax* MSP-1 by using the McDonald and Kreitman test (McDonald and Kreitman, 1991), which compares the intra-and interspecific number of synonymous and non-synonymous sites; significance was assessed by using a Fishers exact test for the 2 × 2 contingency table as implemented in the programs DNAsp Version 4.0 (Rozas et al., 2003). In this analysis, we compare *P. vivax* with *P. cynomolgi* and *P. inui* (see below).

## 3. Results

Table 1 shows the genetic diversity found in the MSP-$1_{42}$ fragments in *P. falciparum* and *P. vivax*. Overall, the genetic diversity of *P. falciparum* is twice that observed in *P. vivax* ($\pi$ of 0.05042 versus 0.02184). Analysis of the genetic diversity of the MSP-$1_{33}$ and MSP-$1_{19}$ fragments confirmed previous observations that the MSP-$1_{19}$ fragment is more conserved than the MSP-$1_{33}$ fragment (Table 1) in both human malarial parasites. *P. vivax* MSP-$1_{19}$ has only one polymorphic site while in *P. falciparum* the substitutions are concentrated in five residues within the epidermal growth factor like domains (EGF). In an extended alignment that included all the MSP-$1_{19}$ sequences reported in the literature at the time of this study ($n = 175$); we found 11 alleles reported based in these five residues, among them, there are four common alleles that have a worldwide distribution: E-KNG-L ($n = 54$), E-TSR-L ($n = 41$), Q-KNG-F ($n = 20$), Q-KNG-L ($n = 33$). It is worth noting that some alleles, although reported in low frequency, have been found in two continents; such are the cases of E-KNG-F ($n = 8$ reported in India and Kenya), E-KSR-L ($n = 4$ reported in Kenya, South Africa, and Vanuatu), and Q-TSR-L ($n = 3$ reported in India and Papua New Guinea). The allele E-TSG-L ($n = 9$) has been reported three times in India (including this study) and is the one observed in *P. reichenowi*, the most closely related species to *P. falciparum* found in chimpanzees (Coatney et al., 1971).

We found two recombination-convergent events using the Rm method (Hudson and Kaplan, 1985); these events are

Table 1
Polymorphism found in the MSP-$1_{42}$ in *P. falciparum* and *P. vivax*

| | $\pi$ | Ds | Dn | Ds − Dn (S.D.) | Z | Tajima $D$ | $F^*$ |
|---|---|---|---|---|---|---|---|
| *P. falciparum* ($n = 120$) | | | | | | | |
| 42 KDa | 0.05042 | 0.0821 | 0.0541 | 0.0280 (0.011) | Ds > Dn ($P<0.05$) | −0.11184 n.s. | 0.13353 n.s. |
| 33 KDa | 0.06551 | 0.1236 | 0.0741 | 0.0494 (0.020) | Ds > Dn ($P<0.05$) | 0.10150 n.s. | 0.86091 n.s. |
| 19 KDa | 0.00884 | 0.0013 | 0.0107 | −0.009 (0.004) | Ds < Dn ($P<0.05$) | −1.72070 ($0.10>P>0.05$) | −4.78810 ($P<0.05$) |
| *P. vivax* ($n = 75$) | | | | | | | |
| 42 KDa | 0.02184 | 0.0125 | 0.0249 | −0.0123 (0.005) | Ds < Dn ($P<0.05$) | 2.19241 ($P<0.05$) | 2.09599 ($P<0.05$) |
| 33 KDa | 0.03249 | 0.0162 | 0.0325 | −0.0160 (0.006) | Ds < Dn ($P<0.05$) | 2.31357 ($P<0.05$) | 2.24458 ($P<0.05$) |
| 19 KDa | 0.0006 | 0.0005 | 0.0006 | 0.0001 (0.000) | Ds = Dn | −1.02018 n.s | −1.02018 n.s |

$\pi$, nucleotide diversity; $n$, number of sequences; Dn is the nucleotide diversity of non-synonymous mutations per non-synonymous sites and Ds is the nucleotide diversity of synonymous mutations per synonymous site using the Nei and Gojobori method; Ds − Dn are the difference of Ds and Dn with their standard deviation, SD, estimated by bootstrap with 1000 pseudo replicates; Z is the Z-test (Nei and Kumar, 2000); Tajima $D$ and $F^*$ are tests for detecting departures from the neutral model.

illustrated using the relative positions of the residues in the allele E-KNG-L, specifically between the position held by the amino acids E and K (separated by 138 bp) and between the positions filled by amino acids K and G (separated by 30 bp). Recombination events have been previously reported in MSP-$1_{19}$ (Qari et al., 1998).

In order to explore the role of natural selection we further analyzed the genetic polymorphism in the MSP-$1_{42}$ as a unit by estimating the number of synonymous (Ds) and non-synonymous (Dn) substitutions per site estimated by the Nei and Gojobori method with the Jukes and Cantor correction. When this comparison is made, both parasites exhibit opposite patterns: MSP-$1_{42}$ in *P. falciparum* shows more synonymous than non-synonymous substitutions while the homologous region in *P. vivax* shows more non-synonymous than synonymous substitutions. In both cases the differences are significant with a *Z*-test (Nei and Kumar, 2000) (Table 1). The Li's method gives identical results. We explore departure from neutrality by using the Tajima's *D* test (Tajima, 1989) and $F^*$ test (Fu and Li, 1993). These tests should to be used with caution since they aim to detect departures from a neutral panmictic population, an assumption that is violated by these geographically and temporally spaced samples. Nevertheless, we used them to explore the distribution of haplotypes in our samples as was used previously to compare *P. vivax* and *P. knowlesi* (Putaporntip et al., 2006). These tests could not detect departure from neutrality in *P. falciparum*, although they did so in *P. vivax* when the complete MSP-$1_{42}$ was considered as a unit.

We explored the diversity in the MSP-$1_{33}$ and MSP-$1_{19}$ fragments separately by comparing the number of synonymous and non-synonymous substitutions in each species. In the case of the MSP-$1_{33}$ of *P. falciparum* there are more synonymous than non-synonymous substitutions ($P < 0.05$) (Table 1), while the contrary was observed in the MSP-$1_{19}$ where there are more non-synonymous than synonymous substitutions ($P < 0.05$). These results suggest that while the MSP-$1_{19}$ is under positive selection in *P. falciparum*, the MSP-$1_{33}$ is under purifying selection; that is, natural selection favors the maintenance of amino acid polymorphism in the MSP-$1_{19}$ while it holds back the rate of amino-acid polymorphism in the MSP-$1_{33}$. Differences between the MSP-$1_{33}$ and MSP-$1_{19}$ were also observed by using the Tajima's *D* and $F^*$ tests (Table 1): there is not a departure from neutrality in the MSP-$1_{33}$ while the MSP-$1_{19}$ polymorphism rejects the expectation under the neutral model. Although the significance level by the Tajima's *D* test is weak for MSP-$1_{19}$ ($0.05 < P < 0.1$), there is almost no synonymous variation, substantiating a departure from the neutrality in this region. It is important to notice that the Tajima's *D* and $F^*$ tests have a negative value indicating that there is an excess of low frequency variants in the sample (Table 1).

In the case of *P. vivax* the pattern is the opposite. There are more non-synonymous than synonymous substitutions in the MSP-$1_{33}$ while there is almost no variation in the MSP-$1_{19}$ (Table 1). The polymorphism in the *P. vivax* MSP-$1_{33}$ is not evenly distributed. Indeed, there is a region of 105 bp out of 848 bp in MSP-$1_{33}$ (35 amino acids) where a clear excess of non-synonymous versus synonymous substitution is observed driving the overall MSP-$1_{33}$ results. In addition, there is a departure from neutrality in the MSP-$1_{33}$ when the Tajima's *D* and $F^*$ tests are applied. However, contrasting with *P. falciparum*, the value of the test is positive as the result of an excess of variants in intermediate frequencies.

We further explore the hypothesis that positive selection is acting on the *P. vivax* MSP-$1_{33}$ fragment by comparing it with its closely related non-human primate malarial parasites (Escalante et al., 2005). The genealogy of the MSP-$1_{42}$ fragments from the species reported in this study is depicted in Fig. 1. *P. cynomolgi* appears as sister taxa of *P. vivax*; however, this clade does not have strong support. *P. cynomolgi* strains are subdivided into two clear clades; no evidence for allele families could be observed with this fragment. *P. inui* and *P. hylobati* are closely related as previously reported (Escalante et al., 2005). The close relationship of these two species was further supported by the presence of a repetitive sequence in the MSP-$1_{33}$ fragment. Specifically, a motif with the residues NE*QEEI* is inserted in some of the *P. inui* isolates while *P. hylobati* has the residues NE*QEEI*KIR*QEEI*. We also found an insertion in *P. knowlesi* that emerged as a duplication of the motif *INNCQIEK* conserved in *P. inui* and *P. vivax* (Fig. 2). Given the lack of

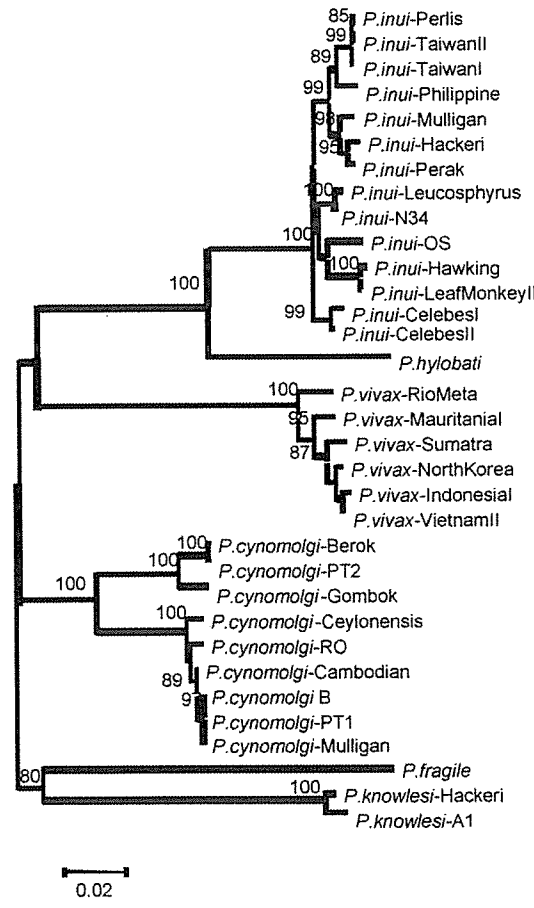


Fig. 1. Neighbor–Joining tree of the MSP-$1_{42}$ alleles using Tamura-Nei's distance. The numbers on the nodes of the tree are percent of bootstrap values based on 1000 pseudo-replications. The sequences reported in this study are identified with their species and strain names.

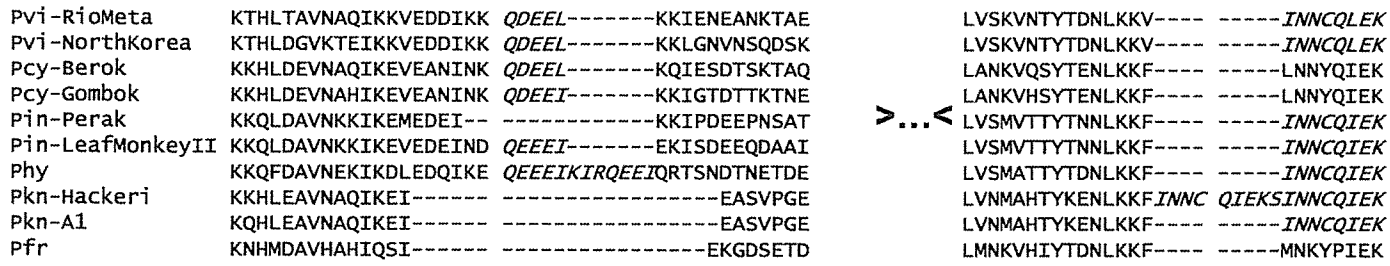


Fig. 2. Repetitive sequences observed in the MSP-$1_{42}$. The observed motifs are in italics. The dots (>...<) are indicating a non-repetitive portion of the protein that is not shown. The first three letters in the sequence codes indicate the species: Pvi, *P. vivax*; Pcy, *P. cynomolgi*; Pin, *P. inui*; *Phy*, *P. hylobati*; Pkn, *P. knowlesi*; Pfr, *P. fragile*.

Table 2
Polymorphism found in the MSP-$1_{42}$ in other non-human *Plasmodium* spp.

| | $\pi$ | Ds | Dn | Ds − Dn (S.D.) | Z |
|---|---|---|---|---|---|
| *P. cynomolgi* ($n$ = 10) | | | | | |
| 42 KDa | 0.03805 | 0.0871 | 0.0287 | 0.0585 (0.015) | Ds > Dn ($P$<0.05) |
| 33 KDa | 0.06551 | 0.1001 | 0.0312 | 0.0687 (0.018) | Ds > Dn ($P$<0.05) |
| 19 KDa | 0.02502 | 0.0469 | 0.0211 | 0.0257 (0.022) | Ds = Dn |
| *P. inui* ($n$ = 15) | | | | | |
| 42 KDa | 0.02416 | 0.0284 | 0.0237 | 0.0049 (0.006) | Ds = Dn |
| 33 KDa | 0.02951 | 0.0358 | 0.0289 | 0.0071 (0.008) | Ds = Dn |
| 19 KDa | 0.0067 | 0.0051 | 0.0073 | −0.0022 (0.005) | Ds = Dn |

$\pi$, nucleotide diversity; $n$, number of sequences; Dn is the nucleotide diversity of non-synonymous mutations per non-synonymous sites and Ds is the nucleotide diversity of synonymous mutations per synonymous site using the Nei and Gojobori method. Ds − Dn are the difference of Ds and Dn with their standard deviation, S.D., estimated by bootstrap with 1000 pseudo replicates. Z is the Z-test (Nei and Kumar, 2000).

resolution of the phylogeny using this region, we used both *P. cynomolgi* and *P. inui* for comparison with *P. vivax*.

Table 2 shows the basic statistics for the MSP-$1_{42}$ in these two non-human primate malarial parasites. As in the cases of the human parasites, the MSP-$1_{33}$ fragment is more diverse than the MSP-$1_{19}$. However, in the case of the non-human primate malarias, there is no excess of non-synonymous substitutions over synonymous substitutions in the MSP-$1_{42}$ as a unit or considering the MSP-$1_{33}$ and MSP-$1_{19}$ fragments separated. Thus, by comparing the rate of non-synonymous versus synonymous substitutions we could not detect evidence for positive selection acting on *P. cynomolgi* or *P. inui* MSP-$1_{42}$. An identical pattern can be observed in *P. knowlesi* when the two complete MSP-$1_{42}$, the one reported in this investigation and the one available in literature (Putaporntip et al., 2006) are compared, specifically Ds = 0.04275 and Dn = 0.00240 for MSP-$1_{42}$.

We then analyzed the genetic diversity of *P. vivax* MSP-$1_{42}$ by using the McDonald and Kreitman test (McDonald and Kreitman, 1991) and compared it with both *P. cynomolgi* and *P. inui* samples. In the case of the complete 42 Kda, there was an overall excess of non-synonymous over synonymous in the *P. vivax* polymorphism when compared with *P. cynomolgi* ($p < 0.05$ using a Fisher's exact test). Similar results were found with *P. vivax* and *P. inui* ($p < 0.001$ using a Fisher's exact test). In both cases, the significance of the MK test was explained by an excess of amino acid replacements in the polymorphism of the *P. vivax* MSP-$1_{33}$. It is worth noting that no departure from neutrality was found when only MSP$1_{19}$ was considered. It is also important to emphasize that no departure from neutrality was observed when *P. cynomolgi* and *P. inui* were compared considering the MSP-$1_{42}$ as a unit, or separating it into the MSP-$1_{33}$ and MSP-$1_{19}$ fragments.

## 4. Discussion

The available data, mostly derived from *P. falciparum*, indicate the importance of the antibody response against block 2 (located in the 83 kDa or MSP-$1_{83}$) and the MSP-$1_{42}$ fragments in developing protective immunity. In this study, we have described the selective forces operating on the polymorphism observed in the MSP-$1_{42}$ fragment in the two major human malaria parasites. We have shown how the MSP-$1_{33}$ and MSP-$1_{19}$ fragments are under different selective pressures in each of the major human malarial parasites by using the rate of non-synonymous versus synonymous substitutions.

In the case of *P. falciparum*, the polymorphism in MSP-$1_{33}$ appears to be neutral or under purifying selection while the polymorphism in MSP-$1_{19}$ is under positive selection. In this case, our results are consistent with immunologic evidence suggesting that the MSP-$1_{19}$ but not MSP-$1_{33}$ elicits a protective immune response, though the latter being highly immunogenic (Ahlborg et al., 2002). Positive selection has been previously proposed as an important mechanism in maintaining the *P. falciparum* MSP-1 polymorphism in the form of balancing selection (Hughes, 1992; Conway et al., 2000); that is, natural selection maintains genetic polymorphism for a longer time than expected under a scenario where only

genetic drift is acting. A polymorphism under balancing selection is expected to have an excess of alleles in intermediate frequencies, a pattern that translates into positives Tajima's $D$ and $F^*$ tests. In the case of MSP-$1_{19}$, however, there is an excess of alleles in low frequency as evidenced by significant and negative values of the Tajima's $D$ and $F^*$ tests, not consistent with balancing selection. This could be the result of several factors. First, we found four alleles that are particularly common while several others are found in low frequency in our sample; low frequency alleles that are found even in different continents suggest an artifact due to a poor sampling effort. Indeed, lack of appropriate sampling could generate negative Tajima's $D$ tests as a result of several sub-populations being analyzed together (Hammer et al., 2003). A second alternative is that a limited number of alleles are increasing in frequency, a scenario expected under a population expansion which coincides with the results reported for mitochondrial data (Joy et al., 2003).

Nevertheless, if the population demographic history and inappropriate sampling were the only factors leading to this result (significant and negative Tajimas's $D$ and $F^*$ tests), then the MSP-$1_{33}$ should have shown a similar trend. The Tajimas's $D$ and $F^*$ tests for MSP-$1_{33}$ are not only non-significant but also have an opposite sign. Interestingly, the MSP-$1_{33}$ also shows more synonymous than non-synonymous substitutions. Therefore, we propose that the negative Tajimas's $D$ and $F^*$ tests, together with the excess of non-synonymous over synonymous substitutions in MSP-$1_{19}$, are the result of directional selection, that is, there are few MSP-$1_{19}$ alleles increasing in frequency because they are positively selected.

Although the immune response against *P. falciparum* MSP-$1_{19}$ is still under intense investigation, there is evidence suggesting that fine specificity rather than prevalence could be an important factor in the observed immune reactivity (Okech et al., 2004). Indeed, only partial cross-reactivity has been found in holoendemic areas among the most common MSP-$1_{19}$ alleles (Udhayakumar et al., 1995; Shi et al., 1996; John et al., 2004). It has been also shown that immunity against MSP-$1_{19}$ in *P. falciparum* has a short lifespan to the extent that its elicited antibody responses allow detecting differences in local transmission (Drakeley et al., 2005). Therefore, the pattern in the genetic polymorphism of MSP-$1_{19}$ could be the result of differences of the most common alleles in their specificity and/or life spans of their elicited immune responses when compared with the less frequent MSP-$1_{19}$ alleles, differences that give them a selective advantage favoring their transmission.

Our hypothesis that directional selection is operating on MSP-$1_{19}$ does not contradict previous claims for balancing selection since they are well supported by the extensive divergence observed in MSP-$1_{83}$, MSP-$1_{30}$, and MSP-$1_{38}$ fragments allowing the identification of two very distinctive allele families (Tanabe et al., 1987) that have been found to be an ancient polymorphism (Hughes, 1992; Polley et al., 2005) as well as evidence derived from population base studies of the MSP-$1_{83}$ (Conway et al., 2000; Takala et al., 2006). Indeed such divergent allele families are not observed when only the MSP-$1_{19}$ is considered.

In the case of *P. vivax*, however, the MSP-$1_{33}$ and MSP-$1_{19}$ fragments appear to be under different selective pressures than the ones just described in the homologous region in *P. falciparum*. We observed an excess of non-synonymous over synonymous substitutions in the MSP-$1_{33}$ and not in the MSP-$1_{19}$; in addition, we found that the Tajimas's $D$ and $F^*$ tests are significant and positive for MSP-$1_{33}$, which is expected under the scenario of balancing selection although it could be the result of population structure, a clear possibility given the origin of the sample analyzed. Nevertheless, when we studied the genetic variation in the MSP-$1_{33}$ and MSP-$1_{19}$ by using the McDonald and Kreitmant test against *P. cynomolgi* and *P. inui* we found an excess of non-synonymous substitutions in the *P. vivax* MSP-$1_{33}$ no matter which species we used to compare it with, suggesting that positive natural selection is operating in this fragment.

Our results support previous observations that *P. vivax* MSP-$1_{33}$ could play an important role in reticulocyte invasion (Espinosa et al., 2003; Rodríguez et al., 2002). However, the polymorphism in the *P. vivax* MSP-$1_{33}$ appears more complicated; indeed, there is a 105 bp fragment with high polymorphism located between regions where peptides with high specific binding activity (HSBA) to reticulocytes have been found (Espinosa et al., 2003; Rodríguez et al., 2002). These regions with HSBA are not only highly conserved among *P. vivax* isolates ($n = 75$) but also show more synonymous than non-synonymous substitutions when compared with *P. cynomolgi* (peptides 1735, 1738 and 1747 *sensu* Rodríguez et al., 2002 have Ks of 0.30, 0.31 and 0.22 versus Kn of 0.16, 0.025, and 0.11, respectively) and a similar pattern is observed when compared with *P. inui* (peptides 1735, 1738 and 1747 *sensu* Rodríguez et al., 2002 have Ks of 0.29, 0.37, and 0.041 versus Kn of 0.17, 0.10, and 0.16, respectively). This overall pattern indicates that these HSBA regions are under selective constraints to accumulate amino acid replacements; as a result, they could be a valuable target for a vaccine against *P. vivax* as has been suggested previously (Espinosa et al., 2003).

There is no information regarding the immunologic role played by the variation observed in *P. vivax* MSP-$1_{33}$. Elucidating whether it hampers effective natural immune responses against these conserved regions with HSBA to reticulocytes or whether it plays any other role requires further investigation. Nevertheless, it seems clear from this comparative analyses that we cannot simply extrapolate information derived from *P. falciparum* into *P. vivax* in the case of MSP-$1_{42}$.

In summary, we have investigated the genetic diversity of the sequence encoding the MSP-$1_{42}$ in the two major human malarial parasites. We found evidence supporting positive natural selection as an important factor in the maintenance and generation of the observed polymorphism. However, we describe how natural selection is acting differently in the MSP-$1_{33}$ and MSP-$1_{19}$ fragments of the MSP-$1_{42}$ in each of the two human malarial parasites. That is, our results suggest that these fragments, MSP-$1_{33}$ and MSP-$1_{19}$, could play different roles in each of the two human malarial parasites.

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