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Short communication

Plasmodium vivax serine repeat antigen (SERA) multigene family exhibits similar expression patterns in independent infections☆

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Plasmodium vivax is the most prevalent form of human malaria accounting annually for 70–80 million cases in the tropics and subtropics [1]. Unfortunately, generally regarded as benign and sympatric with *Plasmodium falciparum*, research on *P. vivax* has lagged disproportionately. However, re-emergence, increased transmission and drug resistance [2] has led to renewed pressure for the development of an effective vaccine to control vivax malaria. Efforts are, currently, hampered by the lack of continuous *in vitro* culture and the scarcity of available information regarding the transcriptome or proteome [3].

The N-terminal 47-kDa domain (SE47′) of *P. falciparum* serine repeat antigen 5 (PfSERA5) has been exploited as a potential vaccine candidate. Recombinant SE47′ elicits specific antibodies that inhibit *in vitro* the parasite intraerythrocytic proliferation [4–6] and confers protective immunity in vaccinated *Aotus* and squirrel monkeys against challenge infection [7,8]. In a malaria-hyperendemic region in Uganda, naturally induced antibody response to the N-terminal domain positively correlated with increased protective immunity in adults; and higher levels of IgG3 anti-SE47′ and IgG anti-SE36 (the recombinant construct without the serine repeat region) were associated, respectively, with the absence of fever and lower parasitemia in the peripheral blood of children aged under 15 [9] and with protection against severe malaria in children under 5 years old [10]. This blood stage antigen is now on Phase I clinical trials in Japan.

PfSERA5 belongs to a large multigene family [11]. All *P. falciparum SERA* (*PfSERA1–9*) are transcribed most actively at trophozoite and schizont stages [12,13]; with PfSERA3, -4, -5 and -6 proteins found to be co-expressed in every single parasite cell [12]. The dominant expression of *PfSERA5* along with *PfSERA6* is consistent with the failure to disrupt these genes during blood stage growth [13]. These observations have led to the premise that only a subset of the family is essential for normal erythrocytic development.

In silico analysis of available *P. vivax* sequence from GenBank (AAKM01000018) have identified six more *SERA* homologues downstream of the five *V-SERA* genes found by Kiefer et al. [14] and flanking one *SERPHvivax* gene identified by Gor et al. [15] (Arisue et al., submitted for publication). In contrast to the co-expression of each *PfSERA* family member in the blood stages, previous studies using RT-PCR and an erythrocytic stage *P. vivax* cDNA library indicate that only a single SERA gene was transcribed from among previously annotated

Abbreviations: SERA, serine repeat antigen; MSP1, merozoite surface protein 1; SNP, single nucleotide polymorphism

☆ The nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB260077–AB260114.

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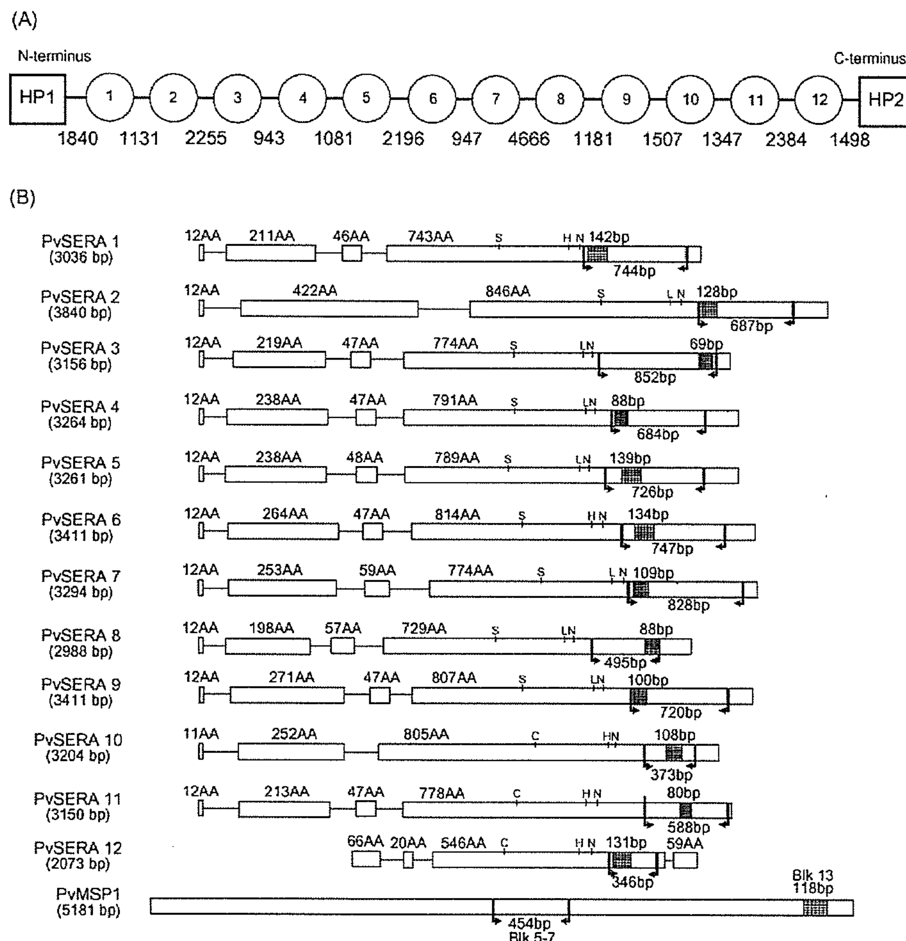
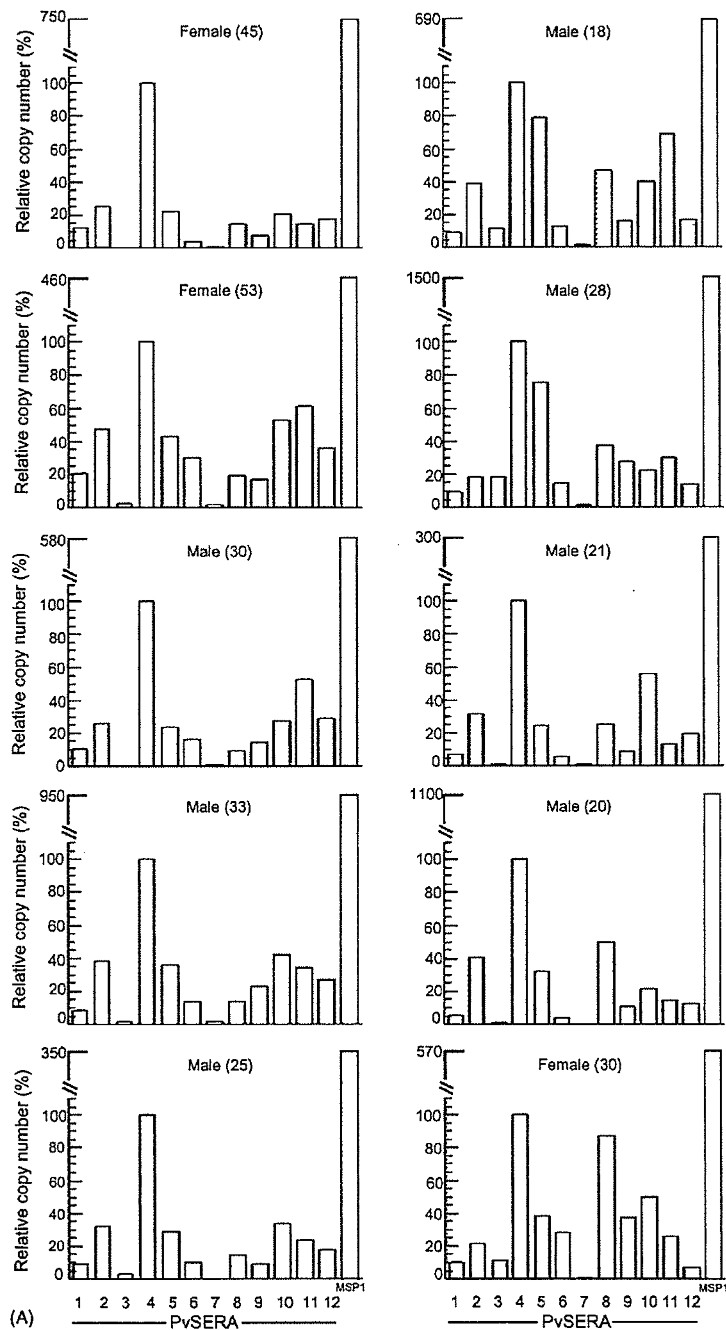
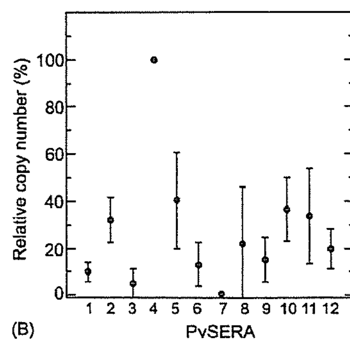


Fig. 1. The *P. vivax SERA* multigene family. (A) The tandem gene cluster arrangement of *SERA* family members in a region between two hypothetical proteins (HP) (Arisue et al., submitted for publication). Individual members are represented by ovals numbered 1–12. *PvSERA*1–9 unshaded ovals possess an active site serine and *PvSERA*10–12 shaded ovals possess an active site cysteine in the central proteinase domain. The length, in base pair, between each member is indicated. (B) SERA gene structure and relative positions of the primers used in the study. Numbers in parenthesis indicates the total length of the exons, in base pair (bp). Exons are denoted by grey boxes and lines linking boxes represent introns. Length of each exon is shown in amino acids (AA). The critical amino acid residues involved in cysteine proteinase activity are indicated. Positions of the primers for real-time PCR and the expected fragment sizes are in cross-striped bars superimposed on the grey exons. Primer positions for genotyping and the expected amplified lengths are symbolized as lined-arrows in the C-terminal portions of *PvSERA* and in the central region in *PvMSP1*. Blk, variable block numbers in *PvMSP1* as described [23].

members [14]. Whether the disparity in expression reflects interspecies difference or dissimilarity of expression patterns *in vivo* and *in vitro* needs to be addressed. Furthermore, because malaria antigens generally exhibit remarkable polymorphisms and their allelic forms differ in the ability to abrogate recognition of the host immune response, the extent of sequence variation is undoubtedly important for malaria vaccine development as well as enabling antigenic diversity-generating mechanisms to become elucidated [16]. Sequence polymorphism of PfSERA5 was virtually restricted to a region in the 47 kDa domain (amino

Fig. 2. Relative transcription of the *PvSERA* genes as assessed by real-time PCR. For quantification purposes, standard curves were generated using serially diluted (1.25 ng–1.25 pg) linearized plasmid DNA for each *PvSERA* gene. Standard curves associating the threshold cycles (C_T) against log amount of starting plasmid were created over a concentration range of standards within which the C_T value showed reproducible linearity [average correlation coefficients, $R^2 = 0.99638$; range = 0.98547–0.9999; $n = 78$; S.D. = 0.00321]. Averages of the slopes and y-intercepts for each gene standard were imported in the formula: $C_T = sn + y$; where s stands for the slope, n for log copy number, and y for y-intercept; to estimate relative copy number based on C_T values of the sample. As reference gene, transcription of *P. vivax* merozoite surface protein 1 (MSP1) was measured in each run. (A) Bar graphs show the copy numbers of individual *PvSERA* genes, relative to the copy number of *PvSERA4*, as assayed in different patient samples (gender and age are indicated). Patient samples were obtained during May 2002 and February 2005. In samples containing predominantly ring-stage parasites, *ex vivo* cultures were performed for 30–36 or 18–24 h when the parasites were at ring or at amoeboid stages, respectively; and processed (as modified from Chotivanich et al. [24]). *P. vivax*-infected erythrocytes were enriched by percoll-gradient centrifugation, frozen and stored at −80° C until analysis. Enriched fractions contained trophozoite/schizont stage parasites (76–93%; mean = 83%) and gametocytes (7–24%; mean = 17%). Representative results of two independent assays are shown. (B) Mean transcription, expressed as relative copy number, of each *SERA* family member ($n = 10$); error bars depict standard deviation.


Female (45)
Male (18)
Female (53)
Male (28)
Male (30)
Male (21)
Male (33)
Male (20)
Male (25)
Female (30)
Relative copy number (%)
MSP1
PvSERA
(A)


Relative copy number (%)
PvSERA
(B)

acids 23–382), due mostly to deletion/insertion events in the octomer repeat units and in the stretch of serine residues; thereby, suggesting that a localized segment is under immune-mediated selection [17]. Virtually, nothing is known for the genetic diversity of the *SERA* genes of *P. vivax* (*PvSERA*). In this study, we investigate the relative transcription levels and the genetic diversity of *PvSERA* family members in field isolates from Thailand.

Blood samples were collected from *P. vivax*-infected uncomplicated adult (≥16 years old) patients consulting the Mae Sot Malaria Clinic, Northwestern Thailand. *P. vivax* infection incidence was 7.06/1000 population based on the 2004 Annual Report of the Rural Vector Borne Diseases (Department of Disease Control, Thailand). Informed consent, under the guidelines of the Ethical Review Committee of Mahidol University, Thai Ministry of Public Health and U.S. Army, was obtained from selected volunteers that have not received or are taking antimalarial drugs at the time of blood collection. Diagnosis for *P. vivax* infection was confirmed using Giemsa-stained blood smears and by real-time PCR using 18S rRNA species-specific primers [18].

Parasite total RNA was isolated using RNeasy Mini column (Qiagen) and the RNase-Free DNase Set (Qiagen). First-strand cDNA was synthesized with Qiagen Omniscript Reverse Transcriptase (RT) in 20 μl reaction volumes of 1 μl total RNA, 0.2 mM each dNTP, 1 μM oligo-dT primer, 10 units RNase inhibitor, and 4 units RT.

Real-time PCR was performed by using Qiagen QuantiTect SYBR Green PCR kit and an ABI PRISM 7900 detection system. The programmed protocol was 95 °C, 15 min; (94 °C, 20 s; 60 °C, 60 s; 57 °C, 15 s) × 40 cycles. Reaction volumes of 20 μl included 1 μl cDNA, 0.2 μM each primer, 10 μl of 2× QuantiTect SYBR Green PCR Master Mix and 0.03 units Pfu polymerase (Promega). All cDNA samples were assayed in duplicate using primers targeting the C-terminal region of each *PvSERA* gene family member (Fig. 1B and Supplementary Table 1). Fidelity of the reactions was confirmed by sequencing. A standard PCR assay using *P. falciparum* (3D7) genomic DNA as template yielded no amplified product, confirming the species specificity of the primers used. RT negative and no cDNA template controls were included in each real-time PCR run.

Genomic DNA was isolated using either DNAzol Reagent (Invitrogen) or QIAamp DNA Blood Mini Kit (Qiagen). Primers for genotyping amplified short C-terminal fragments of 500–800 bp (Fig. 1B and Supplementary Table 1). A 25 μl PCR mixture contained 1 μl template, 0.2 mM each dNTP, 0.2 μM of each primer and 0.6 units Pfu polymerase. The thermal profile was 95 °C, 5 min; (91 °C, 30 s; 53 °C, 30 s; 61 °C, 3 min) × 40 cycles. Amplified products were cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). At least two independent amplifications for each gene locus were used. When singleton mutation was found, bi-directional sequencing was repeated from the original template for verification. Nucleotide and deduced amino acid sequence alignments were performed using GENETYX Ver. 7.0.3 and ClustalW Ver. 1.83. Haplotype diversity or expected heterozygosity was calculated as described [19].

Genes arranged in tandem cluster between two hypothetical proteins are referred to in this paper as *PvSERA1–12*, with *PvSERA1–5*, being the previously described *V-SERA 2* (AAB41485.1), *V-SERA 4* (AAB41486.1), *V-SERA 3* (AAB41487.1), *V-SERA 1* (AAB41488.1), and *V-SERA 5* (AAB41489.1), respectively [14]; and *PvSERA10* corresponds to previously identified *SERPHvivax* (AF052747.1) [15]. Deduced amino acid sequences reveal a highly similar exon/intron organization with *PvSERA1*, *-3* to *-9* and *-11* possessing the characteristic four-exon structure of the family (Fig. 1A and B). *PvSERA2* and *-10* are atypical, due to the fusion of exons III and IV. *PvSERA12* bears a different structural organization, with a truncated N-terminal domain, similar to *PfSERA8* and all other identified *Plasmodium SERA* sequences lying upstream to the second hypothetical protein (Arisue et al., submitted for publication). The canonical Cys His Asn triad of cysteine proteinases is replaced by Ser Leu Asn in seven members: *PvSERA2* to *-5* and *-7* to *-9* (Fig. 1B). In contrast to the *P. falciparum SERA* serine cluster, which was further subdivided into three groups according to the presence of additional mutations to the active site histidine (His to Met; His to Leu; no mutations) [20], only one mutation was observed in *P. vivax* (His to Leu).

Real-time PCR analysis of infected erythrocytes from all patient samples show almost similar transcription profiles, though transcription levels varied to some extent depending on the individual isolates as observed for *PfSERA* family members [12,13]. *PvSERA4* was always more strongly transcribed than other *SERA* genes; *PvSERA3* and *-7*, the least transcribed members (Fig. 2). Relatively high transcription was also noted for *PvSERA2*, *-5*, *-10*, and *-11* (Fig. 2B). The asynchronous parasite populations and the differences in parasitemias likely affected transcription levels as expected when using isolates from natural infections. Attempts to correlate differential expression of *PvSERA* family members with gametocyte levels failed, due to relatively constant proportion of gametocytes in the samples. Although we cannot rule out the possibility that some gene family members might be strongly expressed in the gametocyte stage, the 12 *PvSERA* family members do not appear to be differentially expressed in 10 patient samples collected at various times.

The highest transcription of *PvSERA4* in all isolates parallels that of *PfSERA5*, the predominantly expressed gene member in *P. falciparum*. This contrasts an earlier report that only a single *SERA* (*PvSERA5*) gene was transcribed in *P. vivax* blood stages using a cDNA from Sal-1 infected *Aotus vociferans* [14]. Differences in detection technique and primer locations/sensitivities might explain this discrepancy. The expression profile is clearly different from that observed for the *P. vivax vir* gene family in different patients [21], suggesting that *SERA* does not function as a gene resource for antigenic variation.

In assessing *PvSERA* diversity, C-terminal sequences of *PvSERA4*, *-5*, and *-8*, with *PvMSP1* as control, were used for analyses. These gene family members were arbitrarily chosen based on preliminary screening that showed *PvSERA1*, *-2* and *-12* polymorphisms due mainly to single nucleotide polymorphisms (SNPs); *PvSERA3* to *-11* due to dele-

Table 1
Polymorphisms of *PvSERA4*, *-5*, *-8* and *PvMSP 1* from Thai field isolates

Gene locus	Allele distribution			Haplotype diversity[a]
	Allele	n^b	Frequency	
	4A-1	1	0.111	
	4A-2	1	0.111	
	4B-1	2	0.222	
PvSERA4	4B-2	2	0.222	0.944 ± 0.070
	4B-3	1	0.111	
	4C	1	0.111	
	4R	1	0.111	
	5A-1	8	0.364	
	5A-2	1	0.045	
	5A-3	1	0.045	
	5R	1	0.045	
PvSERA5	5B-a1	2	0.091	0.784 ± 0.063
	5B-a2	1	0.045	
	5B-b1	7	0.318	
	5B-b2	1	0.045	
	8A-1	5	0.312	
	8A-2	1	0.062	
	8A-3	1	0.062	
	8A-4	2	0.125	
	8A-5	1	0.062	
PvSERA8	8A-6	1	0.062	0.900 ± 0.062
	8B-1	2	0.125	
	8B-2	1	0.062	
	8B-3	1	0.062	
	8B-4	1	0.062	
	S1	8	0.267	
	S2	2	0.067	
	S3	1	0.033	
	S4	2	0.067	
	S5	4	0.133	
	S6	1	0.033	
PvMSP1	S7	5	0.167	0.892 ± 0.036
	S8	1	0.033	
	S9	1	0.033	
	S10	1	0.033	
	S11	2	0.067	
	S12	1	0.033	
	B1	1	0.033	

[a] Haplotype diversity index (h) and Student's t-test were calculated as described [19].
[b] n = number of isolates (patient samples) in which the allele was observed.

tion/insertion events compared to Sal-1 sequence type. Table 1 shows alleles at the three *PvSERA* and *PvMSP1* loci. The heterogeneity in the C-termini region of *PvSERA* was in sharp contrast to the complete sequence conservation in the C-termini of *PfSERA5* [5]. At each locus of *PvSERA*s, variable regions can be grouped into 2–3 major allelic forms and each allele in turn shows polymorphism resulting from point mutations or deletions (Supplementary Fig. 1).

PvSERA4 allele groups showed 41–67% pairwise identity with deletions and insertions as well as differing numbers of QG(P/S)(P/S) dispersed repeats. One recombinant type (Allele 4R) was identified. *PvSERA5* alleles had 50–80% pairwise sequence identity between basic sequence types characterized by having either glycine imperfect repeats (Alleles 5A) or GVGVA(P/T) repeats (Alleles 5B). One recombinant type (5R) was also identified. *PvSERA8* alleles had 63–72% pairwise identity, with dimorphic SNPs in one group (Alleles 8A) and another group having a number of substitutions relative to Sal-1 type (Alleles 8B). *PvMSP1* polymorphism was also essentially dimorphic in the region sequenced. Majority of the parasites are of the Sal-1 sequence type. Eight of the observed SNPs were previously reported nucleotide polymorphisms and seven are novel substitutions.

Haplotype diversity of *PvSERA4*, *-5*, and *-8* was substantially high, ranging from 0.78 to 0.94. The haplotype diversity in this three loci did not differ significantly from each other whether the gene family member was highly expressed or not; and their diversity levels were comparable to *PvMSP1*. All infections were complex, having a mean of 2–3 genotypes per infection based on *PvSERA* and *PvMSP1*, respectively. The prevalence of diverse *PvSERA* alleles and multiplicity of genotype infections may well be the source of the recombinant types observed in *PvSERA4* and *-5* from different isolates and sampling periods. Thus, we observed a high level of heterozygosity of *PvSERA* alleles in a *P. vivax* population from Thailand where malaria transmission is low.

In this first analysis of *PvSERA* transcription and genetic diversity in natural infections, we observed that all SERA gene members are transcribed in the blood stage and that the expression profile of the gene family is similar in different patient samples. The significantly dominant transcription of *PvSERA4* parallels the expression profile of PfSERA5, a blood stage vaccine candidate for falciparum malaria. That the SERA multigene family is unique to *Plasmodium* provides a glimpse of the molecules role in parasite survival yet fails to explain the presence of so many homologues, especially in *P. vivax*. Further studies are necessary to determine whether the number of family members is related to the unique aspects of vivax malaria with regard to the invasion of host reticulocytes and/or the presence of hypnozoites; or, similar to *PfSERA*, other members may be substantially upregulated or differentially expressed in another life cycle [22, Arisue et al., submitted for publication]. The sequence diversity of *PvSERA4* is clearly distinct from *PfSERA5*, which shows virtually no diversity in the C-terminal part [5]. This study revealed remarkably high haplotype diversity of *PvSERA* family, a level comparable to that of *PvMSP1*, one of the most polymorphic *P. vivax* antigen genes so far known. In general, the genetic diversity of *P. falciparum* is higher in high transmission areas than in low transmission areas. Here, we observed a high genetic diversity of *P. vivax* in a local area of Thailand with low transmission intensity, indicating a unique feature of antigen diversity of *P. vivax*. The nature and extent of polymorphisms in the C-terminal regions might have important implications for estimating potential positive selection operating at the *PvSERA* gene family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2006.07.006.

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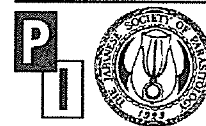

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2 # Allelic diversity in the merozoite surface protein 1 gene of *Plasmodium*
3 # *falciparum* on Palawan Island, the Philippines

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12 **Abstract**

13 Allelic diversity of the *Plasmodium falciparum* merozoite surface protein 1 gene (*msp1*) is mainly generated by meiotic recombination at the
14 mosquito stage. We investigated recombination-based allelic diversity of *msp1* in a *P. falciparum* population from Palawan Island, the Philippines,
15 where malaria transmission is moderate. We identified the 5′ recombinant types, 3′ sequence types and *msp1* haplotypes (unique combinations of
16 5′ recombinant type and 3′ sequence type), and compared them with those of *P. falciparum* from the Solomon Islands, where malaria transmission
17 is high. The mean number of 5′ recombinant types per patient in Palawan was 1.44, which is comparable to the number for the Solomon Islands
18 (1.41). The Palawan parasite population had 15 *msp1* haplotypes, whereas the Solomon Islands population had only 8 haplotypes. The Palawan
19 population showed strong linkage disequilibrium between polymorphic blocks/sites within *msp1*, which is comparable to the results for the
20 Solomon Islands. These findings support our hypothesis that the extent of allelic diversity of *msp1* is determined not only by the transmission
21 intensity but also by the number of *msp1* alleles prevalent in the local parasite population and the extent of mixed-allele infections. Contribution of
22 a high prevalence of the chloroquine (CQ)-sensitive allele of *P. falciparum* CQ resistance transporter (*pfcrt*) to the relatively high *msp1* diversity
23 in the Palawan population is discussed.
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25
26 *Keywords:* Malaria; *msp1*; Polymorphism; Recombination; *pfcrt*

27

28 ## 1. Introduction

29 Malaria caused by *Plasmodium falciparum* is a major public
30 health problem in the tropics. Due to the worldwide spread of
31 drug-resistant malaria, there is an urgent need for the
32 development of malaria vaccines. The 200-kDa *P. falciparum*
33 merozoite surface protein 1 (MSP-1), which plays an important
34 role in erythrocyte invasion by the merozoite, is a target of
35 human immune responses [1,2], and is thus considered a good
36 candidate vaccine target [1,3]. However, the gene encoding
37 MSP-1 (*msp1*) is highly polymorphic, and this polymorphism is
38 a major obstacle to the development of effective malaria
39 vaccines based on MSP-1. In animal models, protective
40 immunity directed against MSP-1 is strain-specific [4–6].
41 *P. falciparum msp1* consists of 17 blocks, with varying degrees
42 of sequence conservation among alleles [7,8]. Sequence
43 variation in *msp1* is dimorphic (*i.e.*, one or the other of two
44 major allelic forms represented by K1 allele and MAD20 allele)
45 in all variable blocks except for block 2, which has three major
46 allelic forms (see Fig. 2). A major source of allelic diversity of
47 *msp1* is meiotic recombination, which only occurs in the *Ano-*
48 *pheles* mosquito stage. These facts suggest that the extent of
49 *msp1* allelic diversity is largely dependent on the frequency of
50 recombination events and the intensity of malaria transmission.

Abbreviations: MSP-1, merozoite surface protein 1; *msp1*, merozoite surface protein 1 gene; CQ, chloroquine; *pfcrt*, *Plasmodium falciparum* chloroquine resistance transporter; LD, linkage disequilibrium; EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol.

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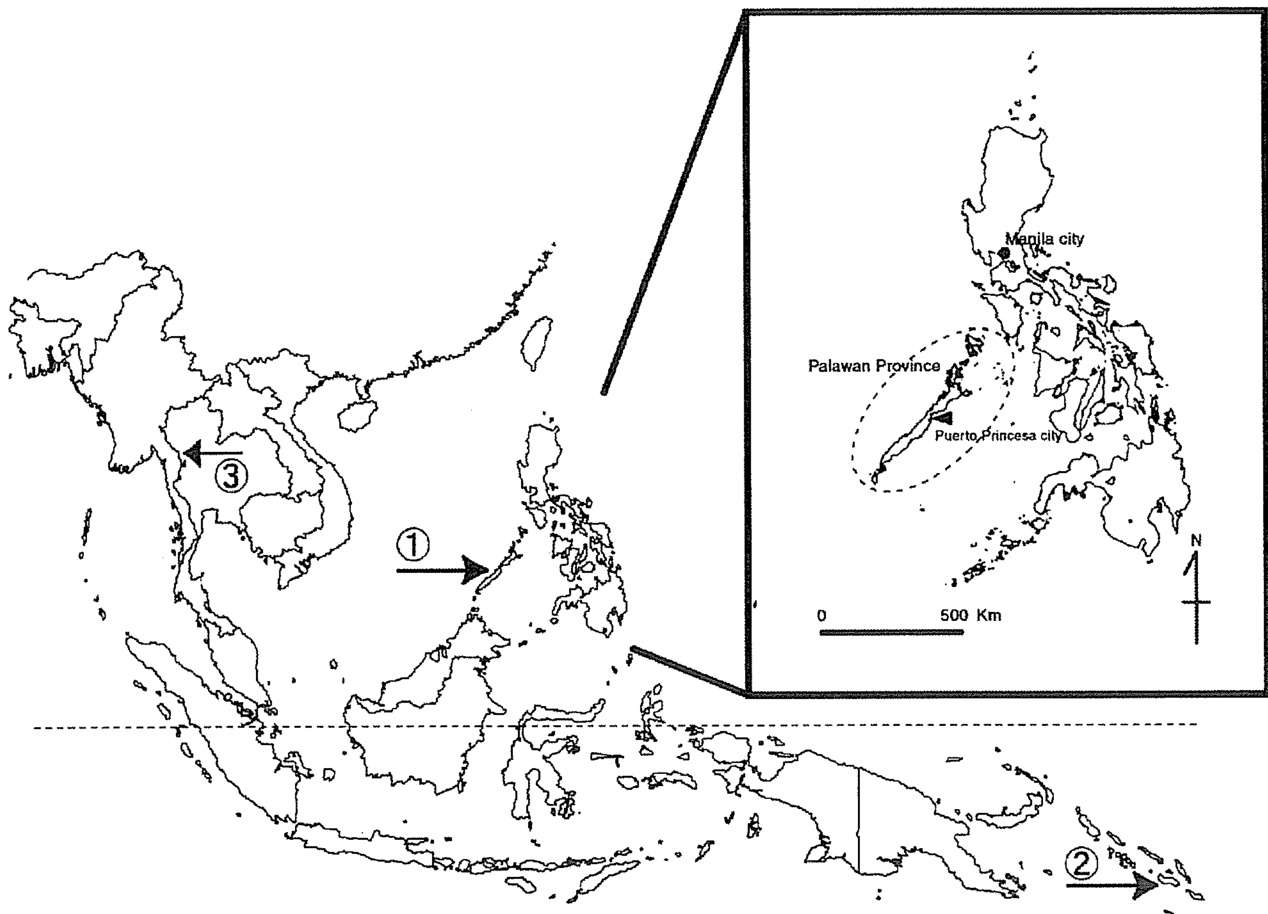

Fig. 1. Location of the study area. (1) Palawan Island in the Philippines, (2) northern Guadalcanal in The Solomon Islands and (3) Mae Sod in northwestern Thailand. Major islands of Palawan Province are circled with broken line in the box. Puerto Princesa is indicated by an arrowhead. Manila City is indicated by a closed circle.

In high-transmission areas in Africa, a high frequency of intragenic recombination events in *msp1* has been observed [9].

To investigate allelic diversity of *msp1* in natural *P. falciparum* populations, we have chosen *msp1* haplotypes as a polymorphic genotype marker [10–12]. *msp1* haplotypes are defined as unique combinations of 5′ recombinant types (combinations of allelic types in blocks 2, 4a, 4b, and 6) and 3′ sequence types (block 17). The extent of *msp1* allelic diversity is estimated by the number of distinct *msp1* haplotypes prevalent in a population and the haplotype diversity (*h*) [13], which is a population genetic index of allelic diversity. The frequency of recombination events in *msp1* can be inferred from analysis of linkage disequilibrium (LD) between polymorphic blocks/sites within *msp1*. Previous studies suggest that the extent of *msp1* allelic diversity and the frequency of recombination events in *msp1* are generally high in high-transmission areas and low in low-transmission areas [12,14,15]. However, not all studies have shown such an association between *msp1* allelic diversity and the intensity of malaria transmission. In a recent study, we observed relatively low allelic diversity of *msp1* in *P. falciparum* populations from the Solomon Islands, where the intensity of malaria transmission is very high [16].

We are interested in *msp1* allelic diversity on islands. *P. falciparum* populations on islands are generally small and have few opportunities to encounter novel *msp1* alleles from other populations, compared with conditions on continents, where novel alleles is recurrently and easily introduced from outside a population because of geographical non-isolation. This suggests that there are differences in population genetic features of *P. falciparum msp1* allelic diversity between island populations and continental populations. We previously observed relatively low frequency of recombination events in *msp1* in populations from the southwestern Pacific islands, compared with populations from northwestern Thailand [16]. In the present study, we investigated recombination-based allelic diversity of *msp1* in a *P. falciparum* population from Palawan, the Philippines, where malaria prevalence is hypo- to mesoendemic. The present results indicate that there is relatively high diversity of *msp1* haplotypes in Palawan, compared with a population from the Solomon Islands, whereas the frequency of recombination events in Palawan is low, which is consistent with our hypothesis that the extent of *msp1* allelic diversity is not always dependent on the intensity of malaria transmission. We also observed a high prevalence of the chloroquine (CQ)-sensitive allele of *P. falciparum* CQ resistance transporter (*pfcrt*) [17] in Palawan. Contribution of this high prevalence of CQ-sensitive *pfcrt* to the relatively high *msp1* diversity in Palawan is discussed.

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2. Materials and methods

2.1. Study area and sample collection

Field isolates of *P. falciparum* were collected between May 1997 and October 1997 on Palawan Island, the Philippines. Palawan Island is the largest and most populated island in Palawan Province, an island province composed of 1768 islands, located 600 km southwest of Manila (Fig. 1). This island is 425 km long and 8.5 to 40 km wide, with a population of 584,490 (according to the year 2000 census [18]). Although malaria in Palawan Province is reportedly hypo- to mesoendemic [19], with a slide positive rate of 5.4% in 1997 [20], the annual parasite incidence was 10.07 (slide-positives per 1000) in 1997, which was the sixth highest of the 78 provinces of the Philippines [20]. The total population of Palawan Province (755,412 in the year 2000 census [18]) has been growing rapidly and continuously due to migration from other areas of the Philippines. The majority of those migrants settle on Palawan Island and account for 63% of its residents. Migrants mainly come from Central Luzon, Southern Luzon and the Visayas, where malaria is less endemic than in Palawan. Malaria transmission is perennial, with a peak during the rainy season from May to December. The main malaria vectors in Palawan are *Anopheles flavirostris*, *A. balabacensis*, *A. litoraris*, and *A. maculatus*; the primary and secondary vectors are *A. flavirostris* and *A. balabacensis*, respectively [21]. Both *P. falciparum* and *P. vivax* are prevalent in Palawan; 71.5% of the confirmed cases in 1997 (3854/5386) were due to *P. falciparum*, in contrast to other areas in the Philippines where *P. vivax* predominates [20]. As of 1997, the first-, second- and third-line drugs used to treat malaria in the Philippines were chloroquine, sulfadoxine–pyrimethamine, and quinine, respectively [22].

A total of 114 *P. falciparum* isolates were collected at Palawan Provincial Hospital and Malaria Control Service Palawan Office located in Puerto Princesa, the capital city of Palawan Province (Fig. 1). The isolates were obtained from 81 out-patients and 33 in-patients with symptomatic uncomplicated malaria; the patients had a mean age of 29.8 years (range, 5–69 years). Most of the patients ($n=84$) were residents of Puerto Princesa city; 15 patients were residents of 6 municipalities outside the city; and 15 patients had unknown addresses. Malaria was diagnosed by microscopic examination of Giemsa-stained blood smears. Venous blood was collected from *P. falciparum*-positive patients and stored frozen at −20 °C. Parasite genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, Germany), as described elsewhere [23,24]. Informed consent was obtained from the donors or their guardians. This study has been approved by the Palawan Provincial Health Office.

For comparison, we also included data previously obtained from *P. falciparum* isolates collected in the Solomon Islands [16] and northwestern Thailand [11]. These isolates are described in detail elsewhere [16]. The isolates from the Solomon Islands ($n=90$) were collected from individuals with clinical malaria or asymptomatic malaria in northeastern Guadalcanal in 1995 and 1996. The isolates from Thailand ($n=111$) were clinical isolates collected from patients attending a malaria clinic in Mae Sot near the Thailand–Myanmar border, in 1995.

In this study, we aimed to investigate recombination-based allelic diversity of *P. falciparum msp1* in Palawan Island and compared with that in the Solomon Islands (northern Guadalcanal) and northwestern Thailand (Fig. 1). For this comparison, the estimates of entomological inoculation rate (EIR) would be suitable for standardization of the intensity of malaria transmission across geographic areas. EIR in Palawan Island has been reported to be 9.7 bites per person per year (based on sampling by human bait catch and microscopic detection of sporozoites) [25]. EIRs in northern Guadalcanal were reportedly 584–1022 bites per person per year [16] (based on sampling by human bait catch and sporozoite detection by enzyme-linked immunosorbent assay (ELISA) [26]), a value comparable with those in high-transmission areas in Africa such as Tanzania [27]. Thus, malaria transmission intensity in Palawan Island seemed to be much lower than in the Solomon Islands. Consistently, the slide positive rate was lower in Palawan Island (5.4%) [20] than in northern

Fig. 2. Structure of *Plasmodium falciparum msp1* and strategy for determination of 5′ recombinant type and 3′ sequence type. Inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For variable blocks, MAD20-type, K1-type, and RO33-type sequences are presented by half-tone, slashed, and closed bars, respectively. The 5′ recombinant type was determined by PCR amplification of blocks 2 to 6 using allelic type-specific primers for blocks 2 and 6, followed by nested PCR between blocks 4a and 4b using allelic-type-specific primers for blocks 4a and 4b. The six amino acid substitutions within block 17, indicated by one-letter codes, were determined by direct sequencing. A previously unidentified amino acid substitution is indicated in italics (see Results). The 3′ sequence type is a unique combination of these substitutions.

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Guadalcanal (39%) [16]. Transmission intensity in Mae Sod, northwestern Thailand is extremely low, as indicated by an estimated "incidence" of 0.8 infections per person per year (in 1991) based on dispensary attendance in a refugee camp located in this area [28].

2.2. Determination of *msp1* haplotypes

msp1 haplotypes are defined as unique combinations of 5′ recombinant types and 3′ sequence types (Fig. 2). 5′ recombinant types are defined as unique combinations of allelic types of blocks 2, 4a, 4b, and 6, which comprise a 1.1 kb region in the 5′ region of *msp1*. Our typing method involves nested PCR, and can distinguish 24 distinct 5′ recombinant types generated by intragenic recombination between *msp1* alleles [11]. Protocols for determination of 5′ recombinant types by PCR using allelic-type-specific primers have been described in previous reports [16]. Preliminary study revealed that there was no significant difference in frequency distribution of 5′ recombinant types between in-patients (n=32) and out-patients (n=65) in Palawan (P=0.927). (Similarly, there was no significant difference in frequency between individuals with clinical malaria and those with asymptomatic malaria in the Solomon Islands [16]). Although the rate of multiple infections of 5′ recombinant type and the multiplicity of infection were slightly higher in in-patients than in out-patients (rate of multiple infections, 34.4% *versus* 32.3%; multiplicity of infection, 1.5 *versus* 1.42), these differences were not significant. Therefore, the data for in-patients and out-patients were combined for further analysis.

The 3′ sequence types are defined as unique combinations of single-nucleotide polymorphism in block 17, which is a 0.4-kb region in the 3′ end of *msp1*. The sequence of block 17 was determined by direct sequencing of isolates that harbored a single 5′ recombinant type. A DNA fragment covering the entire coding region of *msp1* was amplified using Takara LA Taq (Takara Shuzo, Japan) in a 20-μl reaction mixture for 40 cycles essentially as described elsewhere [23,24], except that the primers used were UPF1 (5′-GGCTAATGTAAAATGCAAAAATAAATGT-3′) and DWR1 (5′-ACATGACTAAAATATCACTATTCCTGT-3′). The PCR product was diluted 10-fold, and a 2-μl aliquot was used as the template for nested PCR amplification for 20 cycles in a 50-μl reaction mixture using primers UPF3 (5′-AATAAATGTATACA-TATTTTTGCTAAGTCA-3′) and DWR3 (5′-TTAAGGTAA-CATATTTTAACTCCTACA-3′). The nested PCR product was purified using the QIAquick PCR purification kit (QIAGEN, Germany), and was sequenced from both directions using the BigDye Terminator Cycle Sequencing Kit (ver 3.1) and an ABI 3100 sequencer. The sequencing primers were C17aFs [11] and DWR3. Sequences were verified by re-sequencing a DNA template independently amplified from genomic DNA. Five amino acid substitution sites have previously been identified in block 17 in field isolates from various geographic areas [15,29] (E or Q at residue 1644; T or N at 1691; SR or NG at 1700–1701; and L or F at 1716 [8]). In addition, a new substitution from L to I at residue 1740 was identified in the present study in two isolates from Palawan Island (see Results). We hereafter designate unique combinations of these six residues as 3′ sequence type.

2.3. Genotyping of *pfcrt*

To assess the prevalence of polymorphism of the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) in Palawan Island, we sequenced the 2nd exon of *pfcrt*, which contains a polymorphic region at residues 72 to 76. Isolates harboring a single 5′ recombinant type (n=59) were subjected to direct sequencing. Simultaneously, 71 Thai isolates were sequenced for comparison. Amplification of the 2nd exon and sequencing were performed as previously described [16], except that the dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used. For those samples containing a mixture of both CQ-sensitive and CQ-resistant *pfcrt* alleles, DNA fragments carrying CQ-sensitive and CQ-resistant alleles were separately amplified by semi-nested PCR using two reverse primers (CQ76*K*r [5′-TTTGTTTAAAGTTCTTTTAGCAAAA-*ATTT*-3′], specific to the CQ-sensitive allele; and CQ76*T*r [5′-TTTGTTTAAAGTTCTTTTAGCAAAAA*TTG*-3′], specific to the CQ-resistant allele), in combination with the forward primer CQf4 [16]. In this case, the 1st PCR product, diluted 100-fold, was subjected to nested PCR using AmpliTaq Gold (Applied Biosystems, Foster City, CA) for 12 cycles.

2.4. Statistical analysis

Frequency distributions of *msp1* 5′ recombinant types and *msp1* haplotypes were compared between different parasite populations using the two-tailed χ^2 test, with Yates' correction for data sets of fewer than 10 items, and Fisher's exact probability test. Pairs of polymorphic blocks 2, 4a, 4b and 6 and polymorphic sites in block 17 were subjected to the R^2 test to assess linkage disequilibrium (LD), as described elsewhere [11]. To avoid false detection of LD deriving from inclusions of minor alleles, non-informative pairs (frequency of minor alleles <10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Significance of LD was assessed using the χ^2 test and the two-tailed Fisher's exact probability test. The haplotype diversity index (h) and its standard error (SE) were calculated as described elsewhere [16]. Probability values of $P<0.05$ were considered to indicate statistical significance.

3. Results

3.1. Diversity and multiplicity of 5′ recombinant types (blocks 2 to 6)

Table 1 summarizes diversity profiles of 5′ recombinant types in *P. falciparum* isolates from Palawan, in comparison with those from the Solomon Islands and Thailand. The number of distinct 5′ recombinant types in Palawan (n=9) was close to the number in the Solomon Islands (n=8), but was much lower than the number in Thailand (n=14). The rate of multiple infections of 5′ recombinant types (33%) and multiplicity of infection (*i.e.*, mean number of 5′ recombinant types per isolate) (1.44) in Palawan were also similar to those of the Solomon Islands, but were substantially lower than those of Thailand. These results indicate that the diversity and extent of mixed

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t1.1 Table 1
t1.2 Diversity profiles of *P. falciparum* isolates in Palawan, the Philippines in comparison with the Solomon Islands and Thailand

	Palawan	Solomon Islands [a]	Thailand [a]
Number of isolates	114	90	111
Number of PCR positives	97 (85.1%)	82 (91.1%)	107 (96.4%)
Number of 5′ recombinant types/area	9	8	14
Rate of multiple infections	32/97 (33.0%)	29/82 (35.4%)	103/107 (96.3%)
Multiplicity of infection [b]	1.44	1.41	3.6

t1.9 [a] Data from Sakihama et al. [21].
t1.10 [b] Mean number of *msp1* 5′ recombinant types per isolate.

279 infections of 5′ recombinant types are relatively limited in Palawan and the Solomon Islands.

281 Frequency distribution of 5′ recombinant types greatly differed among the three geographic areas (Fig. 3) ($P<10^{-7}$ for all pairs compared). The observed differences are due to different frequencies of 5′ recombinant types #2, #16, #21, #22, #23 and #24.

286 *3.2. The 3′ sequence types (block 17)*

287 A total of 57 Palawan isolates were successfully sequenced, and six distinct 3′ sequence types were detected (Fig. 4): E/TSR/L (n=17), Q/KNG/L (n=18), E/KNG/L (n=3), Q/TSR/L (n=3), Q/KNG/F (n=14), and E/TSR/L* (n=2). E/TSR/L* is a new type containing a previously unknown mutation from L (codon: TTA) to I (ATA) at amino acid position 1740 (Fig. 4), which is downstream from a putative post-translational cleavage site between residues 1726 and 1727 [30]. 294

3.3. Diversity of msp1 haplotypes 295

296 Table 2 summarizes the distribution of *msp1* haplotypes, unique combinations of 5′ recombinant type and 3′ sequence type in Palawan, in comparison with those of the Solomon Islands and Thailand. The number of *msp1* haplotypes detected in Palawan (n=15) was comparable to the number in Thailand (n=16), but was much greater than the number in the Solomon Islands (n=8). Consistent with this, haplotype diversity (h) in Palawan (0.88) was comparable to that of Thailand (0.89) (P=0.66), but was greater than that of the Solomon Islands (0.80) (P=0.033). 304

305 We observed marked difference in the distribution of *msp1* haplotypes among Palawan, the Solomon Islands and Thailand ($P<10^{-5}$) (Fig. 5). Of the 15 distinct *msp1* haplotypes found in Palawan, seven were unique to Palawan, of which MMMM-Q/KNG/F had the highest frequency. Three haplotypes (KKKK-Q/KNG/L, MMKM-E/TSR/L, and MMMM-E/TSR/L) in Palawan were shared with the Solomon Islands and Thailand. Five haplotypes were shared with the Solomon Islands only, and five haplotypes were shared with Thailand only. These prevalence patterns of *msp1* haplotypes suggest that *msp1* haplotypes are geographically stable in the three regions studied. 315

3.4. Linkage disequilibrium in msp1 316

317 Based on the frequency distributions of *msp1* haplotypes summarized in Table 2, we analyzed linkage disequilibrium 318

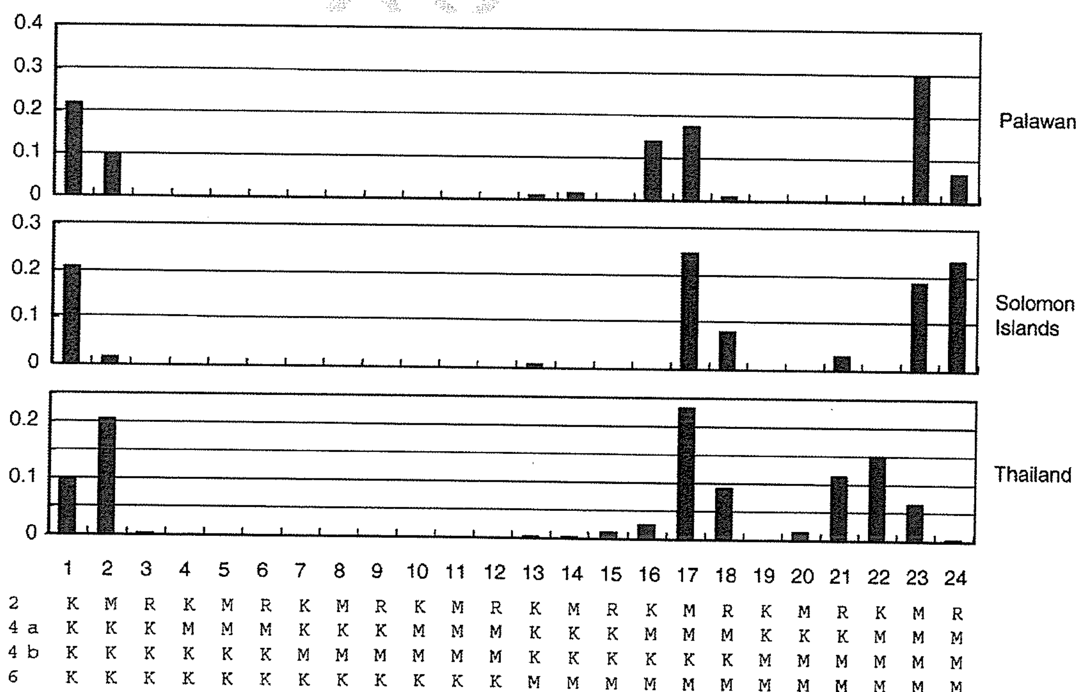

Fig. 3. Frequency distribution of *P. falciparum msp1* 5′ recombinant types in isolates from Palawan, Solomon Islands, and Thailand. Twenty-four potential 5′ recombinant types, defined as unique combinations of allelic types in blocks 2, 4a, 4b, and 6, are shown at the bottom, in which K, M and R are abbreviations of three allelic types: K1, MAD20, and RO33, respectively.

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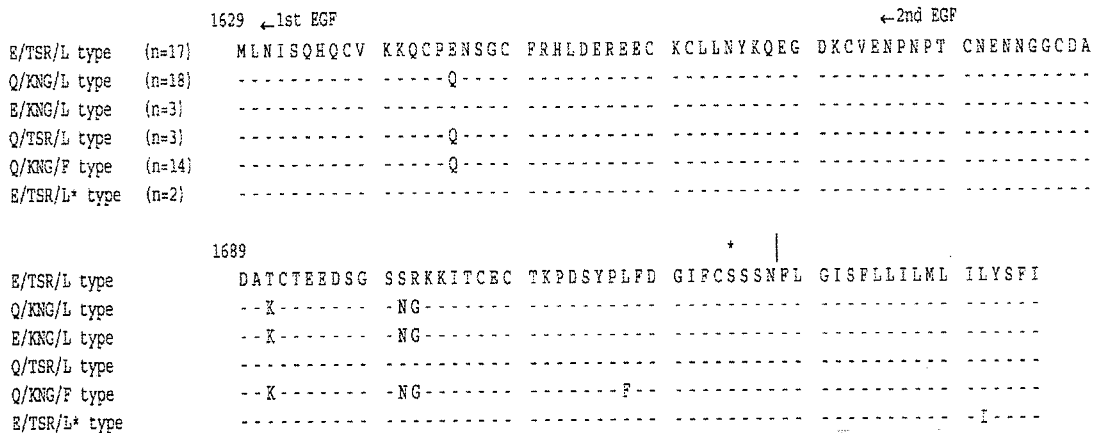

Fig. 4. Amino acid sequence alignment of *P. falciparum msp1* block 17 in isolates from Palawan, Philippines. Arrows indicate the starting points of two epidermal growth factor (EGF)-like domains, and an asterisk indicates a putative attachment site for a glycosylphophatidylinositol (GPI) anchor. A vertical line indicates a putative post-translational cleavage site between residues 1726 and 1727 [30].

(LD) between polymorphic blocks and polymorphic sites in block 17 (Fig. 6). Of the 21 informative pairs within and between polymorphic blocks/sites in Palawan, four pairs (between block 2 and two polymorphic sites in block 17) were not significant. Overall, LD in Palawan was very strong, and was comparable to that of the Solomon Islands (where one out of 15 pairs was not significant), but was stronger than that of Thailand (where seven out of 15 pairs were not significant). These findings suggest that the frequency of recombination events in *msp1* is relatively low in Palawan and the Solomon Islands, compared with Thailand.

3.5. Polymorphism in *pfcrt*

Of the 59 Palawan isolates sequenced, 33 were mono-infections with either the CQ-sensitive *pfcrt* allele ($n=7$) or the CQ-resistant *pfcrt* allele ($n=26$), and 26 had a mixture of both CQ-sensitive and CQ-resistant alleles. The CQ-resistant *pfcrt*

Table 2
Diversity of *P. falciparum msp1* haplotypes in isolates from Palawan, the Philippines incomparison with the Solomon Islands and Thailand

Location	5′ recombinant type	3′ sequence type						No. of *msp1* haplotype [a]	$h\pm$SE
		Q/KNG/L	Q/KNG/F	E/KNG/L	Q/TSR/L	E/TSR/L	(Total)		
Palawan	KKKK	9	0	0	0	0	(9)		
	MKKK	5	0	0	0	0	(5)		
	MKKM	1	0	0	0	0	(1)		
	KMKM	1	0	3	1	5	(10)		
	MMKM	1	0	0	0	9 [b]	(10)		
	MMMM	1	14	0	1	5	(21)		
	RMMM	0	0	0	1	0	(1)		
	(Total)	(18)	(14)	(3)	(3)	(19)	(57)	15	0.88±0.02
Solomon Islands	KKKK	9	0	0	0	0	(9)		
	MMKM	0	1	0	0	13	(14)		
	RMKM	0	0	0	0	1	(1)		
	MMMM	1	0	5	0	3	(9)		
	RMMM	0	0	0	0	14	(14)		
	(Total)	(10)	(1)	(5)	(0)	(31)	(47)	8	0.80±0.03
Thailand	KKKK	2	0	0	0	0	(2)		
	MKKK	9	0	4	0	0	(13)		
	KMKM	0	0	0	0	1	(1)		
	MMKM	0	2	12	0	2	(16)		
	RMKM	0	0	3	0	0	(3)		
	RKMM	0	0	3	0	1 [c]	(4)		
	KMMM	1	1	2	0	3	(7)		
	MMMM	0	0	1	0	1	(2)		
	(Total)	(12)	(3)	(25)	(0)	(8)	(48)	16	0.89±0.03

[a] *msp1* haplotypes are defined as unique associations of 5′recombinant type and 3′ sequence type.
[b] Includes two isolates with a new amino acid substitution at residue 1740.
[c] One isolate with E/TSG/L type.

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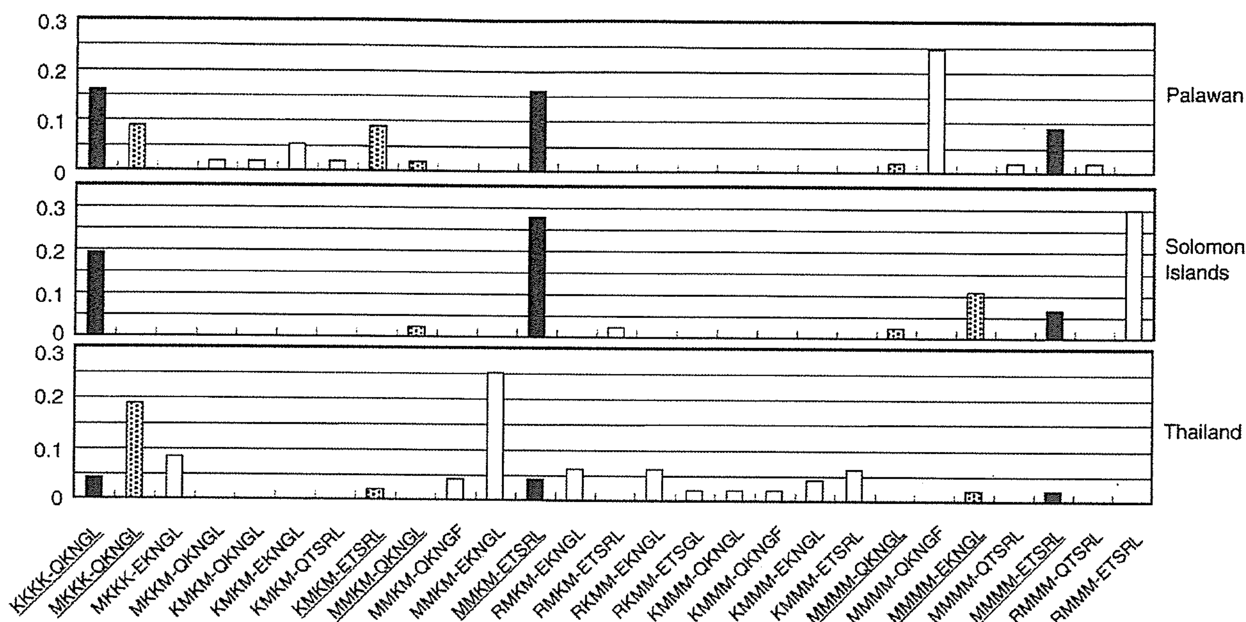

Fig. 5. Frequency distribution of *P. falciparum msp1* haplotypes in Palawan, the Solomon Islands, and Thailand. *msp1* haplotypes, defined as unique combinations of 5′ recombinant types and 3′ sequence types, are indicated along the horizontal axis. MMKM-E/TSR/L type includes two isolates with a new amino acid substitution at residue 1740 (see Fig. 4). Closed column, haplotype detected in all three areas; half-tone column, haplotype detected in two of the three areas; open column, haplotype detected in only one area.

allele was of Papua New Guinean type, with the amino acid sequence SVMNT at residues 72 to 76 [31]. We did not detect the Southeast Asian type with the amino acid sequence CVIET at residues 72 to 76 [32]. In contrast, all of the Thai isolates ($n=71$) had the CVIET-type *pfcrt* allele. Strikingly, the wild-type CQ-sensitive *pfcrt* allele, with the amino acid sequence CVMNK at residues 72 to 76, was detected in 33 Palawan isolates, with a prevalence rate of 38.8%. This is in sharp contrast to the monomorphic prevalence of the Papua New Guinean type CQ-resistant *pfcrt* allele in the Solomon Islands ($n=57$) and Vanuatu ($n=142$) [16,33]. There was no significant difference in the distribution of *msp1* haplotypes between isolates harboring the CQ-sensitive *pfcrt* allele and those with the CQ-resistant *pfcrt* allele (data not shown), suggesting that there is no genetic linkage between the gene *msp1* in chromosome 9 and the gene *pfcrt* in chromosome 7.

4. Discussion

It is to be expected that the extent of allelic diversity of *P. falciparum msp1* would be low in a low-transmission area such as Palawan Island, because *msp1* allelic diversity is mainly generated by meiotic recombination events that takes place solely in the mosquito stage of the *P. falciparum* life cycle. In the present study, the frequency of recombination events in *msp1*, as inferred from LD, was relatively low on Palawan Island. However, the extent of *msp1* allelic diversity on Palawan Island was higher than that of the Solomon Islands, where malaria transmission is very high. These findings support our hypothesis that the extent of *msp1* allelic diversity is not always dependent on the intensity of malaria transmission. This hypothesis was originally raised in our previous study of parasite populations from the Solomon Islands, in which *msp1* allelic diversity was very limited despite a high level of malaria transmission. In contrast, the present study of the Palawan

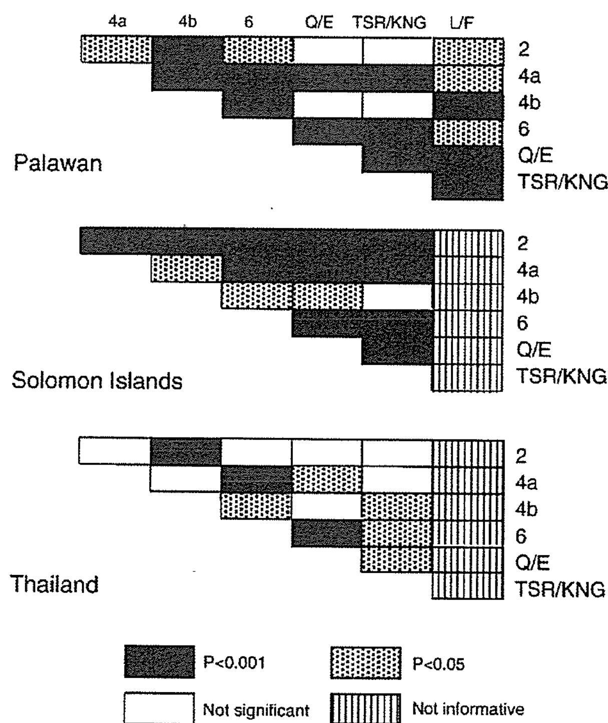

Fig. 6. Linkage disequilibrium in *P. falciparum* merozoite *msp1* in populations from Palawan, the Solomon Islands, and Thailand. Pairs of polymorphic blocks (2, 4a, 4b, and 6) and three polymorphic residue sites (Q/E, TSR/KNG, and L/F) in block 17 were subjected to the R^2 test.

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368 population shows the opposite situation: *i.e.*, relatively high
369 *msp1* allelic diversity with a low level of transmission.
370 Therefore, it is likely that different mechanisms are involved
371 in the lack of association between the extent of *msp1* allelic
372 diversity and the intensity of malaria transmission.

373 The frequency of recombination events in *msp1* is determined
374 not only by the frequency of malaria transmission but also by other
375 variables: the frequency of mixed-allele infections, the mean
376 number of alleles per person, and the repertoire of alleles in a local
377 area [12]. Mixed-allele infections are required for recombination
378 events to occur between heterologous *msp1* alleles. Therefore,
379 those variables would affect the rate of inbreeding, and a high
380 inbreeding rate could lead to limited recombination events *de facto*
381 in spite of extensive genetic shuffling during meiosis in the
382 mosquito stage in a high transmission area. In the parasite
383 populations from the Solomon Islands studied previously, values
384 of those variables are rather low, suggesting a high inbreeding rate,
385 which in turn can limit recombination events (and subsequently
386 lower the allelic diversity of *msp1*). In the present study, the
387 Palawan parasite population had low rates of mixed 5′ recombinant
388 type infections and multiplicity (the mean number of 5′
389 recombinant types per person), with levels similar to those of the
390 Solomon Islands. However, diversity profiles, the number of *msp1*
391 haplotypes, and haplotype diversity (*h*) were higher in Palawan
392 than in the Solomon Islands. Therefore, the observed relatively high
393 allelic diversity of *msp1* in Palawan may be due to a mechanism not
394 directly related to the frequency of malaria transmission.

395 Then, a question arises: why *msp1* allelic diversity is
396 relatively high in Palawan, where recombination events are
397 limited? We consider that the high prevalence of CQ-resistant
398 *pfcrt* allele observed in Palawan may be partly involved in the
399 *msp1* diversity. It is generally believed that the parasites having
400 CQ-resistant *pfcrt* allele does not necessarily mean that they are
401 resistant to CQ, and that first line treatment of CQ might have
402 minor influence on parasite transmission by asymptomatic
403 individuals, where parasites are known to persist for long
404 periods. These arguments may be valid in the situation in highly
405 endemic areas of malaria such as African tropics, where
406 repeated malaria infections and asymptomatic cases are
407 common and CQ is still effective in asymptomatic individuals
408 harboring CQ-resistant *pfcrt* allele, who are immune or semi-
409 immune to malaria due to synergistic effect of immunity and the
410 drug. However, these arguments do not always apply to
411 geographic areas other than African tropics, particularly to
412 islands, where *P. falciparum* populations are isolated and the
413 influx of new genotypes from outside is very limited. In
414 addition to this limited gene flow, malaria-immune asymptom-
415 atic cases are rare in areas with low or moderate malaria
416 transmission such as Palawan Island and northwestern Thai-
417 land. (Asymptomatic cases are exceptionally not rare in the
418 Solomon Islands, where malaria transmission is intense).
419 Taking these malaria epidemiological settings into considera-
420 tions, it is likely that frequent and extensive mass administra-
421 tions of CQ on islands as done in the Solomon Islands would
422 cause severe bottleneck of local parasite populations and
423 subsequent reduction in the parasite genotypes. Therefore, the
424 100% prevalence of CQ-resistant *pfcrt* allele would reflect
425 selective pressure of CQ in the past and reduction in parasite
426 genotypes including *msp1* alleles. Conversely, a high preva-
427 lence of CQ-sensitive *pfcrt* allele would reflect less intense
428 selective pressure, which is not so strong to cause population
429 bottleneck and reduction in the diversity of *msp1* alleles. We
430 observed here that the wild-type CQ-sensitive *pfcrt* allele
431 remained at a substantially high level (39%) in Palawan. No
432 wild-type *pfcrt* allele was found in the Solomon Islands, where
433 nation-wide mass administration of CQ was implemented in the
434 1980s (in Guadalcanal, 1984–1987) [34]. The population
435 bottleneck caused by mass CQ administration in the Solomon
436 Islands would presumably have been intense, and thereby
437 caused a marked reduction in the diversity of *P. falciparum*
438 genotypes in the area. Consistently, we observed a low level of
439 *msp1* allelic diversity. Additionally, continued usage of CQ as
440 the first-line drug, while it is still effective against *P. vivax*,
441 might have contributed to persistence of a low level of parasite
442 genetic diversity. On the other hand, no such mass drug
443 administration has ever been performed in Palawan. Thus,
444 persistence of a high prevalence of CQ-sensitive parasites may
445 have contributed to the relatively high allelic diversity of *msp1*
446 observed in Palawan.

447 In contrast to malaria epidemiological settings on islands,
448 monomorphic prevalence of a CQ-resistant *pfcrt* allele did not
449 apparently limit the genetic diversity of *msp1* in the Thai
450 parasite population, a situation opposite to on islands. We
451 consider a likely explanation as follows: the Thai parasite
452 population is not isolated within a limited land area and subject
453 to recurrent gene flows of novel genotypes from outside, most
454 probably from Myanmar where malaria transmission is higher
455 than in Thailand. Multiplicity of infection was very high in
456 northwestern Thailand (3.6), whereas the intensity of malaria
457 transmission was extremely low with expected EIR of 0.8 [28].
458 Elevated levels of mixed genotype infections may well increase
459 the frequency of recombination events of *msp1* alleles in the
460 mosquito and subsequently a high diversity of *msp1* alleles
461 would be maintained in the Thai population. Rohr et al. have
462 recently demonstrated that, in the Thai–Myanmar border
463 area, the majority of multiple or polyclonal infections with
464 *P. falciparum* are generated by the bite of a single mosquito
465 infected with multiple parasite genotypes [Rohr C, Singlam S,
466 Pumpieng K, Nosten F, Anderson TJC. Kinship relationships in
467 polyclonal malaria infections. In: Abstracts of the 11th
468 International Congress of Parasitology (Glasgow, Scotland 6–
469 11th Aug 2006). Abstract No. a1960], consistent with the high
470 multiplicity of infection frequently reported in this area despite
471 of an extremely low transmission intensity [35].

472 The present study provides the first molecular evidence that the
473 prevalence of CQ resistance in *P. falciparum* in Palawan did not
474 reach 100% in 1997. CQ resistance in Palawan was first reported
475 by Baird et al. [19], who conducted a field survey in April to May
476 1995 and found that 23 to 39% of *P. falciparum* isolates were CQ-
477 resistant in an *in vitro* test. In addition, Bustos et al. [36,37]
478 observed a cure rate of only 30.4% (7/23) in the control CQ
479 monotherapy group during a clinical trial of antimalarial drug
480 therapy conducted at Palawan Provincial Hospital between
481 October 1994 and February 1995. These reports suggest that

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CQ-sensitive and CQ-resistant parasite populations co-existed in Palawan in the mid-1990s. Here, we have confirmed this co-existence in 1997, with a prevalence rate of 39% for the CQ-sensitive *pfcrt* allele. Identification of the Papua New Guinea-type *pfcrt* allele in Palawan as the sole CQ-resistant *pfcrt* allele is consistent with a recent report on the distribution of *pfcrt* alleles in the Philippines: all twelve *P. falciparum* isolates collected at Malaria Control Service Palawan Office between 1997 and 1999 (four isolates per year) had the CQ-resistant *pfcrt* allele of Papua New Guinea type, and the Southeast Asia type CQ-resistant *pfcrt* allele was only detected in the vicinity of a refugee processing center that accommodated refugees from Southeast Asia at the time of sample collection (between 1989 and 1993) [38]. Further study is required to monitor subsequent change in the prevalence of CQ resistance in *P. falciparum* in Palawan (*e.g.*, whether the prevalence of CQ resistance has since reached 100%) and elucidate how this change has influenced recombination-based diversity of the *msp1* gene. Follow-up monitoring of the prevalence of CQ resistance will also contribute to optimization of drug-based malaria control in Palawan.

In summary, the present study shows relatively high allelic diversity of *msp1* in Palawan, compared with the Solomon Islands, whereas the frequency of recombination events in *msp1* (as determined by LD analysis) was limited, presenting additional evidence for our hypothesis that allelic diversity of *msp1* is not always dependent on the intensity of malaria transmission. Persistence of a substantially high level of the wild-type CQ-sensitive *pfcrt* allele may have contributed to the higher allelic diversity of *msp1* in Palawan, where the parasite population is isolated and gene flow is limited.

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2-Cys Peroxiredoxin TPx-1 is involved in gametocyte development in *Plasmodium berghei*

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Abstract

Peroxiredoxins (Prxs) constitute a ubiquitous family of antioxidant enzymes involved in diverse cellular functions including cell proliferation and differentiation. To investigate the physiologic role of typical 2-Cys Prx in malaria parasites (TPx-1), we disrupted this gene in the rodent malaria parasite *Plasmodium berghei* (*pbtpx-1*). The gene-disrupted parasite (Prx KO) developed normally in mouse erythrocytes and multiplied at a rate similar to that of the parent strain (WT) during the experimental period. The normal growth rate was not altered after 10 passages, and the level of 8-hydroxy-2′-deoxyguanosine, which accumulates in the parasite genome during the cell cycle, was similar between Prx KO and WT. These results suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx, and that it is not essential for asexual parasite growth in mouse erythrocytes. However, Prx KO produced up to 60% fewer gametocytes, sexual-stage parasites involved in the transition between the mammalian host and the mosquito, than WT did. The peak of gametocytemia was also delayed; however, the male/female ratio of gametocytes and the exflagellation activity of male gametocytes were normal. These results suggest that TPx-1 is required for normal gametocyte development but does not affect the male/female gametocyte ratio or male gametogenesis. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, these findings suggest, for the first time, the involvement of Prx in the sexual development of the malaria parasite.
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Keywords: Gametocyte; Peroxiredoxin; *Plasmodium berghei*; Thioredoxin peroxidase

1. Introduction

As *Plasmodium* spp. actively proliferate within erythrocytes of their vertebrate hosts, large quantities of reactive oxygen species (ROS), which damage biologic macromolecules, are generated [1,2]. A major source of ROS in parasite cells is heme, a byproduct of hemoglobin digestion for amino acid procurement [3,4]. ROS are also generated when the organism is exposed to various stress conditions such as that induced by the host immune system [5]. *Plasmodium* spp. (the malaria parasites) are highly susceptible to oxidative stress, and their antioxidant defenses are considered to play essential roles in their asexual development. They are thus potential targets for chemotherapy [6,7].

To protect biologic macromolecules from the effects of ROS, aerobes have evolved efficient defense systems composed of nonenzymatic and enzymatic antioxidants [8]. The four major cellular antioxidant enzymes are superoxide dismutase, catalase, glutathione (GSH) peroxidase, and peroxiredoxin [9]. Peroxiredoxins (Prxs) constitute a family of proteins structurally homologous to the thiol-specific antioxidant of yeast [10] and have been identified in all living organisms, from bacteria to human [11,12]. There are three subtypes of Prxs, 1-Cys Prx, typical 2-Cys Prx, and atypical 2-Cys Prx. Although the cellular function of 1-Cys Prx and electron donor for the molecule remain controversial [12,13], 2-Cys Prxs have been found to act

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GSH, glutathione; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Prx, peroxiredoxin; ROS, reactive oxygen species

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as a terminal peroxidase that reduces hydrogen peroxide and organic hydroperoxides with the use of electrons donated by the thioredoxin (Trx) system [11,12]. With respect to the cellular functions of 2-Cys Prx, diverse findings have been reported in mammals, including modulation of cytokine-induced hydrogen peroxide levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation, and apoptosis [9,12,14].

Malaria parasites do not possess catalase or genuine GSH peroxidase in their genome, but the parasites are equipped with a 1-Cys Prx and two typical 2-Cys Prxs [1,2]. Thus, it is believed that GSH itself is the major redox buffer for transient H_2O_2 exposure and that the basal cellular peroxide flux is dealt with by the Trx system, which includes Prxs [7]. The 1-Cys Prx and one of the 2-Cys Prxs are expressed in the cytosol, and the other 2-Cys Prx is localized in mitochondria [15]. We recently reported that disruption of the gene encoding the cytosolic 2-Cys Prx (PlasmoDB; PF14_0368; PfTPx-1) in *P. falciparum* renders parasites hypersensitive to ROS and reactive nitrogen species, although it did not affect parasite growth under normal culture conditions [16]. This suggests that this Prx is not essential for parasite survival under culture conditions and that physiologic function of Prx in parasite cells remains to be elucidated. This Prx is constitutively expressed during the asexual development as well as in the gametocyte both in *P. falciparum* [17] and in the rodent malaria parasites [18,19]. To determine the cellular function of 2-Cys Prx in malaria parasites, we disrupted the gene encoding TPx-1 in the rodent malaria parasite *P. berghei* and analyzed the phenotype of the gene-disrupted parasite in infected mice. The disruptant showed normal asexual proliferation in mouse erythrocytes but showed a defect in gametocyte development, the sexual stage for transition to mosquitos that is induced by host environmental factors.

2. Material and methods

2.1. Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The parasite was maintained by mosquito transmission in *Anopheles stephensi* interspersed by a maximum of two serial passages in DBA/2 or Balb/c mice (Clea Japan, Tokyo, Japan). The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation of the International Medical Center of Japan.

2.2. Plasmid construction

For disruption of the PbTPx-1 gene, 5′ and 3′ portions of the gene were amplified by polymerase chain reaction (PCR) and cloned into the targeting vector pMD204 [20], which was supplied by the Malaria Research and Reference Reagent Resource Center; MR4/ATCC, Manassas, VA, USA. Each fragment contained part of the coding sequence and flanking region was amplified with sequence-specific primers and parasite genomic DNA. The primers used for the 5′ fragment were 5′-GG <u>GGG CCC</u> TCA CCA GCC TTA TTA AG-3′ and 5′-CC CCC <u>GTC GAC</u>AAT ATA TTT CTT TCC-3′ (*Apa*I and *Hinc*II sites are underlined). The primers used for the 3′ fragment were 5′-CG <u>GAA TTC</u> CGA GTT TGT AAA AGA AC-3′ and 5′-TT <u>CTG CAG</u> TCA TTT AAA ATA AAG-3′ (*Eco*RI and *Pst*I sites are underlined). The primers were designed on the basis of sequences in the *P. berghei* genome database provided by the *Plasmodium* Genome Resource (PlasmoDB; PB_5804.1 and PB000037.01.0). PCR products were purified and cloned into upstream or downstream of the pyrimethamine-resistant form of *P. berghei* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, which was used as a selectable marker. For gene targeting experiments, the plasmid was digested with *Apa*I and *Pst*I to separate the linear targeting construct from the plasmid backbone.

2.3. Transfection and selection of disruptant

Transfection and selection were performed essentially according to the protocols of Ménard and Janse [21]. Blood-stage parasites used for transfection were prepared from Jcl:Wistar rats (Clea Japan) that had been infected by intraperitoneal (i.p.) injection of 5×10^6 parasitized erythrocytes per animal. Animals were killed when parasitemia reached 3%. Blood was drawn from the animal into heparinized syringes (100 μg/ml of blood) by cardiac puncture under ether anesthesia. Samples were washed for 10 min at $160 \times g$ at room temperature (RT) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM HEPES and 20% heat-inactivated fetal calf serum. The packed cells were then resuspended in 40–50 ml (10 volumes of the blood sample) of the medium and cultured in glass petri dishes (20–25 ml of the blood suspension/dish) in a AnaeroPack® microaerophilic culture system, which provided 5% CO_2 and 8% O_2 gases condition (Mitsubishi Gas Chemical, Tokyo, Japan), for 16 h at 37 °C with gentle rotation (50 rpm). Blood suspensions in the dishes were then layered onto 7.5 ml Nycoprep™ density gradient 1.077 medium (Axis-Shield, Oslo, Norway) and centrifuged first at $160 \times g$ for 5 min and then at $360 \times g$ (by shifting to high speed before stop) for 15 min at RT. Schizonts at the interface were collected and washed with 40 ml culture medium for 5 min at $360 \times g$ at RT and then with 3 ml phosphate-buffered saline (PBS). Schizont pellets were resuspended in 400 μl PBS containing 50 μg linearized targeting vector, then transferred to 0.4 cm electroporation cuvettes for transfection. Mixtures were subjected to an electric pulse (800 V, 25 μF) with a Gene Pulser® II System (Bio-Rad, Hercules, CA, USA) and were immediately inoculated into two rats (200 μl electroporated schizont suspension/animal) by intravenous (i.v.) injection. Animals were treated with pyrimethamine (12.5 mg/kg body weight) at 30 h after inoculation every 12 h until parasites were no longer detected in the blood circulation. When the parasitemia increased again to 1–2%, rats were treated with the same dose of pyrimethamine, and resistant parasites were transferred to new rats in which the parasites multiplied in the absence of drug. When parasitemia had reached 1%, the parasite population in

each rat was separated into wild-type and disruptant by limiting dilution. Both parasite genomes were determined by PCR and Southern blot analysis.

2.4. Nucleic acid techniques

Parasite-infected blood was passed through a CF11 (Whatman, Maidstone, Kent, UK) column and span with Lymphoprep™ density gradient medium (Axis-Shield, Oslo, Norway) to remove leukocytes. Parasite-infected erythrocytes were then washed with PBS and lysed with PBS containing 0.05% saponin. Parasite pellets were washed several times with PBS, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Nucleic acids were extracted from parasite pellets. For Southern blot, genomic DNA was extracted by the standard phenol–chloroform method and incubated with *Spe*I. For Northern blot, total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Nucleic acids were separated by agarose gel electrophoresis and transferred onto HyBond N^+ membranes (Amersham Biosciences UK Ltd., Little Chalfont, UK). Membranes were probed with a 368-bp fragment spanning the 5′ sequence of *pbtpx-1* for Southern blot (Fig. 1B) and the entire coding sequence for Northern blot (Fig. 2B). The probes were labeled, hybridized, and detected with the AlkPhos Direct Labeling and Detection System (Amersham Biosciences).

2.5. Western blot analysis and indirect immunofluorescence assay

For Western blot analysis, parasite pellets were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 5% 2-mercaptoethanol [22]. After separation by SDS-PAGE (12.5%), parasite proteins were transferred electrophoretically to polyvinylidene difluoride sheets (Immobilon; Millipore, Bedford, MA, USA) and incubated with anti-recombinant PfTPx-1 (rPfTPx-1) rabbit IgG (25 μg/ml). Interspecific crossreactivity between PbTPx-1 and anti-rPfTPx-1 rabbit IgG has been reported [18]. Immune complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH, USA). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Indirect immunofluorescence assay was performed as described previously [15]. The IgG fraction of rabbit antisera to rPfTPx-1 was used at 20 μg/ml. Parasite cells on slides were mounted with Prolong AntiFade Solution (Molecular Probes, Eugene, OR, USA) and observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

2.6. Analysis of parasite development in mice

Five-week-old female Balb/c and C57B6 mice (Clea Japan) were infected with *P. berghei* (10^6 parasitized cells per mouse) by i.p. injection. Parasitemia and gametocytemia were determined by microscopic examination of Giemsa-stained thin blood films. Male and female gametocytes were distinguished by size and coloration. Numbers of exflagellation centers were counted according to a previously described method [23]. Accumulation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the parasite genome was evaluated with a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan) [24]. DNA for ELISA was extracted from parasites collected from infected mice (blood from 10 infected mice was pooled) when

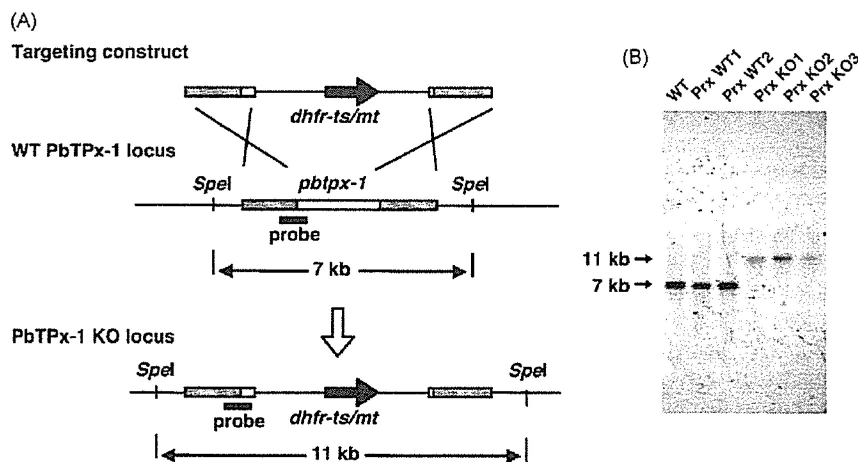

Fig. 1. Targeted disruption of the TPx-1 gene in *P. berghei*. (A) The targeting construct, composed of the 5′ and 3′ regions (hatched boxes) and partial open reading frame (open box) of the PbTPx-1 gene (*pbtpx-1*) and the pyrimethamine-resistant variant of DHFR-TS gene of *P. berghei* (*dhfr-ts/mt*) as a selectable marker (filled arrow), was integrated into the wild-type (WT) PbTPx-1 locus by double-crossover homologous recombination. Recombination disrupts *pbtpx-1* and creates the locus containing *dhfr-ts/mt* (PbTPx-1 KO locus), which confers pyrimethamine resistance to disruptants. The *Spe*I restriction sites and the position of the probe used for Southern blot analysis in B are indicated. (B) Southern blot analysis of genomic DNA samples from the parent parasite (WT), two wild-type parasite populations with pyrimethamine resistance (*dhfr-ts/mt* at the DHFR-TS locus) (Prx WT), and three *pbtpx-1* disruptant populations (Prx KO). DNA samples were digested with *Spe*I, separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized with probe. The single 11-kb band in the Prx KO populations indicates *pbtpx-1* disruption, whereas the single 7-kb band in the WT and Prx WT populations indicates an intact PbTPx-1 locus.