Table 4. Chemoprophylaxis of military soldiers of South Korea, US Army, and North Korea

	Number of soldiers									
	1997	1998	1999	2000	2001	2002	2003	2004	2005	Total
ROK Army[a)]	15,981	37,529	61,772	90,000	90,000	140,000	160,000	190,000	200,000	985,282
US Army	35	2,485	8,510	1,159	ND[c)]	ND	ND	ND	ND	12,189
North Korea[b)]	0	0	0	0	100,000	350,000	300,000	300,000	ND	1,050,000
Total	16,016	40,014	70,282	91,159	190,000	490,000	300,000	300,000	200,000	2,047,471

[a)]Republic of Korea Army.
[b)]Figures are based on the amount of anti-malarial drugs used for chemoprophylaxis and treatment supported by South Korea.
[c)]ND = no available data.

Table 5. Financial support for malaria control in South Korea and North Korea

Group/Year		Expenditures (in USD)				
		2001	2002	2003	2004	Total
South Korea	Mosquito control[a)]	689,100	734,900	816,600	2,914,100	5,154,700
North Korea[†]	Supported by South Korea					
	Anti-malarial drugs, mosquito control, etc.[b)]	490,000	620,000	700,000	700,000	2,510,000
	Education[c)]	38,450	26,900	26,900	26,900	119,150
	Supported by IFRC[d)]					
	Anti-malarial drugs, etc.	21,000	21,000	158,000	321,500	521,500
	Subtotal	549,450	667,900	884,900	1,048,400	3,150,650
Total		1,238,550	1,402,800	1,701,500	3,962,500	8,305,350

[a)]For insecticide purchase and equipments purchase for insecticide spraying in Kyonggi-do, Kangwon-do, and Incheon city, South Korea.
[b)]Anti-malarial drugs included chloroquine and primaquine (for treatment of 100,000-300,000 patients per year), and mosquito control included nsecticides like permethrin, devices for insecticide spraying, and insecticide-treated bed nets and clothes. Others included lancets, pH meters, staining reagents for blood smears and microscopes. Data are from World Health Organization, (WHO) and Korea Center for Disease Control and Prevention, South Korea.
[c)]For training laboratory technicians, entomologists, and health workers (total 70 persons per year) to help build a sustainable national ability to control malaria by WHO.
[d)]International Federation of Red Cross and Red Crescent Societies.

(more than 30% of all patients during the period 1998-2004) have been reported in Pusan, Taegu, Kyongsangbuk-do, and Kyongsangnam-do regions, which are considerably removed from major outbreak areas. Such cases may include retired soldiers, travelers to major outbreak areas, and locally infected civilians. In the case of retired soldiers living in these areas, the majority were probably infected while working in major outbreak areas, and developed febrile illness after a long incubation period of 5-13 mo (Chai, 1999), whereas travelers may have developed fever after a short (within 1 mo) or a long incubation period. It is unfortunate that no study has yet reported firm evidence of local malaria transmission in areas remote from the major outbreak areas.

There is no doubt that vivax malaria reemergence in South Korea was originally caused by infected mosquitoes originating from North Korea and the DMZ (Chai, 1999; Park et al., 2003). In this regard, it is worth mentioning that genotypes of circumsporozoite protein (Kho et al., 1999), merozoite surface protein (MSP)-1 (Zakeri et al., 2003), Duffy-binding protein

(Kho et al., 2001), apical membrane protein antigen-1 (Han et al., 2002), and MSP-3α (Han et al., 2004) of the reemerging vivax malaria in South Korea are similar to those found in the North Korean (NK) strain. Infected mosquitoes probably constantly migrate from North to South Korea (Cho et al., 2002), and we suggest that a large proportion of malaria cases in South Korea have resulted from this influx. Moreover, it is evident that the malaria situation in northern South Korea will be influenced by that in North Korea.

With regard to the malaria situation in North Korea, no data was available before 1997. However, recently some occurrence data has become available (Global Funds to Fight AIDS, Tuberculosis and Malaria, 2003; United Nations, 2003; United Nations Office for the Co-ordination of Humanitarian Affairs DPRK, 2003, 2004; World Health Organization, 2004; Chol et al., 2005). Indigenous cases have now been reported from 1997 (Chol et al., 2005), and nationwide patient numbers increased sharply prior to 2001, but then dramatically reduced to 2004. Several factors may have facilitated the increase in malaria cases during 1999-2001 in North Korea. Such factors may include changes in agricultural practices, such as, reduced use of pesticides and changes in rice field irrigation, intermittent big flooding, increased vector host densities, and inadequate health care delivery system.

However, a malaria control program was implemented in endemic areas of North Korea, in South and North Hwanghae-do (Provinces) during 2001-2003 by the National Program Office of WHO, in North Korea (Chol et al., 2005). It has been stated that the prevalence of malaria began to decline immediately after implementing this control program, and individual awareness regarding malaria increased rapidly. However, more precise data are required to better assess the situation of vivax malaria in North Korea.

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ORIGINAL ARTICLE

Further investigations of glucose-6-phosphate dehydrogenase variants in Flores Island, eastern Indonesia

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Abstract We conducted field surveys for malaria and glucose-6-phosphate dehydrogenase (G6PD) deficiency in the eastern part of Flores Island, East Nusa Tenggara Province, Indonesia. A total of 1,108 volunteers (642 males and 466 females) belonging to three ethnic groups (Sikka, Ende and Bajo) were examined, and 55 G6PD-deficient individuals (38 males and 17 females) were detected. Among them, 50 samples were analyzed molecularly, in addition to three deficient cases in a Bajo family. In the Sikka population, G6PD Kaiping (1388G>A), one of the two common variants in the Chinese population, was unexpectedly found as the most dominant variant (11/22, 50.0%), followed by G6PD Chatham (1003G>A, 36.4%), G6PD Coimbra (592C>T, 9.1%) and G6PD Vanua Lava (383T>C, 4.5%). Frequency of G6PD Kaiping in the Sikka might be the highest among non-Chinese populations reported so far. In the Ende population, G6PD Vanua Lava (9/14, 64.3%) was the highest, followed by G6PD Kaiping (14.3%), G6PD Chinese-5 (1024C>T, 14.3%) and G6PD Chatham (7.1%). In the Bajo population, a total of 18 deficient cases were analyzed, and a novel mutation (844G>T) in exon 8 with a predicted amino acid change of 282 Asp>Tyr was found in a 7-year-old boy at a Bajo village near Maumere. This new Class II (mild type) variant was also confirmed in his mother and sister, and designated as G6PD Bajo Maumere. The missense mutation at the same nucleotide 844 has been known as G6PD Seattle/Lodi/Modena/Ferrara II, but this mutation is caused by a G>C substitution (282 Asp>His). In the Bajo population, G6PD Viangchan (871G>A, IVS 11 nt93 T>C, 1311C>T), the most common variant in continental Southeast Asian populations, was found to be the dominant (11/18, 61.1%), followed by G6PD Vanua Lava and the new variant (each 16.7%), and G6PD Coimbra (5.6%). These results strongly suggest that the Bajo peoples may have different ancestors from those for Sikka and Ende, and may be much closer to continental Southeast Asian populations. It is interesting that G6PD Canton (1376G>T), another common variant in Chinese, was not seen in the Flores population.

Keywords G6PD deficiency · New variant · Bajo · Sikka · Ende · Maumere · Flores Island · Indonesia

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most frequent hereditary disorders in the world. This enzyme is encoded in 13 exons with an open reading frame of 1,545 bp (Chen et al. 1991). Most G6PD deficiency is caused by a single nucleotide mutation, resulting in one amino-acid change among 515. To date, more than 400 G6PD biochemical variants have been described, and at least 140 mutations of G6PD deficiency have been discovered (Beutler and Vulliamy 2002).

The major G6PD variants reported in Southeast Asian countries are G6PD Viangchan (871G>A, IVS 11 nt93 T>C, 1311C>T) and G6PD Mahidol (487G>A), but the distribution of G6PD mutation differs in each country. For instance, almost all of G6PD deficient cases detected in Laos (Iwai et al. 2001) and Cambodia (Louicharoen and Nuchprayoon 2005; Matsuoka et al. 2005) are G6PD Viangchan. This variant is also the most common in Thais (54%, Nuchprayoon et al. 2002; 31.3%, Laosombat et al. 2005) and Malaysian Malays (37.2%, Ainoon et al. 2003), suggesting that this is a common ancestral origin of continental Southeast Asian populations. In Myanmar, however, G6PD Viangchan has never been reported, and more than 90% of variants are G6PD Mahidol (Iwai et al. 2001; Matsuoka et al. 2004; Than et al. 2005). This type is also common in Thailand (8%, Nuchprayoon et al. 2002; 17.2%, Laosombat et al. 2005) and among Malaysian Malays (15.1%, Ainoon et al. 2003), but not reported in Laos and Cambodia.

In Thailand and Malaysia, many other variants derived from Chinese immigrants such as G6PD Canton (1376G>T), G6PD Kaiping (1388G>A), G6PD Gaohe (95A>G) are also distributing. Furthermore, variants derived from Europe (G6PD Mediterranean, 563C>T), the Middle East to India (G6PD Chatham, 1003G>A) and Oceania (G6PD Vanua Lava, 383T>C; G6PD Union, 1360C>T), are commonly detected in both countries.

In Indonesia, numerous ethnic groups are present, and thus many G6PD variants might be included as seen in Thailand and Malaysia. In fact, five cases of G6PD Mediterranean, three of G6PD Canton, two of G6PD Mahidol and one of G6PD Coimbra (592C>T) have been reported in Javanese (Soemantri et al. 1995; Iwai et al. 2001). Furthermore, we have reported 11 cases of G6PD Vanua Lava in Amboinese on Buru and Halmahera Islands, and five mixed variants of G6PD Canton, G6PD Kaiping, G6PD Gaohe, G6PD Chatham and G6PD Surabaya (1291G>A) in ethnic Chinese on Surabaya (Iwai et al. 2001). In the Sikka population on Flores Island, we have also identified five different variants (G6PD Chatham, G6PD Kaiping, G6PD Coimbra, G6PD Vanua Lava, G6PD Viangchan) in a small area (Matsuoka et al. 2003).

Following these previous studies in Indonesia, we conducted further field surveys for malaria and G6PD deficiency in Sikka and Ende districts, eastern Flores Island. Here, we report seven G6PD variants from three ethnic groups, including a novel mutation found in a family of the Bajo (Sea Gypsy) who have their own language and have never married into other tribes until recently.

Materials and methods

This study was approved by the Health Departments of Sikka and Ende districts, East Nusa Tenggara Province, and by the Ethical Committees of Jichi Medical University and Oita University Faculty of Medicine, Japan.

During March 2004 to December 2005, we surveyed malaria and G6PD deficiency in villages near Maumere (Sikka district; population ca. 260,000) and Ende (Ende district; population ca. 240,000), Flores Island (Fig. 1). Informed consent was obtained from each febrile volunteer before diagnoses of malaria and G6PD deficiency.

Febrile volunteers were first registered by name, age, gender, and ethnicity. Then, three drops of blood were collected from the fingertip: one for malaria diagnosis, one for hemoglobin concentration, and one for G6PD test. Malaria was diagnosed by acridine orange staining method (Tantular et al. 1999; Matsuoka et al. 2003), and hemoglobin concentration was measured using a battery-powered, HemoCue machine (Angelhorm, Sweden). For diagnosis of G6PD deficiency, we used a rapid diagnostic method (Tantular and Kawamoto 2003; Jalloh et al. 2004). When malaria patients or G6PD deficient individuals were detected, informed consent was again obtained, and 0.2–2.0 ml of venous blood was taken for further molecular analysis.

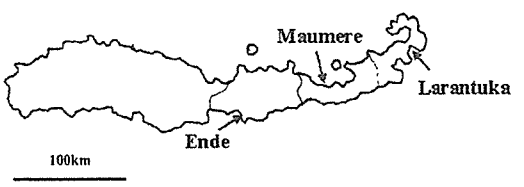

Fig. 1 Map of Sikka and Ende districts, Flores Island

Blood samples collected were stored at 4°C and brought back to Jichi Medical University, and G6PD activity was re-confirmed by another G6PD test developed by Fujii et al. (1984). Then, G6PD mutation was identified by sequencing both strands of G6PD gene (Matsuoka et al. 2004; 2005). Epidemiological data for malaria infection obtained in this study will be reported in elsewhere.

Results and discussion

On Flores Island, eight major ethnicities, Manggarai, Riung, Ngada (Bajawa), Nage-Keo, Palue, Ende (Ende-Lio), Sikka and Larantuka (see Fig. 1), are present as indigenous islanders, and they have their own languages. Except for the Manggarai (the westernmost) and the Larantuka (the easternmost), the other six tribes are thought to be very close each other.

In Sikka and Ende districts, a total of 1,108 volunteers (642 males and 466 females) belonging to ethnic groups of Sikka, Ende and Bajo were examined, and 38 males and 17 females were identified as G6PD deficient (Table 1). Among them, we obtained informed consent from 50 volunteers for further analysis of the G6PD gene. As shown in Table 2, a total of seven different variants, including G6PD Vanua Lava, G6PD Coimbra, G6PD Viangchan, G6PD Chatham, G6PD Chinese-5, G6PD Kaiping and a new variant, G6PD Bajo Maumere (844G>T), were identified.

In our previous study (Matsuoka et al. 2003), we reported the five different variants in the Sikka population at Maumere and Talibura (40 km east from Maumere), and a similar combination of variants was again observed in this survey at Nangahure and Magepanda, 10–30 km west from Maumere. Four variants among the five, except G6PD Viangchan, were detected (Table 2), but the frequency distribution of G6PD variants was little different from that in the previous study. This may be caused either because of a small sample size and/or because of a possible heterogeneity within the Sikka tribe. Surprisingly, however, G6PD Kaiping, one of the two common variants in Chinese population, was dominant (50.0%), followed by G6PD Chatham (36.4%). The presence of G6PD Kaiping in non-Chinese populations has been reported in Thais (2/39, 5% in Bangkok, Nuchprayoon et al. 2002; 27/134, 20.1% in southern Thai, Laosombat et al. 2005; 1/9, 11.1% in southern island, Ninokata et al. 2006), Malays (2/86, 2.3%, Ainoon et al. 2003), and Papuans (1/4, 25%, Wagner et al. 1996). Total frequency of G6PD Kaiping in the Sikka population reached 42.4% (14/33), and this value may be the highest among non-Chinese populations reported so far. From historical and religious backgrounds, it is easily presumed that Chinese traders or immigrants have merged closely with Christian Sikka women and so introduced this mutated gene into the Sikka population.

Table 1 The incidence of glucose-6-phosphate dehydrogenase deficiency in various ethnic groups in eastern Flores Island

Ethnic group	Sikka	Ende	Bajo	Total
Male				
Number of samples	224	177	241	642
Number of deficiency	19	7	12	38
Incidence (%)	8.5	4.0	5.0	5.9
Female				
Number of samples	161	186	119	466
Number of deficiency	5	9	3	17
Incidence (%)	3.1	4.8	2.5	3.6

In the Ende population, the prevalence of G6PD deficiency was higher in females than in males for unknown reasons (Table 1). G6PD Kaiping and Chatam were also detected among them, but G6PD Vanua Lava was the most common (64.3%). G6PD Chinese-5 was also detected in two sisters (Table 2): a 6-year-old girl was heterozygous with double variants of G6PD Chinese-5 and Vanua Lava, whereas her 7-year-old sister was heterozygous with G6PD Chinese-5. As they have a Chinese father and an Ende mother, two mutated genes of G6PD Chinese-5 and Vanua Lava were apparently derived from their father and mother, respectively. The Ende are neighbors to the Sikka, and they are thought to be very close to each other. However, their territories are separated by steep mountains, and they have different languages. Our results on frequency distribution of G6PD variants between these two tribes suggest that their origins may be more complicated than we expected.

High frequency distribution of G6PD Kaiping in the Sikka is a rather unexpected finding. In contrast, G6PD Canton, another common variant in Chinese population, was never seen in the Sikka nor in the Ende. Many surveillances of G6PD deficiency in Chinese, Thai, and Malay populations, conducted in China, Taiwan, Thailand, Malaysia and Singapore, have revealed that frequency of G6PD Canton is higher than that of G6PD Kaiping (Chang et al. 1992; Huang et al. 1996; Chiang et al. 1999; Ainoon et al. 1999; Nuchprayoon et al. 2002) or they are almost equal (Chiu et al. 1993; Quak et al. 1996; Ainoon et al. 2003, 2004; Jiang et al. 2005, 2006). Therefore, it is interesting why G6PD Canton was not detected in the Sikka or Ende populations. In addition, another remarkable finding observed in our studies was that G6PD Viangchan or

Table 2 Glucose-6-phosphate dehydrogenase variants detected in the present and previous studies

Nucleotide change	Name of variant	Number identified			
		Sikka	Ende	Bajo[a]	Total
383T>C	Vanua Lava	1 (1)[b]	9[c]	3	13
592C>T	Coimbra	2 (5)	0	1	3
844G>T	Bajo Maumere (new)	0 (0)	0	3	3
871G>A	Viangchan[d]	0 (1)	0	11	11
1003G>A	Chatham	8 (1)	1	0	9
1024C>T	Chinese-5	0 (0)	2[c]	0	2
1388G>A	Kaiping	11 (3)	2	0	13
Total		22 (11)	14	18	54

[a] Three deficient individuals in a family of a boy with G6PD Bajo Maumere were included (see text)

[b] Numbers in parentheses indicate those of each G6PD variant detected in our previous study (Matsuoka et al. 2003)

[c] A girl was heterozygous with G6PD Chinese-5 and Vanua Lava, and her sister was heterozygous with G6PD Chinese-5 (see text)

[d] Two more non-sense mutations at 1311C>T in exon 11 and at nt93T>C in intron 11 were confirmed

Mahidol, the most common variants in continental Southeast Asian populations, was very rare in both populations (only 1/47; 2.1%), indicating that the Sikka and Ende (and also Amboinese) may have different ancestors from those of continental Southeast Asian populations.

On the other hand, the Bajo (Bajau) are one of the sea nomads (Sea Gipsy), inhabiting the area between eastern Malaysia (Sabah State, Kalimantan Island) and central to eastern Indonesia (Sulawesi, Sumbawa and Flores Islands). This ethnic group also belongs to the Malayo-Polynesians, the same as Javanese, Amboinese, Sikka and Ende, and are thought to have originated from south Mindanao, Philippines (Fox 1997). Over the last 300 years, they have migrated from south Mindanao, and scattered into small settlements in many coastal areas. Until quite recently, the Bajo peoples were very isolated, since their settlements were apart from native villages, they spoke only Bajo (or Samal), and never married with any other tribe. Therefore, we expected the existence of a unique G6PD variant or unique combination of G6PD variants in the Bajo population, differing from those of the Sikka, Ende, Amboinese or Javanese.

As expected, we found a novel variation from a 7-year-old boy in a Bajo village (Wuring village, 4 km west of Maumere; population ca. 4,000). A missense mutation was seen at nucleotide 844 (G to T) in the exon 8 with a predicted amino-acid change of 282 Asp>Tyr (Fig. 2a). To confirm this new variant, we further examined G6PD activities in his family. His mother (31 years old) and one (5 years old) of two young sisters were found to be heterozygous with this variant (Fig. 2b), whereas a cousin of his mother was identified as hemizygous with G6PD Viangchan. The new variant was categorized as Class II (mild type), and we designated this new variant as G6PD Bajo Maumere.

The missense mutation at the same nucleotide 844 has been known as G6PD Seattle/Lodi/Modena/Ferrara II, but this mutation is caused by a substitution from G to C (282 Asp>His, Beutler and Vulliamy 2002). Sharing two different G6PD variants by two

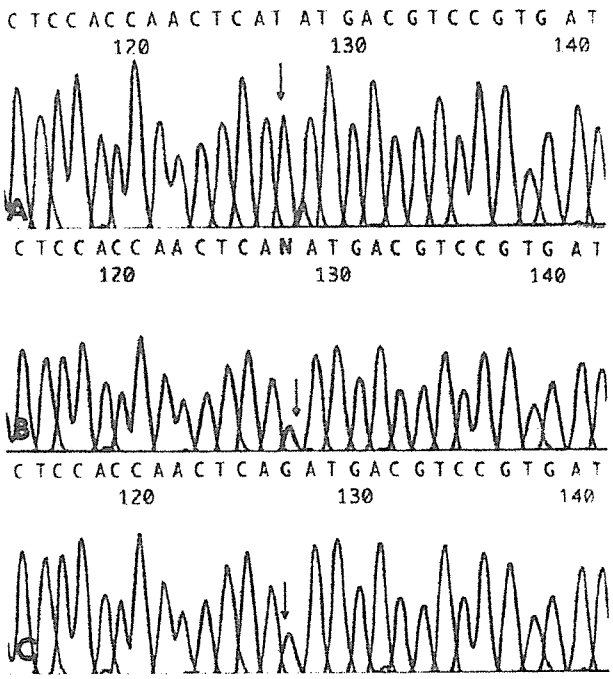

Fig. 2 **a** Part of the DNA sequence in the exon 8 from a hemizygous boy with G6PD Bajo Maumere. **b** Part of the DNA sequence in the exon 8 from a heterozygous mother with G6PD Bajo Maumere. **c** Part of the DNA sequence in the exon 8 from his young sister with normal G6PD. *Arrows* show 844 T, 844 T/G, and 844 G, respectively

different mutations at the same nucleotide has been found between G6PD A- (680G>T, 376A>G) and Mexico City (680G>A), G6PD Chinese-1 (835A>T) and Haikou (835A>G), G6PD Loma Limda (1089C>A) and Aachen (1089C>G), G6PD Olomouc (1141T>C) and New York (1141T>A), G6PD Kawasaki (1229G>C) and Japan (1229G>A), G6PD Canton (1376G>T) and Cosenza (1376G>C) (Beutler and Vulliamy 2002).

Among a total of 18 deficient cases analyzed in the Bajo population, G6PD Viangchan was the highest (61.1%), followed by G6PD Vanua Lava and the new variant (each 16.7%), and G6PD Coimbra (5.6%). All these results taken together indicate that the Bajo is absolutely different from the Sikka, Ende, Amboinese or Javanese, and might be closer to ethnic groups in continental Southeast Asian populations, having the most common variant of G6PD Viangchan. It is of interest to note that, in the Moken population, another Sea Gypsy living in costal areas in Myanmar and Thailand, G6PD Mahidol (11/17, 64.7%) is the most common variant, followed by G6PD Viangchan (4/17, 23.5%) (Ninokata et al. 2006).

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MOLECULAR & BIOCHEMICAL PARASITOLOGY

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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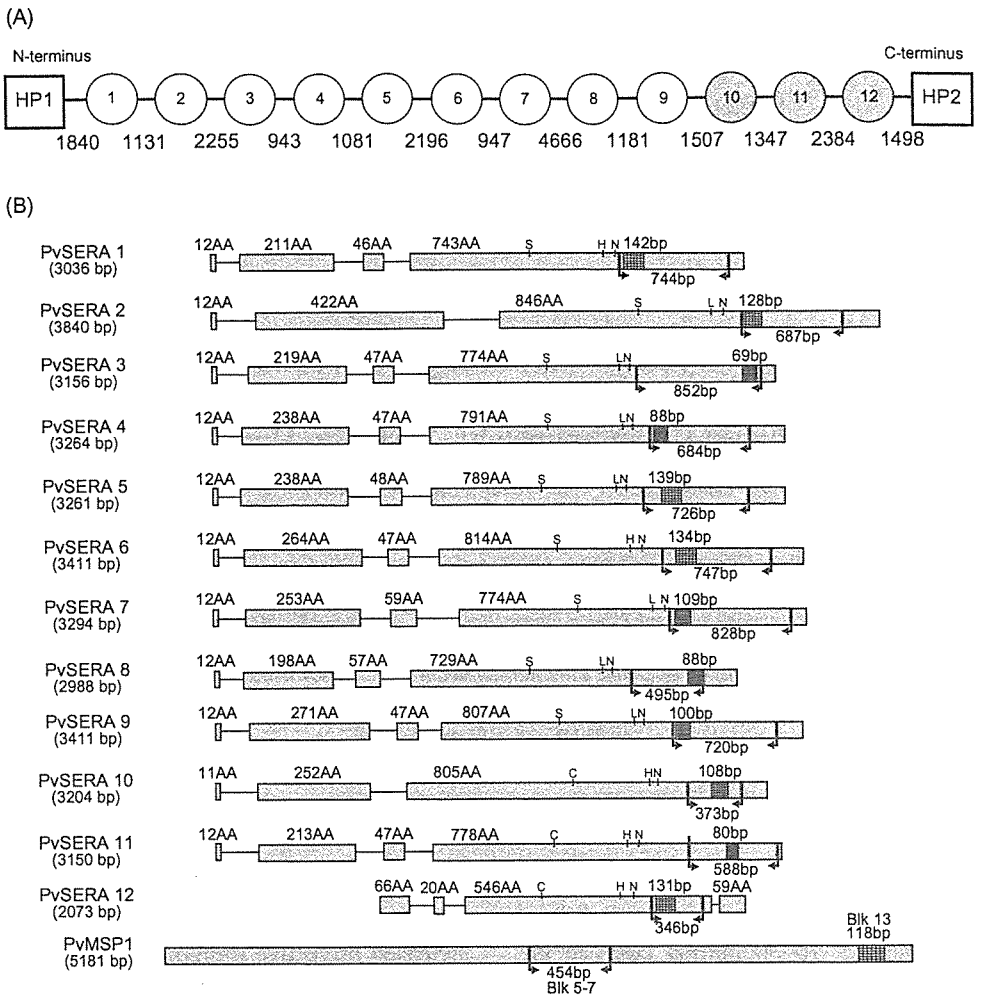
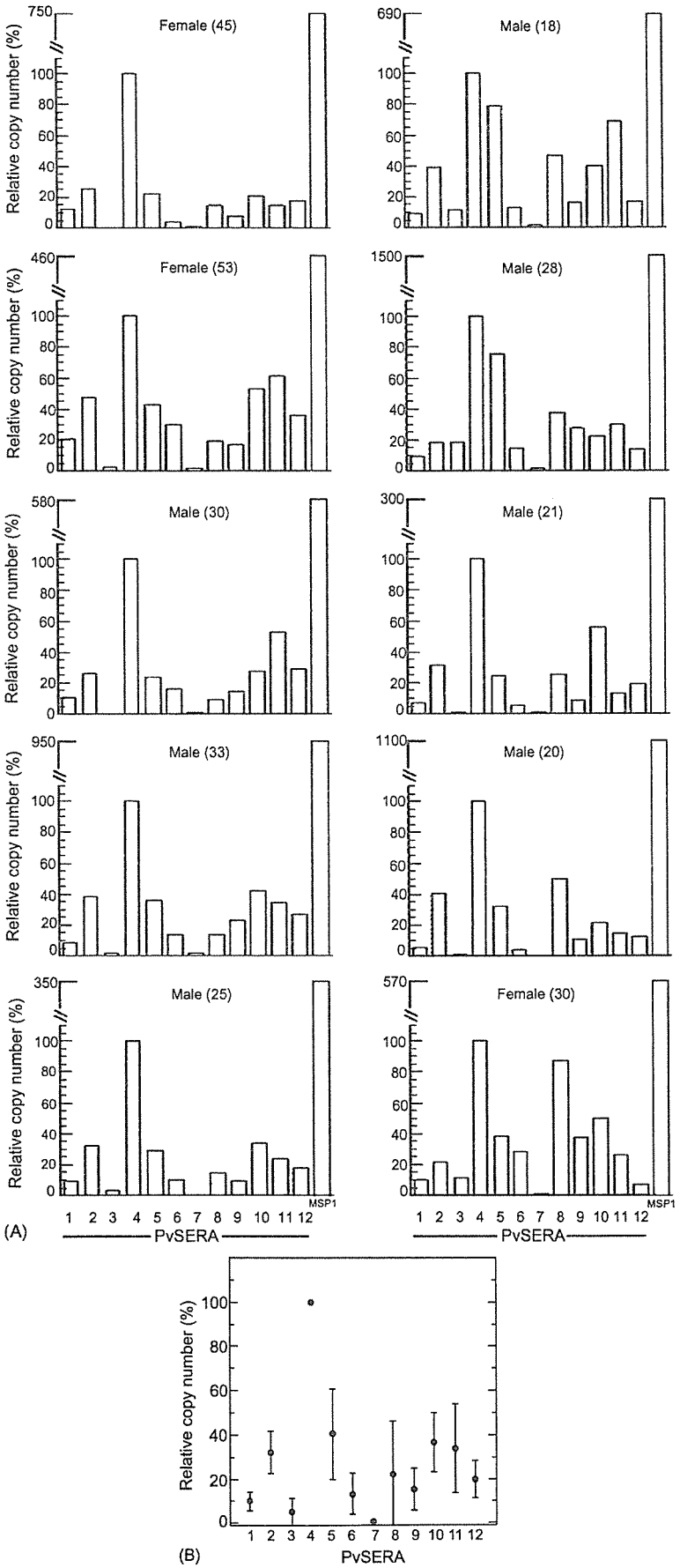


Fig. 1. The *P. vivax SERA* multigene family. (A) The tandem gene cluster arrangement of *SERA* family members in a region between two hypothetical proteins (HP) (Arisue et al., submitted for publication). Individual members are represented by ovals numbered 1–12. *PvSERA*1–9 unshaded ovals possess an 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
1 2 3 4 5 6 7 8 9 10 11 12
PvSERA
(A)
(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	
PvSERA4	4A-1	1	0.111	0.944 ± 0.070
	4A-2	1	0.111	
	4B-1	2	0.222	
	4B-2	2	0.222	
	4B-3	1	0.111	
	4C	1	0.111	
	4R	1	0.111	
PvSERA5	5A-1	8	0.364	0.784 ± 0.063
	5A-2	1	0.045	
	5A-3	1	0.045	
	5R	1	0.045	
	5B-a1	2	0.091	
	5B-a2	1	0.045	
	5B-b1	7	0.318	
	5B-b2	1	0.045	
PvSERA8	8A-1	5	0.312	0.900 ± 0.062
	8A-2	1	0.062	
	8A-3	1	0.062	
	8A-4	2	0.125	
	8A-5	1	0.062	
	8A-6	1	0.062	
	8B-1	2	0.125	
	8B-2	1	0.062	
	8B-3	1	0.062	
	8B-4	1	0.062	
PvMSP1	S1	8	0.267	0.892 ± 0.036
	S2	2	0.067	
	S3	1	0.033	
	S4	2	0.067	
	S5	4	0.133	
	S6	1	0.033	
	S7	5	0.167	
	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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Short communication

The *Plasmodium vivax* homolog of the ookinete adhesive micronemal protein, CTRP ☆

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Abstract

The *Plasmodium* circumsporozoite protein/thrombospondin-related anonymous protein-related protein (CTRP) is expressed at the mosquito midgut ookinete stage and is considered to be a transmission-blocking vaccine candidate. CTRP is composed of multiple von Willebrand factor A (vWA) and thrombospondin type 1 domains in the extracellular portion of the molecule, and a short acidic cytoplasmic domain that interacts with the actomyosin machinery. As a means to predict functionally relevant domains within CTRP we determined the nucleotide sequences of CTRP from the *Plasmodium vivax* Sall and the *Plasmodium yoelii* 17XL strains and characterized the conservation of domain architectures and motifs across *Plasmodium* genera. Sequence alignments indicate that the CTRP 1st to 4th vWA domains exhibit greater conservation, and thereby are perhaps functionally more important than the 5th and 6th domains. This point should be considered for the development of a transmission-blocking vaccine that includes CTRP recombinant subunit. To complement previous cellular studies on CTRP, we further determined the expression and cellular localization of CTRP protein in *P. vivax* and *P. yoelii*.

Keywords: *Plasmodium vivax*; Ookinete; CTRP; von Willebrand factor A domain

Malaria parasites possess an apicomplexan-specific class of molecules, termed the TRAP/MIC2 family, which mediate adhesion onto host cell and tissue surfaces, gliding motility, and invasion of host cells. Members of this diverse family of transmembrane proteins are typically composed of one or more von Willebrand factor A (vWA) and thrombospondin type 1 (TSP1) domains in the extracellular portion of the molecule, and a short acidic cytoplasmic domain that interacts with the actomyosin machinery. Family members include the prototypic thrombospondin-related anonymous protein (TRAP) [1,2] that is expressed in *Plasmodium* sporozoite micronemes; the circumsporozoite protein/thrombospondin-related anonymous protein-related protein (CTRP) [3–7], localized to *Plasmodium* ookinete micronemes, and the *Toxoplasma gondii* tachyzoite micronemal protein, TgMIC2 [8]. TRAP/MIC2 family proteins are found across the apicomplexan clade, including NcMIC2 in *Neospora caninum*, Et100 in *Eimeria tenella*, and Em100 in *Eimeria maxima major* [9–11], and predicted homologs within the genome sequence of *Theileria annulata* (TA07755) and *Theileria parva* (TP04_0306). The *Cryptosporidium parvum* genome sequence lacks extracellular examples of the vWA domain and in this pathogen the predicted TRAP functional homolog (*Cp*TRAP-C1) is composed of Apple domains and TSP1 domains [12] (Fig. 1A).

Abbreviations: CTRP, circumsporozoite protein/thrombospondin-related anonymous protein-related protein; MIDAS, metal ion-dependent adhesion site; TSP1, thrombospondin type 1; vWA, von Willebrand factor A.

☆ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL, and DDBJ databases under the accession numbers: AB247368–AB247370.

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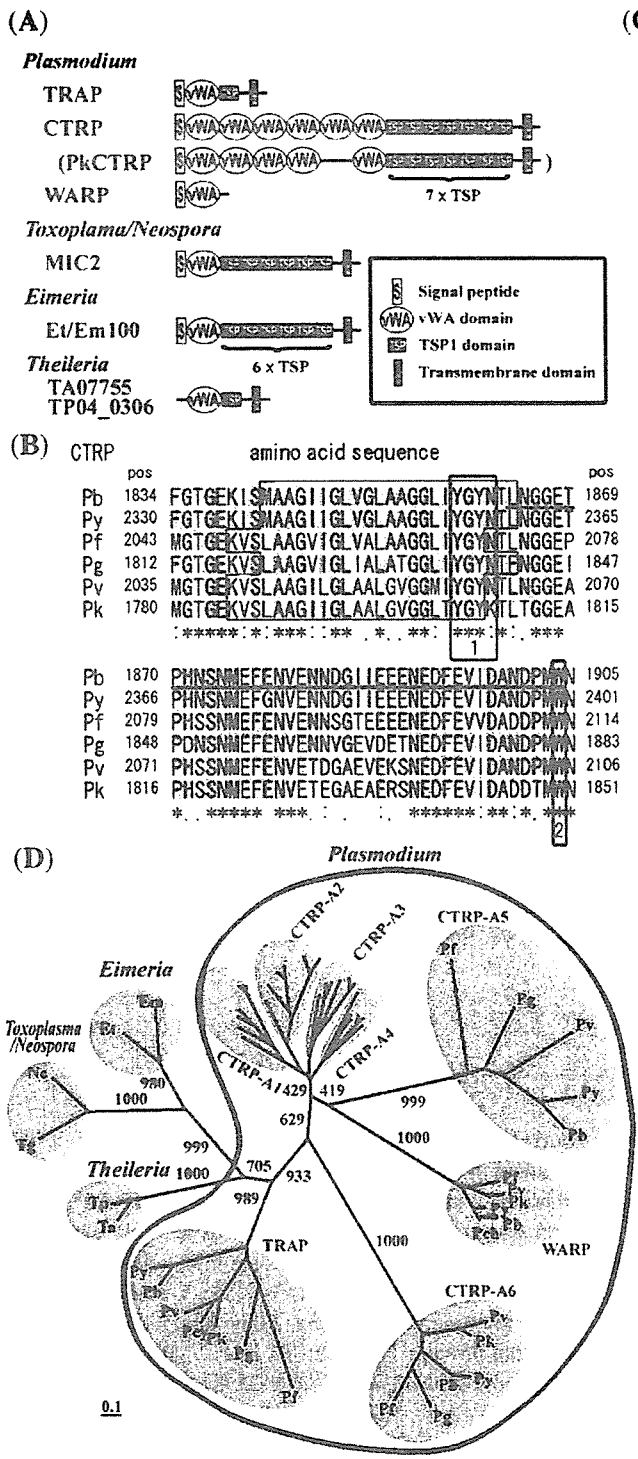

Fig. 1. (A) Schematic diagram of apicomplexan proteins containing vWA and TSP1 domains. Tg, *Toxoplasma gondii*; Et, *Eimeria tenella*; Em, *Eimeria maxima major*; TA, *Theileria annulata*; TP, *Theileria parva*. (B) Amino acid alignment of the transmembrane (boxed with thin line) and cytoplasmic regions of *Plasmodium* CTRP. The underlined region corresponds to that used to generate anti-*Pb*CTRP immune sera recognizing the cytoplasmic tail of *Pb*CTRP (amino acid position 1864–1904). Amino acids identical to those of *Pb*CTRP are shaded. Asterisks indicate the positions where amino acids are identical in all species, and similar amino acids are indicated with colons or periods under the alignments. The tyrosine-based motif involved in cellular trafficking (1) and the tryptophan residue that interacts with the motility actomyosin machinery (2) are boxed with thick lines. (C) Alignment of the MIDAS motif in the apicomplexan vWA domain superfamily. Numbers indicate intervening amino acids separating the three components of MIDAS (DxSxS, T and D, shaded). (D) Phylogenetic analysis of vWA domain of CTRP, TRAP, and WARP. Predicted amino acid sequences were aligned using the MUSCLE multiple sequence alignment program. Phylogenetic trees were constructed with deduced amino acid sequences by using neighbor joining method with Kimura's correction.

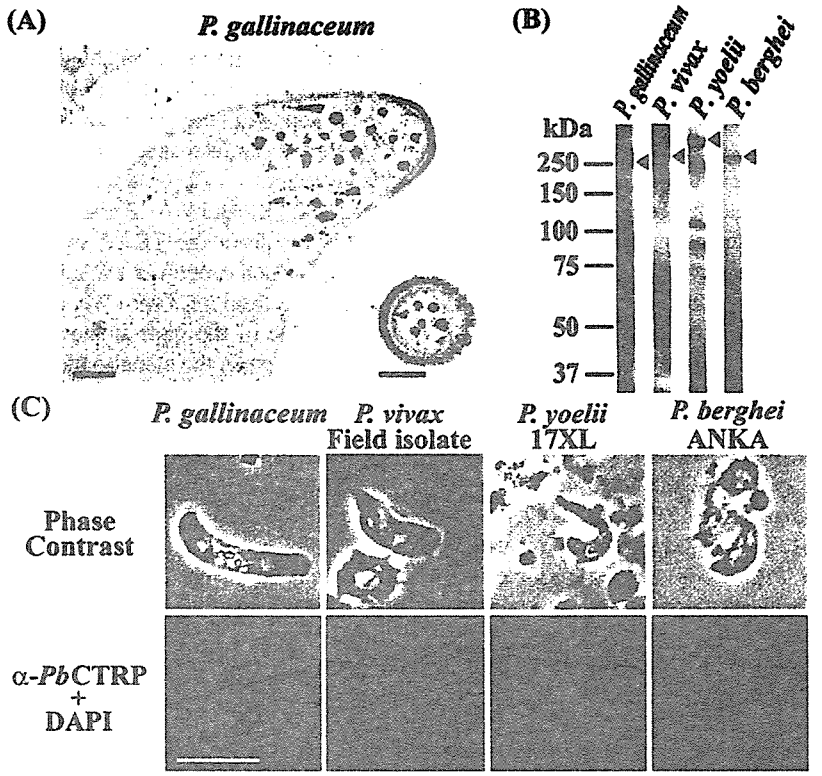

Fig. 2. (A) Immunoelectron microscopy of *P. gallinaceum* ookinetes using rabbit anti-*Pb*CTRP antibody. The immuno-gold particles associated with micronemes and the subpellicular region at the anterior portion. Bars indicate 0.5 μm. (B) Western immunoblot analysis of CTRP expression in *P. vivax* and *P. yoelii*. SDS-PAGE was performed with a 7.5% gel under reducing conditions. Proteins were transferred onto a PVDF membrane and reacted with rabbit anti-*Pb*CTRP antibody, followed by horseradish peroxidase-conjugated anti-rabbit IgG, then visualized by chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate; Millipore, Billerica, MA). A band greater than 250 kDa was observed for *P. vivax* and *P. yoelii*. Anonymous bands smaller than 250 kDa were observed for *P. yoelii*, which may represent degraded *Py*CTRP protein. As a comparison, bands for *Pg*CTRP and *Pb*CTRP were visualized. An anonymous 50-kDa band was detected for *P. berghei*, which was also seen previously [6]. (C) Visualization of *Pv*CTRP and *Py*CTRP within the ookinete cytoplasm by confocal microscopy. Ookinete-stage parasites of *Plasmodium* parasites were stained with anti-*Pb*CTRP rabbit antibody, followed by FITC-conjugated anti-rabbit IgG. Phase contrast images are shown in the upper panels, and CTRP expression is seen in the cytoplasm of the parasites when FITC images were overlaid with DAPI-stained nuclei (lower panel). Bar indicates 10 μm.

The *Plasmodium* ookinete micronemal protein, CTRP, is essential for translocation of mosquito midgut ookinetes to the oocyst stage [4–7], and partial transmission-blocking activity was shown with antibodies recognizing CTRP in *Plasmodium gallinaceum* [13]. CTRP is thereby considered to be a transmission-blocking vaccine candidate. To understand the conservation of CTRP domain architectures and motifs across *Plasmodium* genera, we determined the nucleotide sequences of CTRP from the *Plasmodium vivax* SalI and the *Plasmodium yoelii* 17XL strains, for comparison with previously reported CTRP sequences and those retrieved from the genome sequence databases. To complement cellular studies on CTRP we determined the expression and cellular localization of CTRP protein in *P. vivax* and *P. yoelii*.

A partial DNA sequence of the putative *P. vivax* CTRP (*Pv*CTRP) gene was identified by a TBLASTN search of the *P. vivax* Gene Sequence Tag Project Website [14; accession number, AZ568294] using the amino acid sequence of *Pb*CTRP (AJ238798) as a query. The deduced amino acid sequence of this fragment had homology with the amino acid positions 1295–1464 of *Pb*CTRP, a region that spans the 6th vWA domain and the first TSP1 domain. Full-length *Pv*CTRP nucleotide sequence was obtained by anchored PCR gene walking as described [15], using four distinct *P. vivax* (SalI) genome DNA splinkerette libraries. PCR-amplified DNA fragments were inserted into the pGEM-T Easy plasmid (Promega, Madison, WI), and nucleotide sequences were determined (AB247369). The gene encoding the *P. yoelii* CTRP (*Py*CTRP) was isolated from the *P. yoelii* 17XL strain (AB247368), independently from the genome project, using a panel of degenerate oligonucleotides designed based upon alignments of the *pfctrp* (U34363) and *pbctrp* nucleotide sequences. Genomic DNA sequence for the *P. knowlesi* CTRP (*Pk*CTRP) was identified within the contig 4777 at the Sanger Centre website (http://www.sanger.ac.uk) following TBLASTN search using *Pv*CTRP amino acid sequence as a query. The reported genomic DNA sequence for the *P. gallinaceum* CTRP (*Pg*CTRP) was incomplete at the carboxy terminus [13; AB247370], and completion of the sequence was achieved using sequence identified within the *P. gallinaceum* genome database at the Sanger Centre website (2570384.c000412673.Contig1).

All CTRP genes possess predicted signal peptide sequences and single transmembrane regions, followed by a short (less than 50

amino acid), acidic cytoplasmic domain at the carboxy terminus (Fig. 1B). Similar to TRAP family members, a tyrosine residue is conserved in all species within the cytoplasmic domain near the transmembrane region; as well as a tryptophan residue at the penultimate amino acid position of the carboxy terminal (Fig. 1B). The tyrosine-based motif is proposed to target trafficking to the micronemes [16]; whereas the tryptophan residue is predicted to be involved in a hydrophobic interaction that is essential for interaction with the subpellicular actomyosin motility network [17]. The overall CTRP domain architecture, composed of six contiguous vWA domains followed by seven contiguous TSP1 domains, is conserved across species boundaries; with the exception that *Pk*CTRP lacks the 5th vWA domain and possesses only five vWA domains (Fig. 1A). Interdomain regions between adjacent vWA domains are easily discernable by characteristic insertions of low complexity proline-rich stretches of charged amino acids. The nucleotide sequence of *Py*CTRP of 17XL strain was identical to that of the 17X nonlethal strain that was used for the *P. yoelii* genome project. *Py*CTRP is notable for a greater than 420 amino acid long insert between the 5th and 6th vWA domains that is composed almost entirely of a repeat of glycine and asparagine residues. This insert is not shared with *Pb*CTRP, despite greater than 86% amino acid similarity throughout the protein.

The vWA domain metal ion-dependent adhesion site (MIDAS) motif [18] is relatively conserved in the first 4 vWA domains of CTRP, but has degenerated in the 5th and 6th vWA domains, and is additionally absent in the *Plasmodium* von Willebrand factor A domain-related protein (WARP) [13,19]. The MIDAS motif consists of a DxSxS consensus at approximately amino acid position 12–16 within each CTRP vWA domain, and threonine and aspartate residues conserved at approximately 89 and 121, respectively (Fig. 1C). This motif is well conserved across apicomplexan parasites and is thus likely to be important for domain function, such as contributing to the correct conformation for the receptor recognition by associating with the divalent metal ion. The MIDAS motif is degenerated in the WARP vWA domain, and because this molecule was found to be a multimer it does not appear that metal binding is essential for the multimerization.

To determine the evolutionary relationships of vWA domains in *Plasmodium* species, the amino acid sequences of this domain were aligned using the MUSCLE algorithm [20] with manual correction (Supplemental figure S1) and a phylogenetic tree was constructed by the neighbor joining method with Kimura's correction with bootstrap value of 1000. As out groups, apicomplexan proteins possessing both vWA and TSP1 domains were included: namely, TgMIC2, NcMIC2, Et100, Emt100, TA07755, and TP04_0306 (Fig. 1D). The amino acid diversities amongst the CTRP vWA domains are markedly different; for example, the 5th and 6th domains are more divergent compared to the first 4 domains as described [3], but also more diversified among *Plasmodium* species. Thus the functional constraints appear to be relaxed with respect to the 5th and 6th domains in comparison to the 1st through 4th domains, based on the following observations: 1) *Pk*CTRP lacks the 5th vWA domain, 2) the 5th and 6th domains are the most diverse amongst the *Plasmodium* species, and 3) the MIDAS motif is degenerated in these domains and the ability of receptor recognition is potentially lost. Alternatively, the first 4 vWA domains of CTRP might have evolved by concerted evolution with the gene conversion event between domains before *Plasmodium* speciation. Concerted evolution is known to reduce the diversity between homologous sequences.

The CTRP and WARP vWA domains form a single clade that is distinct from the TRAP vWA domain. This suggests that WARP, which contains a vWA domain but lacks TSP1 domains, originated from a CTRP vWA domain.

Because of the high amino acid sequence similarity (80 to 97.5%) between the cytoplasmic domains of CTRP (Fig. 1B), it was anticipated that cross-species reactivity would be observed for the described purified rabbit antibody to *Pb*CTRP cytoplasmic domain [6]. To evaluate this we performed immunoelectron microscopy using *P. gallinaceum* ookinetes. Indeed, gold particles were found associated with micronemes of mature ookinetes, especially localized at the periphery of each microneme and concentrated in the electron-dense subpellicular region just beneath the apical end (Fig. 2A). The gold particle associated electron-dense region appears to be circumferentially distributed around the apical pole and is consistent with the observed CTRP localization using antibodies specific to *Pg*CTRP [13]. Thus the cross-species reactivity of anti-*Pb*CTRP antibody was verified for *Pg*CTRP. The *Pb*CTRP antibody was further used to detect CTRP orthologs in *P. vivax* and *P. yoelii*. Western immunoblot analyses of ookinetes showed a band with the size slightly greater than 250 kDa for *P. vivax* and excessively greater than 250 kDa for *P. yoelii*. The protein sizes of *Pv*CTRP and *Py*CTRP were greater than those estimated from the amino acid sequences (229 kDa for *Pv*CTRP and 256 kDa for *Py*CTRP, after removing putative signal peptide sequences), which is frequently observed for the malaria proteins. Indeed, control *Pg*CTRP and *Pb*CTRP bands appeared around 250 kDa, which were estimated to be 210 kDa and 213 kDa, respectively (Fig. 2B). By confocal microscopy, anti-*Pb*CTRP antibody demonstrated a cytoplasmic patchy pattern in ookinetes of *P. vivax* and *P. yoelii* with deviated distribution, similar to the pattern for *Pb*CTRP and *Pg*CTRP (Fig. 2C).

In summary, we determined the *Pv*CTRP and *Py*CTRP nucleotide sequences and show protein expression in ookinete stages that is localized to the apical region. Sequence alignments suggest that the CTRP 1st to 4th vWA domains are functionally more important than the 5th and 6th domains. This point should be considered for the development of the recombinant subunits of a transmission-blocking vaccine based on CTRP.

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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.parint.2006.04.003.

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