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# The genetic polymorphism of *Plasmodium vivax* genes in endemic regions of Thailand

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ABSTRACT

**Objective:** To investigate the genetic polymorphism of *Plasmodium vivax* (*P. vivax*) PvCSP and PvMSP1 genes from field isolates at four endemic regions (North, East, West and South) of Thailand. **Methods:** The 152 *P. vivax* infected cases from dried blood spots were DNA extracted and confirmed by species-specific primer sets using multiplex PCR method. PvMSP1 fragments F2 and F3; PvCSP were genotyped using RFLP-PCR method. **Results:** Totally amplified DNA which was multiple genotypes for PvMSP1 F2 and PvMSP1 F3 were 12.50% and 8.55%, respectively while PvCSP was 3.95%. The overall frequency of multiple genotypes was 25%. There were 12 allele types of PvMSP1 F2 using AluI enzyme digestion and 8 size variations were found in PvMSP1 F3. The isolates from western region was highly genetic diverse when compare among all isolates. The predominant variant type of PvCSP gene was VK210 type. **Conclusions:** The multiple genotypes are common found in Thailand and it might hide the real genotype. PvCSP does not have extensive genetic diversity in this study. However, PvMSP1 marker due to multiple genotypes is difficult to be analyzed. The multiple genotypes findings might stem from population migration and vector species findings.

## 1. Introduction

Malaria remains a potent threat to public health especially along the Thailand border regions, with *Plasmodium vivax* (*P. vivax*) being one of the human malaria species causing significant malaria morbidity. Drug treatment is still effective for this parasite in Thailand, meanwhile malaria vaccines are being developed and a key factor determining their likely efficacy is the extent of genetic variation in the population[1]. The genetic diversity of *P. vivax* is less well studied than that of *P. falciparum* but studies have recently started to redress this imbalance since completion of the genome project for *P. vivax* in 2008[2]. Data on genetic structure are important because gene flow between malaria transmission sites and recombination of different allele types are involved in the spread of drug resistance and, potentially, in the evolution of drug sensitivity. The investigation and comparison of parasite genetics between populations can quantify the geographical distribution of genetic polymorphisms, which can be used to monitor existing interventions or inform vaccine formulation based on malaria endemicity. Genetic studies of *P. vivax* in Asia showed more diversity than South-American populations[3], whereas African populations of *P. falciparum* are highly genetically diverse[4].

Several genetic markers in *P. vivax* have been identified, mainly through their role in vaccine development, that can be used to study genetic diversity such as apical membrane antigen 1(AMA1), Merozoite surface protein 1 (MSP1), merozoite surface protein 3 alpha, beta, gamma (MSP3 $\alpha$, MSP3 $\beta$, MSP3 $\gamma$), and circumsporozoite protein (CSP). However PvAMA1 and PvMSP3 genes show limited sequence polymorphism and display limited genetic diversity within a geographic region[5,6] PvMSP1 is highly polymorphic so is a suitable marker for molecular analysis and also for detecting multiple infections[7]. Previous studies of the PvCSP gene have revealed three variant types; VK210[8],

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VK247[9] and *P. vivax*-like[10]. The most predominant type was VK210 with most of the remainder distributed between VK210 and VK247, and *P. vivax*-like variant type was rarely found[11-15]. The variation of PvCSP gene in different regions may be due to parasite/host interactions in the Anopheline vectors and human population[16]. Analysis of PvMSP1, revealed six variable blocks[17]. Imwong *et al*. found size variation in PvMSP1 block 2 and 10 and a total of 23 allelic types for blocks 6-8[18]. Genetic diversity studies of PvMSP1 and PvCSP genes have been performed in India, Iran and Afghanistan as well as Thailand[18-21], but a comparison between regions in Thailand has not previously been undertaken. Both PvCSP and PvMSP1 genes are currently vaccine candidates for *P. vivax* and were selected in this study to investigate *P. vivax* genetic diversity in Thailand. Knowledge of *P. vivax* allelic polymorphism is needed to define the epidemiologic distribution of *P. vivax* strains and to aid the development of an anti-*P. vivax* vaccine because the presence of variant forms of *P. vivax* parasites has implications for the efficacy of vaccine formulation: if these two genes are seen to be more conserved, this would strengthen the case for further study for vaccine development and treatment in Thailand. The aim of this study was to investigate the genetic diversity of PvCSP and PvMSP1 genes from field isolates of malaria parasites from four different regions of Thailand.

## 2. Materials and methods

### 2.1. Study samples

The finger prick blood collections on filter paper (Protein saver card, Whatman Inc.) from February 2008 to February 2009 were extracted using a DNA mini blood kit (QIAGEN, Germany) according to the manufacturer's instructions and diagnosed for *P. vivax* by multiplex PCR[23]. The 152 infected *P. vivax* isolates from the highly endemicity at Tak (West) ($n$=66), Chanthaburi (East) ($n$=60), the lower endemicity at Prachuap Kriri Khun (South) ($n$=21), and Mae Hong Son (North) ($n$=5) Provinces were designated by regions (Figure 1A). Only study site from East is on Thai-Cambodia bordering whereas other three sites are closed to Thai-Myanmar border. *P. vivax* infection has been increased for the last few years and found more than half of total malaria incidence in Thailand[22]. The samples were collected from areas where West, East were the high endemicity and North, South are low endemicity (when compare between this study). Permission for this study was obtained from the ethical review committee for research in human subjects, Ministry of Public Health, Thailand (Reference no. 101/2550).

### 2.2. Gene amplifications

Oligonucleotide primers were designed using published sequences of PvMSP1. The assay conditions, including mastermix, were adapted and separately determined in the different primer sets[18]. All amplification reactions were carried out in a total volume of 50 μL and in the presence of 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 250 nM of each oligonucleotide primers, 125 μM of each of the four dNTPs, and 0.4 units HotStar Taq polymerase (QIAGEN, Germany). Primary amplification reactions were initiated with 2 μL of the template genomic DNA prepared from the blood samples, and the 2 μL of the product of these reactions was used to initiate the secondary amplification reactions. PvMSP1 F2 PCR products were digested by AluI enzyme. For the multiple clones found in PvMSP1 F2 were excluded from PCR-RFLP reaction.

The CSP gene amplification protocol was adapted from previous study[24]. This protocol can detect three variant types of CSP gene, VK210, VK247, and *P. vivax*-like.

### 2.3. Data analysis

All PCR products were analyzed by 1.5% agarose gels electrophoresis staining with ethidium bromide. Electrophoresis was performed in TBE buffer, and then was visualized on ultraviolet transilluminator. The size of the amplified fragments was estimated by comparison to a 100 bp ladder. Allele frequencies were estimated from PCR products in each gene. The proportion of single and mixed infections was calculated and confidence intervals were obtained using the modified Wald method of the on-line GraphPad program at http://www.graphpad.com/quickcalcs/ConfInterval1.cfm.

## 3. Results

The genotyping data are summarized in Figure 1. There were no mixed genotypes in North and South for PvMSP1 F2 and F3, respectively. However, mixed genotypes were found at relatively high frequencies (up to 40%) in North for PvMSP1 F3. In contrast most of the isolates (>90%) showed a single genotype for the PvCSP gene. This underestimates the true proportion of mixed samples because some may appear single at the genotyping site but are known to be mixed at other sites. The overall level of mixed genotypes, defined as heterozygous at any marker, was 25% (38/152). The PvMSP1 gene was genotyped in two fragments; PvMSP1 F2 and PvMSP1 F3. The number of alleles found in PvMSP1 gene is shown in Figure 1B. PvMSP1 F2 had 12.50% (19/152) of total amplified samples as multiple genotypes (95% CI 8.07 to 18.78%) and PvMSP1 F3 had 8.55% (13/152) of total amplified samples as multiple genotypes (95% CI 4.95 to 14.19%). PvCSP was found as multiple genotypes in 4.6% (6/152) of samples (95% CI 1.63 to 8.53%) (Table 1).

The allele frequencies of PvCSP gene is shown in Figure1C. The results showed that the predominant variant was the VK210 type at a frequency of 93.42% (142/152). However, VK247 variant type and mixed genotypes were also found at frequencies of 2.63% (6/152) and 3.95% (4/152), respectively. *P. vivax*-like type was also found 1% (1/231). Pairwise test between the populations and genetic analysis were investigated whether all population differed, or whether the result was due to a single, genetically-distinct population

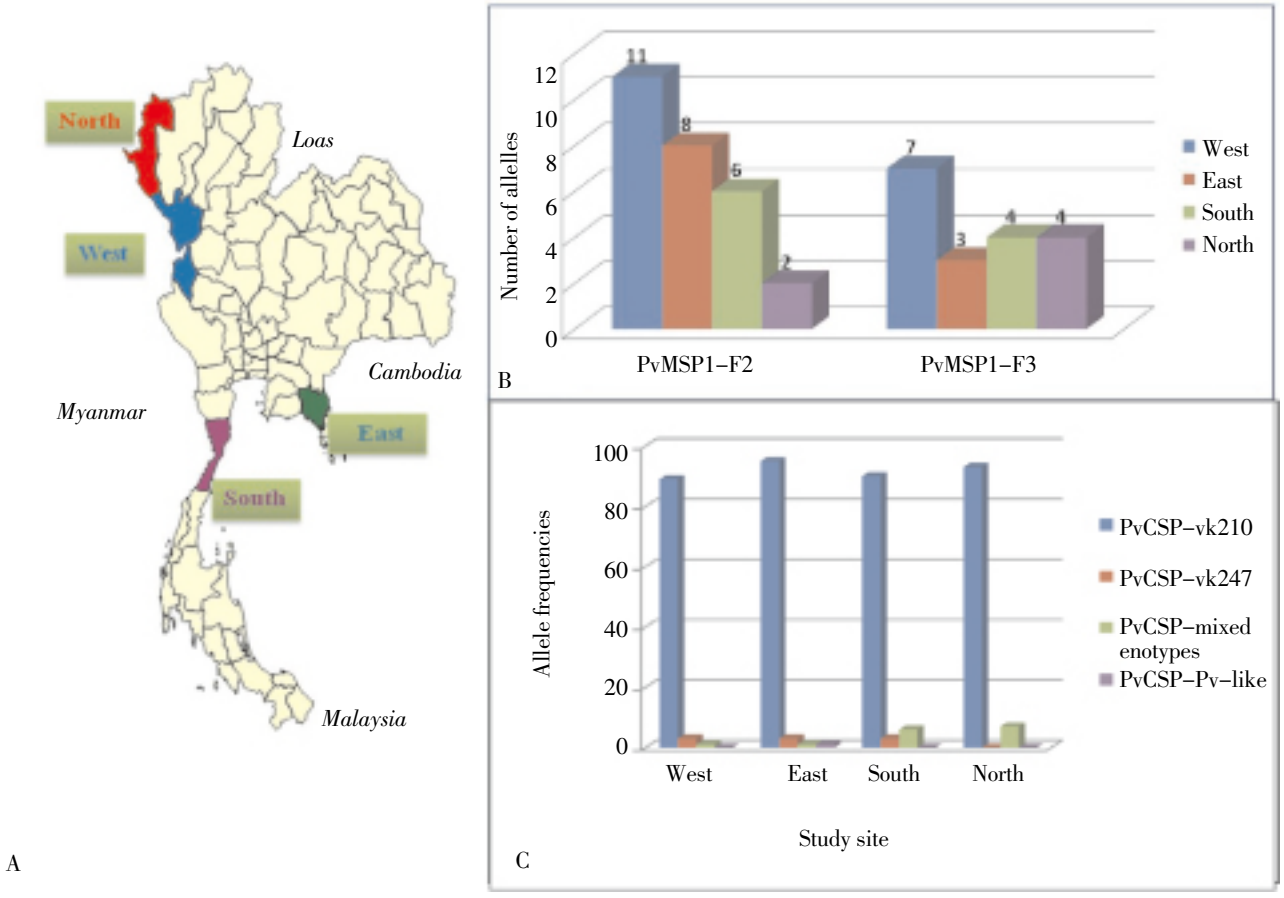


**Figure 1.** (A) Four study regions (north=Mae Hong Sorn, west=Mae Sod, east=Chanthaburi, south=Prachuap Khiri Khun), (B) number of PvMSP1 allelic types in each study site, (C) the allele frequencies of PvCSP gene in each study site.

**Table 1**

The overall cases of single and multiple infections in PvMSP1 and PvCSP.

| Study site | Total samples available | MSP1 F2 genotype | | MSP1 F3 genotype | | CSP genotype | |
|---|---|---|---|---|---|---|---|
| | | Single | Mixed | Single | Mixed | Single | Mixed |
| West | 66 | 59 | 7 | 57 | 9 | 61 | 5 |
| East | 60 | 53 | 7 | 58 | 2 | 60 | 0 |
| South | 21 | 16 | 5 | 21 | 0 | 20 | 1 |
| North | 5 | 5 | 0 | 3 | 2 | 5 | 0 |
| Total | 152 | 133 | 19 | 139 | 13 | 146 | 6 |

Note: West = Tak, East = Chanthaburi, South = Prachup Kriri Khun, North = Mae Hong Sorn province.

**Table 2**

PvMSP1 F2 fragments patterns from PCR RFLP.

| Type | Fragment size (bp) | | | | |
|---|---|---|---|---|---|
| A | 140 | 280 | 470 | – | – |
| B | 140 | 210 | 470 | – | – |
| C | 140 | 240 | 270 | – | – |
| D | 140 | 330 | 440 | – | – |
| E | 140 | 280 | 440 | 590 | – |
| F | 140 | 170 | 280 | 430 | – |
| G | 160 | 210 | 280 | 430 | – |
| H | 210 | 280 | 320 | 440 | – |
| I | 170 | 190 | 210 | 250 | 290 |
| J | 210 | 270 | 300 | 390 | – |
| K | 190 | 210 | 320 | – | – |
| L | 140 | 170 | 190 | 210 | 240 |

The size (bp) of the amplified fragments was estimated by comparison to a 100 bp ladder, there were 12 alleles showing A–L.

(data not shown).

Two sizes differences were found in PvMSP1 F2, identical to a previous study[18] (Figure 2A). Twelve alleles were reported after digested with Alu I enzyme (Figure 2B). Size of 12 patterns was shown in Table 2. Size variations were determined for PvMSP1 F3, from 250 bp to 390 bp (Figure 2C). There were size variations in PvMSP1 F3 in every study sites except in east isolates. There were high frequency distribution of curtain genotype with size 270 bp suggested that the parasite population are likely to be under selective pressure than other parasites in three areas.

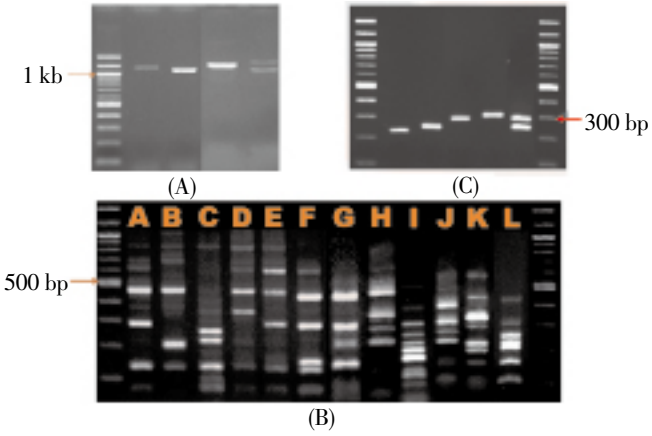


**Figure 2.** Size variations in (A) PvMSP1 F2 (B) PvMSP1 F3 (C) PvMSP1 F2 RFLP.

## 4. Discussion

This report is one of a series of studies designed to bring our understanding of *P. vivax* genetic structure up to the level of that for *P. falciparum*. The results for PvMSP1 indicated that there was genetic diversity in Thailand with more variants seen than previous studies[18,19].

Even though PvMSP1 in Salvador-1 and Belem were successfully amplified as the positive controls, polymorphism in the region targeted by the PvMSP1 F1 forward primer[17] is the reason for the failure to amplify this marker in many of the isolates we tested. However, PvMSP1 F1 primer was excluded as they might hinder effective PCR amplification. This suggested that this particular marker has limited utility for genetic diversity studies in Thailand.

The major limitation in this study was the use of dried blood spots on filter papers for the source of P. vivax DNA. Although the use of dried blood spot filters has many practical advantages for field work (i.e collected directly from patient finger prick and no need for cold reservation), the small quantity of blood (~50 $\mu$L) and the presence of inhibitors significantly limits the DNA extraction yield. To limit this problem, parasitemia > 0.1% was included for sample collection. However this in itself represents a source of potential bias, nonetheless one that is difficult to avoid. Despite the above limitations, our approach for assessing the genetic diversity of *P. vivax* in Thailand was relatively successful and compares well to similar studies in India[19]. However, many studies on *P. vivax* genetic diversity determined many markers on this Plasmodium sp. In 2011, they found two new alleles and several unidentified dimorphic substitutions and size polymorphism in PvMSP1[25]. In 2010, Zeyrek *et al*. found the limited polymorphism of PvMSP1 in Turkey isolates which showed a remarkably lower diversity in *P. vivax* population than in populations in Thailand and Brazil[26]. Recently PvMSP5 was studied for genetic diversity in Colombian and comparative within Thai, Brazilian, Indonesian isolates[27,28]. They found high polymorphism in PvMSP5 exon I which was detected positive selection in all isolates[28]. Furthermore, PvMSP3 and PvMSP3 were found to be reliable polymorphic marker for population genetic study in Thailand by using PCR-RFLP without sequencing[29]. Many studies used PCR-RFLP for genetic diversity study and they can confirm the polymorphic pattern without doing sequencing. For these reason, we concluded that PvMSP gene family can be used as a polymorphic marker and PCR-RFLP technique can be reliable method for genetic diversity study.

Even the PCR-RFLP analysis might not be easy to interpret the allelic polymorphisms in the population but this method is also popular used. The size variation can be inaccurate when different alleles coexist in the sample. Despite recent advances in sequencing technology, it is still not practical for public health laboratories in malaria endemic regions to conduct regular sequencing for large scale diversity studies. Consequently, full sequencing was not conducted in this study, primarily focusing on the distribution of previously characterized genetic markers in Thai *P. vivax*, using appropriate molecular techniques readily available to researchers from limited resource settings. In summary, no single genetic marker is inherently good or bad makers vary in attributes and in their most appropriate application.

Undoubtedly, PvCSP variant type found in this study was similar found to those obtained in Papua New Guinea, Indonesia, Brazil, Guyana, and Madagascar[9,30], but contrast with the results of an earlier study by Gopinath et al. in 1994 that did not find *P. vivax*-like type[31]. Most of variant types of PvCSP were VK210, as previously observed in India, Thailand, Azerbaijan, Guyana and Brazil[11,14,19,31,32]. Especially, mix subtypes (VK210 and VK247) were found in Myanmar[33] by serological test as same as in the present study. This implied that mixed genotypes may be distributed from Myanmar to Thailand. This shows that PvCSP type does not have extensive genetic diversity, which could be useful for vaccine development in Thailand.

The present of VK247 type may be related to the prevalence of different mosquito species that can contain different parasite genotypes or strains. In general, the mechanism of malaria vector and parasite interaction are different among the species, in addition, it could reflect parasite avoidance of the host immune response[34], the sampling bias or immune selection by the host. Interestingly, mixed genotypes were found in Northern isolates using PvMSP1 F3 but not with PvMSP1 F2. In contrast, isolates from the south found mixed genotypes in PvMSP1 F2 but not in PvMSP1 F3. This would indicate that parasites may contain mix clones that can be transferred into hosts which show different clones in one time biting of mosquito. Moreover, the protein variation of CSP is also dependent on the susceptibility of

each species of mosquito, which probably occurs during the ookinete and/or early oocyst stages[35]. Other work has confirmed that parasite population structure can result from the adaptation of malaria parasites to their vectors[36]. Furthermore, multiple genotypes may be caused by the time of biting of mosquitoes, which has been related to the range of genetic diversity exhibited by mosquitoes and malaria field populations[37-48]. Moreover, the problem when analyzing human blood samples is that humans in areas of moderate to high malaria transmission are often infected by several genetically distinct malaria clones; the number of clones is known as their multiplicity of infection (MOI). Consequently, many blood samples will have MOI>1 and be genetically ambiguous. Furthermore, the low sensitivity of genotyping should be recognized and noted that 'minor' clones (i.e. those with low parasitaemia) may be missed in genotyping, leading to an underestimated MOI[49].

These studied genes are recognized by host's immune system. Analyzes of polymorphism and population diversity of *P. vivax* have focused on parasite molecules that are under selection by host immunity, particularly antigens homologous to those of *P. falciparum*, such as MSP, CSP or erythrocyte binding antigens[11]. However, some population parameters are better determined by using DNA markers that are neutral, such as microsatellite loci[50].

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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