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▣ ORIGINAL ARTICLE

Genetic Diversity of *Plasmodium vivax* in Clinical Isolates from Southern Thailand using *PvMSP1*, *PvMSP3* (*PvMSP3α*, *PvMSP3β*) Genes and Eight Microsatellite Markers

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Abstract: *Plasmodium vivax* is usually considered morbidity in endemic areas of Asia, Central and South America, and some part of Africa. In Thailand, previous studies indicated the genetic diversity of *P. vivax* in malaria-endemic regions such as the western part of Thailand bordering with Myanmar. The objective of the study is to investigate the genetic diversity of *P. vivax* circulating in Southern Thailand by using 3 antigenic markers and 8 microsatellite markers. Dried blood spots were collected from Chumphon, Phang Nga, Ranong and, Surat Thani provinces of Thailand. By PCR, 3 distinct sizes of *PvMSP3α*, 2 sizes of *PvMSP3β* and 2 sizes of *PvMSP1* F2 were detected based on the length of PCR products, respectively. PCR/RFLP analyses of these antigen genes revealed high levels of genetic diversity. The genotyping of 8 microsatellite loci showed high genetic diversity as indicated by high alleles per locus and high expected heterozygosity (H_E). The genotyping markers also showed multiple-clones of infection. Mixed genotypes were detected in 4.8% of *PvMSP3α*, 29.1% in *PvMSP3β* and 55.3% of microsatellite markers. These results showed that there was high genetic diversity of *P. vivax* isolated from Southern Thailand, indicating that the genetic diversity of *P. vivax* in this region was comparable to those observed other areas of Thailand.

Key words: *Plasmodium vivax*, malaria, genetic diversity, antigenic marker, microsatellite marker

INTRODUCTION

Malaria remains one of the significant global health problems. Despite enormous control efforts over many decades, about 40% of the world's population who lives in more than 140 countries are at risk of malaria [1]. South-East Asia suffers the highest burden for *Plasmodium vivax*. 74% of the *P. vivax* cases are in South-East Asia followed by 11% in Eastern Mediterranean Region, and 10% African Region, respectively [2]. In Thailand, *P. vivax* is the most prevalent and accounts for 80% of the total infection [3]. Unlike *P. falciparum*, *P. vivax* has a unique dormant stage that can cause relapse in weeks or months after the initial infection. These latent hypnozoites complicate the ability to classify as re-infection or recurrent infection and could cause treatment failure due to the relapse of hypnozoites. This phenomenon contributes to the parasite resistance to standard antimalarial regimens, especially the emergence of chloroquine resistance [4] and the use of primaquine, an anti-hypnozoite drug against *P. vivax* relapse, especially in glucose-6-phosphate dehydrogenase deficiency patients [5]. Furthermore, *P. vivax* infected only reticulocytes [6] and represented only in the blood circulation about 0.5-2% [7]. At present, cultivation of *P. vivax* is challenging to maintain in vitro, resulting in a limitation on molecular research. Only blood samples from *P. vivax* infected patients are the source for molecular studies. Multiple clone infections are often observed with *P. vivax* infection, which are caused by a single mosquito bite carrying a mixture of parasites or different mosquitoes bite each taking a single clone [8-10]. The multiple parasitic she infection usually poses a higher risk of treatment failure [11]. Hence, understanding the genetic diversity of parasite populations would reveal their population dynamics and epidemiology in different regions which could help in assessment of the effectiveness of malaria control.

PCR/RFLP technique is a reliable genotyping method for large-scale genetic analysis of *P. vivax* even though the tech-

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nique demands time-consuming investigation of restriction fragments. The most polymorphic markers frequently used for PCR/RFLP analysis of *P. vivax* are members of Merozoite surface protein (MSP) genes, *MSP1*, *MSP3α*, and *MSP3β*. On the contrary, sequencing usually offers higher resolution at the nucleotide sequence level, but it is not applicable for multiple-clone of infections. Recently, microsatellite analysis is the method for detection of size polymorphism using capillary electrophoresis and subsequent analysis by software such as GeneMapper or GeneMarker. This method uses highly polymorphic and reliable markers for analysis of *P. vivax* population [12-16] owning to its capability to detect differences among closely related species of *P. vivax* [17-19].

The detailed knowledge of the genetic diversity of *P. vivax* is essential for the understanding of the dynamics of malaria disease transmission in this region. The high genetic diversity of *P. vivax* population has been reported in the Thai–Myanmar border [20,21]. However, little is known about the genetic diversity of *P. vivax* circulating strains in endemic areas of Southern Thailand. This study evaluates the genetic diversity of *P. vivax* isolated from Southern Thailand using 3 merozoite surface genes markers; *MSP1* F2, *MSP3α*, and *MSP3β* genes, and highly polymorphic 8 microsatellite markers; Pv1.501, Pv3.27, Pv6.34, Pv8.504, Pv14.297, Pv3.502, Pv11.162, and MS1.

MATERIALS AND METHODS

Study sites and blood collection

One hundred and forty-seven patients who attended malaria clinics of the Office of Disease Prevention and Control 11, Thailand, from 2012 to 2015 were involved in this study. Fig. 1 shows sample collecting sites and the number of samples collected from each area. *P. vivax* infected individuals were treated with 25 mg/kg chloroquine and 0.5 mg/kg primaquine for 14 days as the first-line drugs according to the treatment protocol of the Ministry of Public Health, Thailand. Approximately 80 µl of blood that had been microscopically confirmed for *P. vivax* infection was collected by finger-prick and spotted on 3M filter paper, (Whatman International Ltd., Maidstone, UK) and let the blood spots air dry at room temperature before keeping in a plastic zip bag. This study was approved by the Ethics Committee of Faculty of Medicine, Prince of Songkla University (REC57-0077-19-2). Written informed consent was obtained from all the participants.

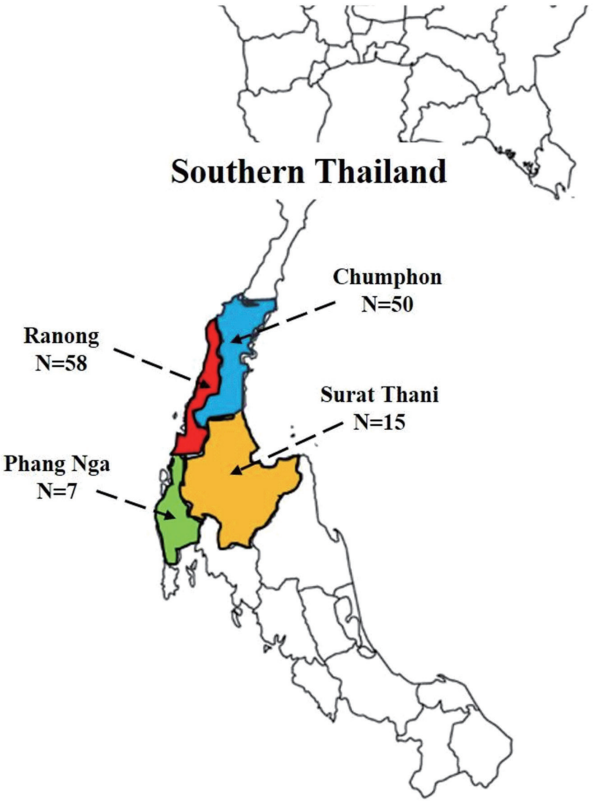

Fig. 1. Map of the study sites in Southern Thailand.

DNA extraction and confirmation of malaria species

P. vivax DNA from dried blood spot was extracted by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The final volume of the DNA solution was eluted in volume of 100 µl. Nested PCR was used to confirm human malaria species based on the small subunit (SSU) 18S ribosomal RNA (18S rRNA) gene as described by the previous study [22].

Plasmodium vivax genotyping

Amplification of *P. vivax MSP3α*, *MSP3β*, and *MSP1* F2 genes were performed as previously described [13,16,23]. The final reaction volume of 20 µl PCR comprised 0.2 µl of each primer, 10×of PCR buffer, 0.2 mM of deoxynucleotides (dNTPs), 1 mM of $MgCl_2$ and 0.5 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA). Primers for PCR amplification are shown in Table 1. Two µl of genomic DNA was added in the first round PCR and 1.5 µl of the primary PCR product was used in the second round of PCR. The concentration of $MgCl_2$ for *PvMSP3* genes amplification was 2.5 mM and for *PvMSP1* F2 gene was 1 mM. Ten µl of the amplified

Table 1. Primer sequences for *Plasmodium vivax* genotyping

Gene	Primer	Sequence (5'→3')	Reference
Merozoite surface protein gene markers			
PvMSP3α (N1)	3α -OF	CAGCAGACACCATTTAAGG	Bruce et al., 1999 [23]
	3α -OR	CCGTTTGTTGATTAGTTGC	
PvMSP3α (N2)	3α -NF	GACCAGTGTGATACCATTAAC	
	3α -NR	ATACTGGTTCTTCGTCTTCAGG	
PvMSP3β (N1)	3β -OF	GGTATTCTTCGCAACACTC	Yang et al., 2006 [16]
	3β -OR	GCTTCTGATGTTATTTCCAG	
PvMSP3β (N2)	3β -NF	CGAGGGGCGAAATTGTAAACC	
	3β -NR	GCTGCTTCTTTTGCAAAGG	
PvMSP1 F2 (N1)	VM1-O2F	GATGGAAAGCAACCGAAGAAGGGAAT	Imwong et al., 2005 [13]
	VM1-O2R	AGCTTGTACTTTCCATAGTGGTCCAG	
PvMSP1 F2 (N2)	VM1-N2F	AAAATCGAGAGCATGATCGCCACTGAGAAG	
Microsatellite markers			
Pv1.501	Forward	TCCTGTAACTCCTGCTCTGT	Imwong et al., 2007 [18]
motif: GGTGAGA	Reverse	CTTACTTCTACGTGCCCACT	
	Forward, s-n	*6FAM*-AATTGTAGTTCAGCCCATTG	
Pv3.27	Forward	AAGCTGCACTGAATTATGCT	
motif: AAAC	Reverse	TTCCAAATGTATGTGCAGTC	
	Forward, s-n	*6FAM*-AGCACAAGCATATGCAAAA	
Pv3.502	Forward	CCATGGACAACGGGTTAG	
motif: AACGGATG	Reverse	TCCTACTCAGGGGGAATACT	
	Forward, s-n	*HEX*-GTGGACCGATGGACCTAT	
Pv6.34	Forward	CAAATCATGGTAGCCTCCTA	
motif: AC	Reverse	GCTATGCATGTGTGGATGT	
	Forward, s-n	*6FAM*-TTAAGCTTCTGCATGCTCTT	
Pv8.504	Forward	AAAAGACTAGGCAGTTGACG	
motif: TGACCAA	Reverse	AGTGTGTGTAGTGGGTGGAG	
	Forward, s-n	*HEX*-TCTTCTCGTTCTCCTTTTCTG	
Pv14.297	Forward	TGACATCTTTCAAATATTCCTTT	
motif: AAG	Reverse	TGAAAAATGTTCCGCTACTT	
	Forward, s-n	*HEX*-TACACCCTTTAGGTCCTCGT	
Pv11.162	Forward	GTAGGAACACGCCACGTT	
motif: ATAC	Reverse	TAAATGACACTTTGGCTTCC	
	Forward, s-n	*HEX*-TTTGTTAGGAGATCCGTCTG	
MS1	Forward	*6-FAM* TCAACTGTTGGAAGGGCAAT	Karunaweera et al., 2007 [24]
motif: GAA	Reverse	ctgtcttTTGCTGCGTTTTTGTTTCTG	

N1 = Nest 1 (Primary) reaction; N2 = Nest 2 (Secondary) PCR reaction.
s-n, seminested.

PCR product was mixed with 2 µl of loading buffer and applied to 1.8% agarose gel.

Genotyping *P. vivax* antigenic markers were done by nested PCR/RFLP assays using restriction enzyme *Hha*I, *Pst*I and, *Alu*I (NEB Inc, Beverly, Massachusetts, USA), respectively. Five µl of the final product was applied to 2% agarose gel electrophoresis. The size of the amplified fragments was estimated by comparison with a 100 bp ladder marker set.

Microsatellite markers containing 8 polymorphic markers, i.e., Pv1.501, Pv3.27, Pv6.34, Pv8.504, Pv14.297, Pv3.502, Pv11.162, and MS1 were analyzed using the methods as described previously [18,24]. The microsatellite primers are summarized in Table 1. Two µL of genomic DNA were used as a template for the first PCR amplification, and 1 µl of the primary amplification product was carried out as a template for the secondary amplification. ABI 3130 Genetic Analyzer and GeneMapper® software version 4.0 (Applied Biosystems, Foster City, California, USA) was used to measure an allele in each locus compared to LIZ-500 size standards. The multiplicity of infection (MOI) of a given isolate was measured by calculating the number of different alleles at each of the 8 loci. Single infections were those with only 1 peak per locus in electrophero-

gram in any of the genotyped loci, while multiple-clone infections were defined as more than one peak at each locus and the height of minor peak was at least 1/3 of the height of the predominant allele present for each locus [25]. The genetic diversity was measured using the frequency of the predominated allele at each locus to calculate i) the mean number of alleles (*A*), which were calculated from all detected alleles at each locus divided by the total number of samples, and ii) the expected heterozygosity (H_E) at a given locus, which $H_E = [n/(n-1)][1-\Sigma pi^2]$, where "n" is the number of samples and pi is the frequency of the i^{th} allele. H_E ranges between 0 and 1; a value close to 1 indicated high genetic diversity levels in the population [10]. Both parameters were computed using version 2.9.3 of FSTAT software [26]. Multilocus linkage disequilibrium (LD) was calculated using a standardized index of association (I^S_A) [27,28]. Only the dominant alleles were considered to verify linkage. This test compares the variance (*VD*) of the number of alleles shared between all pairs of haplotypes observed in the population (*D*) with the variance expected under random association of alleles (*VE*) as follows: $I^S_A = (VD/VE-1)(r-1)$, where *r* is the number of loci analyzed. The analysis was performed using the LIAN 3.7 software [29].

RESULTS

Out of the 147 microscopically confirmed *P. vivax* cases recruited from various regions of Southern Thailand, nested PCR results indicated that 130 were mono-*P. vivax* infection, 4 were mono-*P. falciparum*, 3 were mixed infection of *P. vivax* and *P. falciparum*, and 10 samples were unable to be amplified due to minimal quantities of parasite DNA. Total of 130 *P. vivax* samples were isolated from 4 provinces: 50 from Chumphon, 7 from Phang Nga, 58 from Ranong, and 15 from Surat Thani, respectively.

Characterization of *Pvmsp1* F2, *Pvmsp3α* and *Pvmsp3β*

PvMSP3α gene of *P. vivax* was successfully amplified in 62 out of 130 samples (47.70%) (Chumphon = 21/50, Phang Nga = 5/7, Ranong = 35/58, and Surat Thani = 1/15). Three different allele-types were detected in different allele sizes of 1.9, 1.5, and 1.1 kb which were categorized as types A, B, and C, respectively. These 3 genetic types have been recognized as a previously described study [38]. Type A which corresponded to the sequence of the Belem strain and types B and C were the deletions close to the N-terminus of the central alanine-rich domain. In this report, 49/62 (79%) was found in type A, 3/62 (3.3%) was type B, and type C was 11/62 (17.7%), respectively. No mixed genotypes were identified. After *Hha*I digestion, the conservation of the large fragment was seen (1,000 bp) and fragments less than 100 bp was not disregarded [23]. In this study, a total of 14 patterns were found with DNA fragment sizes between 150-600 bp (Fig. 2A). There were 11 haplotypes of type A (A1-A11), 2 haplotypes of type B (B1-B2), and one variant of type C. The highest frequency was 16% observed with haplotype A3 (fragment size 500/280/210 bp). Mixed genotypes were found in 3 isolates (4.8%) including al-

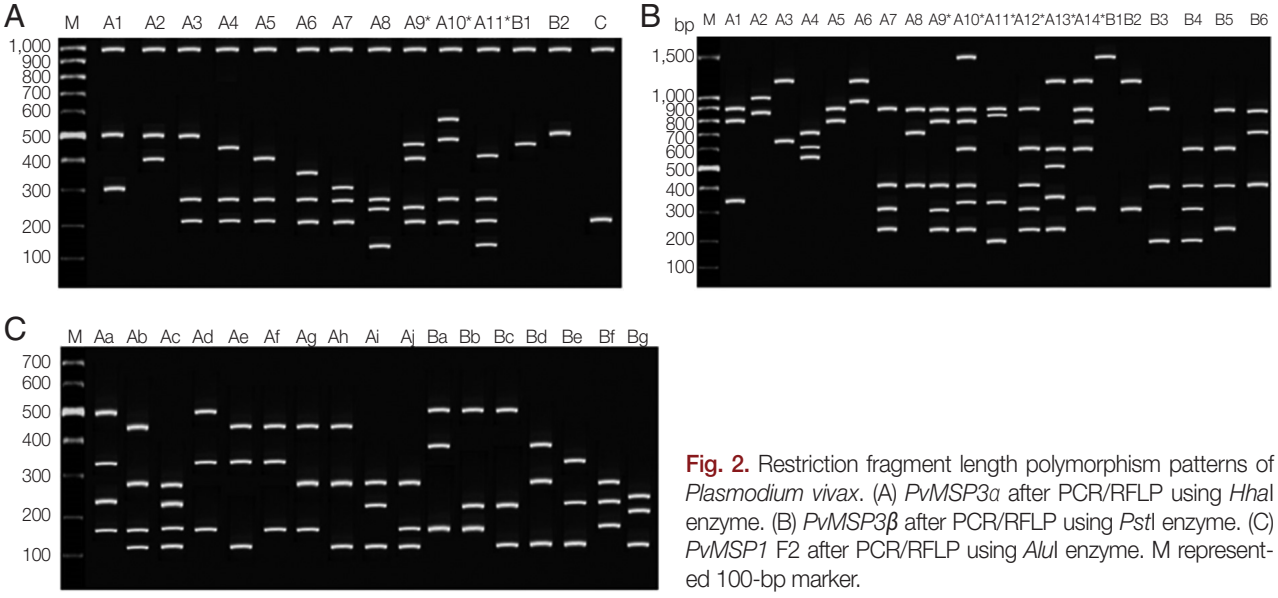

Fig. 2. Restriction fragment length polymorphism patterns of *Plasmodium vivax*. (A) *PvMSP3α* after PCR/RFLP using *Hha*I enzyme. (B) *PvMSP3β* after PCR/RFLP using *Pst*I enzyme. (C) *PvMSP1* F2 after PCR/RFLP using *Alu*I enzyme. M represented 100-bp marker.

lele type A9-A11 when the summed size of the restriction amplicon exceeded the size of the PCR products. The frequencies of each pattern are shown in Table 2.

For *PvMSP3β* gene, 60/130 (46.15%) of the samples were successfully amplified (Chumphon = 17/50, Phang Nga = 3/7, Ranong = 35/58, Surat Thani = 1/15). The alleles differed radically into 2 types: type A showed size polymorphism with ~1.7-2.2 kb, which corresponds to the insertions of sequences

Table 2. Frequencies of each allelic fragment pattern of *PvMSP3α* gene in 62 isolates of *Plasmodium vivax* from Southern Thailand as identified by PCR/RFLP after digested with *Hha*I restriction enzyme

Genotype (kb)	Allele type	*Hha*I restriction fragments (bp)	No. of samples of each Province				Total No. of samples	Frequency (%)
			Chumphon	Phang Nga	Ranong	Surat Thani		
A (~1.9)	A1	1,000+500+300	2	0	0	0	2	3.23
	A2	1,000+500+400	0	0	2	0	2	3.23
	A3	1,000+500+280+210	1	0	9	0	10	16.13
	A4	1,000+450+280+210	1	1	7	0	9	14.53
	A5	1,000+400+280+210	5	0	3	0	8	12.9
	A6	1,000+350+280+210	2	1	3	1	6	9.68
	A7	1,000+300+280+200	0	0	3	0	4	6.45
	A8	1,000+300+250+150	1	2	2	0	5	8.06
	A9*	1,000+450+400+250+200	0	0	1	0	1	1.61
	A10*	1,000+550+480+280+200	0	0	1	0	1	1.61
	A11*	1,000+400+280+210+150	1	0	0	0	1	1.61
B (~1.5)	B1	1,000+500	0	0	1	0	1	1.61
	B2	1,000+500	0	0	1	0	1	1.61
C (~1.1)	C1	1,000+200	8	1	2	0	11	17.74
Total			21	5	35	1	62	100

*Mixed genotype.

Table 3. Frequencies of each allelic fragment pattern of *PvMSP3β* gene in 55 isolates of *Plasmodium vivax* from southern Thailand as identified by PCR/RFLP after digested with *Pst*I restriction enzyme

Genotype (kb)	Allele type	*Pst*I restriction fragment (bp)	No. of samples of each Province				Total No. of sample	Frequency (%)
			Chumphon	Phang Nga	Ranong	Surat Thani		
A (~1.7-2.2)	A1	900+800+350	4	0	5	0	9	16.36
	A2	1,000+850	2	0	3	0	5	9.09
	A3	1,200+650	4	0	1	1	6	10.91
	A4	700+600+550	1	0	2	0	3	5.45
	A5	900+800	0	0	1	0	1	1.82
	A6	1,200+980	0	1	0	0	1	1.82
	A7	900+400+300+250	0	0	1	0	1	1.82
	A8	900+700+400	0	0	1	0	1	1.82
	A9*	900+800+400+300+250	0	0	4	0	4	7.27
	A10*	1,500+900+800+600+400+350+250	0	0	1	0	1	1.82
	A11*	900+850+350+150	0	0	3	0	3	5.45
	A12*	900+600+400+300+250	1	0	1	0	2	3.64
	A13*	1,200+600+500+380+200	0	1	0	0	1	1.82
	A14*	1,200+900+800+600+300	0	1	1	0	2	3.64
B (~1.4-1.5)	B1	1,500	2	0	4	0	6	10.91
	B2	1,200+300	0	0	1	0	1	1.82
	B3	900+400+200	0	0	1	0	1	1.82
	B4	600+400+300+200	0	0	1	0	1	1.82
	B5	900+600+400+250	0	0	3	0	3	5.45
	B6*	900+700+400	3	0	0	0	3	5.45
Total			17	3	34	1	55	100

*Mixed genotype.

Table 4. Frequencies of each allelic fragment pattern of *PvMSP1* F2 gene in 67 isolates of *Plasmodium vivax* from southern Thailand as identified by PCR/RFLP after digested with *Alu*I restriction enzyme

Genotype (kb)	Allele type	*Alu*I restriction fragment (bp)	No. of samples of each Province				Total No. of sample	Frequency (%)
			Chumphon	Phang Nga	Ranong	Surat Thani		
A	Aa	170+230+320+450	1	0	0	0	1	1.49
1,150 pb	Ab	140+170+280+450	1	0	2	4	7	10.45
	Ac	140+170+230+280	1	0	1	0	2	2.99
	Ad	170+320+500	2	0	0	0	2	2.99
	Ae	140+320+480	0	0	4	0	4	5.97
	Af	170+320+480	3	0	0	2	5	7.45
	Ag	170+280+480	0	1	0	1	2	2.99
	Ah	140+280+480	3	1	5	0	9	13.42
	Ai	140+230+280	7	0	3	0	10	14.92
	Aj	140+170+280	0	0	1	0	1	1.49
B	Ba	170+380+500	1	0	2	0	3	4.48
	Bb	170+230+500	0	0	2	0	2	2.99
	Bc	140+230+500	0	0	3	1	4	5.97
1,090 pb	Bd	140+280+380	4	0	0	0	4	5.97
	Be	140+230+320	1	0	1	0	2	2.99
	Bf	170+230+280	0	0	2	1	3	4.48
	Bg	140+210+240	5	0	1	0	6	8.96
Total			29	2	27	9	67	100

Table 5. All microsatellite fragment sizes and allele frequency of *Plasmodium vivax* isolates from Southern Thailand

Marker (size, bp)	Microsatellite analysis							
	Pv1.501 (76-195)	Pv 3.27 (85-240)	Pv 3.502 (128-265)	Pv 6.34 (136-200)	Pv 8.504 (191-317)	Pv 11.162 (172-228)	Pv 14.297 (180-229)	MS1 (228-246)
Samples amplified	95	100	99	97	100	94	102	47
All detected alleles	128	125	109	104	104	97	109	49
Microsatellite fragments[a] (%)	76 (0.8)	92 (12.6)	134 (2.8)	134 (1.9)	198 (9.5)	176 (1.0)	180 (6.4)	225 (12.2)
	83 (5.4)	96 (3.1)	142 (4.6)	136 (1.0)	205 (10.5)	180 (70.1)	183 (2.8)	228 (36.7)
	90 (10.8)	100 (5.5)	150 (27.5)	138 (3.8)	212 (21.9)	184 (16.5)	186 (9.2)	231 (22.4)
	97 (16.2)	104 (7.9)	158 (8.3)	140 (4.8)	219 (26.7)	188 (4.1)	189 (12.8)	234 (10.2)
	104 (14.6)	108 (5.5)	166 (15.6)	142 (22.1)	226 (6.7)	192 (3.1)	192 (22.0)	237 (2.0)
	111 (13.1)	112 (7.1)	174 (9.2)	144 (6.7)	233 (8.6)	196 (5.2)	195 (35.8)	240 (16.3)
	118 (6.9)	116 (6.3)	182 (3.7)	146 (11.5)	247 (2.9)		198 (10.1)	
	125 (3.1)	120 (4.7)	190 (0.9)	148 (12.5)	254 (3.8)		201 (0.9)	
	132 (7.7)	124 (1.6)	198 (12.8)	150 (3.8)	261 (1.0)			
	139 (4.6)	128 (3.1)	206 (7.3)	152 (10.6)	268 (2.9)			
	146 (4.6)	132 (8.7)	222 (1.8)	154 (5.8)	275 (2.9)			
	153 (4.6)	136 (7.9)	246 (5.5)	156 (4.8)	289 (2.9)			
	160 (1.5)	144 (2.4)		158 (4.8)				
	160 (1.5)	148 (0.8)		160 (1.0)				
	167 (1.5)	152 (1.6)		166 (3.8)				
	181 (1.5)	156 (2.4)		198 (1.0)				
	188 (1.5)	160 (3.1)						
		164 (2.4)						
		176 (0.8)						
		188 (0.8)						
		204 (0.8)						
		208 (0.8)						
		212 (0.8)						
		236 (2.4)						
		240 (7.1)						
No. of alleles (*A*)	15	24	12	16	12	6	8	5
No. of alleles per locus	1.35	1.25	1.1	1.07	1.04	1.03	1.07	1.04
H_E	0.897	0.938	0.87	0.896	0.859	0.494	0.804	0.768

[a]All microsatellite sizes in base pair were detected in this study which collected both predominant peaks and minor peaks.
Calculation of no. of alleles (*A*) and H_E values were obtained from only the predominant alleles data set at each locus.
No. of alleles per locus values were calculated from all detected alleles at each locus and divided by the total number of samples amplified.

in the central Ala-rich domain of the gene. Another type was the amplicon size ~1.4-1.5 kb, which categorized as type B. This type is considered to be concordant with the Belem reference strain [16]. The PCR products of *PvMSP3β* showed type A in 43/60 (71.67%) and 16/60 of type B (26.67%). Another one isolate (1.67%) was considered a mixed infection because more than one PCR products of different sizes were observed. After digestion with *Pst*I restriction enzyme, a total of 20 restriction patterns with DNA fragment sizes between 150-1,500 bp were found from 55/60 samples (Table 3; Fig. 2B). Among them, alleles A1 (900+800+350) was the most frequent (16.4%). Mixed infection was detected in 16 isolates (29.1%), including allele type A9-A14.

PvMSP1 F2 gene could be amplified from 67/130 (51.54%) samples (Chumphon=29/50, Phang Nga=2/7, Ranong =27/58 and Surat Thani=9/15). Two distinct size variants were type A (1,150 bp) and type B (1,090 bp). These 2 classified types were based on the polymorphic in size of 100 Thai *P. vivax* isolates which described in the previous study [13]. After *Alu*I restriction enzyme digestion, PCR/RFLP revealed distinct 17 patterns with fragments containing between 140-500 bp (Fig. 2C). The allele frequencies of *PvMSP1* F2 gene are shown in Table 4. No mixed genotyped was noticed.

Microsatellite genotyping of *P. vivax*

Eight microsatellite loci of *P. vivax* were successfully genotyped from 103/130 (79.2%) samples, (38/55 from Chumphon, 4/7 from Phang Nga, 46/ 58 from Ranong and 15/15 from Surat Thani). The microsatellite characteristics of the 8 microsatellite loci used in *P. vivax* genotyping are described in Table 5. A total of 102 different alleles, included predominate and minor peaks, were observed in all the samples and markers. The predominant alleles data set at each locus were used to calculate the genetic diversity with an average H_E=0.82 (SD=±0.14) for all 8 microsatellite markers. Only Pv11.162 was the least polymorphic markers (H_E=0.49). The average number of alleles (*A*) was 12.25 (SD=±6.2), ranged from 5 (locus MS1) to 24 (locus 3.27). The average number of distinct alleles per locus values were calculated from all detected alleles at each locus and divided by the total number of samples amplified was 1.12 (SD=±0.12). A total of 103 samples, these were 46 samples (Chumphon=16/38, Phang Nga=2/2, Ranong=24/46,

Table 6. Multiple of allele sizes at eight loci from *Plasmodium vivax* isolates from Southern Thailand

Marker (size, bp)	Microsatellite analysis							
	Pv1.501 (76-195)	Pv 3.27 (85-240)	Pv 3.502 (128-265)	Pv 6.34 (136-200)	Pv 8.504 (191-317)	Pv 11.162 (172-228)	Pv 14.297 (180-229)	MS1 (228-246)
No. of multiple alleles at each locus	30	25	8	7	4	7	3	2
Multiple of allele sizes at particular locus (%)	97/132 (6.7)	96/132 (4)	150/166 (50)	140/146 (14.3)	205/212/219 (25)	186/189 (14.3)	180/188 (33.4)	227/230 (50)
	97/118 (3.3)	96/104 (4)	150/166/174 (12.5)	142/150 (14.3)	205/212 (25)	189/192 (14.3)	180/196 (33.3)	227/236 (50)
	90/97 (13.3)	92/132/152 (4)	150/166/198 (12.5)	142/158 (14.3)	212/219 (25)	189/195 (14.3)	180/184 (33.3)	
	90/118 (3.3)	92/132 (8)	166/174 (12.5)	144/148 (14.3)	212/247 (25)	192/198 (14.3)		
	90/111/132 (3.3)	100/156 (4)	166/198 (12.5)	146/158 (28.6)		186/195 (14.3)		
	90/111 (23.3)	100/108 (4)		148/154 (14.3)		180/195 (14.3)		
	83/76 (3.3)	104/132 (4)				183/192 (14.3)		
	83/104 (3.3)	108/100 (4)						
	167/188 (3.3)	108/112 (4)						
	139/160 (3.3)	108/112 (4)						
	132/153 (6.7)	108/188 (4)						
	125/146 (3.3)	112/236 (4)						
	125/139 (3.3)	116/120 (4)						
	111/132 (3.3)	116/132 (4)						
	111/104 (3.3)	116/144 (4)						
	104/125 (3.3)	116/160 (4)						
	104/111/132 (3.3)	116/240 (4)						
	104/111/118 (3.3)	120/128/132 (4)						
	104/111 (3.3)	124/132 (4)						
		160/208 (4)						
		132/240 (8)						
		236/240 (8)						

Table 7. The genetic diversity based on eight microsatellite markers of *Plasmodium vivax* isolated from four different provinces of Southern Thailand

Genetic diversity	Province					*P*-value*
	Chumphon n=38	Phang Nga n=4	Ranong n=46	Surat Thani n=15	Mean	
No. of alleles	9.2	2.8	9	5.4	6.6	<0.05*
H_I	0.769	0.771	0.785	0.757	0.771	0.935
Multiple-clone infections, % (isolated)	58% (22/38)	50% (2/4)	48% (22/46)	73% (11/15)	57.25	0.104
MOI**	1.67	1.5	1.53	1.8	1.63	0.408

**P*-value according to 1-way ANOVA, **Multiple clone of infection.

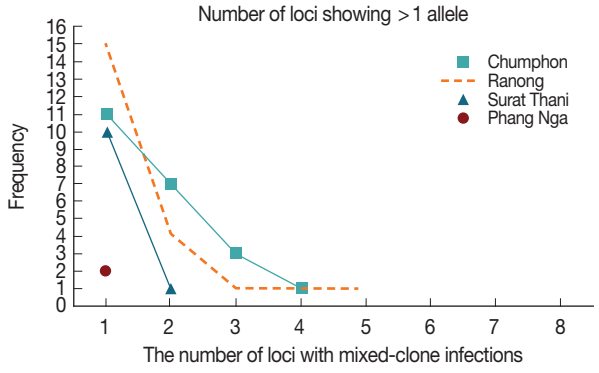

Fig. 3. Frequency distribution of the number of loci with mixed-clone infections isolated from Southern Thailand. Fifty-seven multiple-clone infections were detected for Chumphon, Ranong, Surat Thani and Phang Nga provinces, respectively.

and Surat Thani=4/15) determined to have single clone *P. vivax* infection and the 57 samples determined to have multiple clone *P. vivax* infections. The highest multiple clone infections were found in loci Pv1.501 (30/95 samples) and Pv3.27 (25/100 samples), while Pv 3.502, Pv6.34 and 11.162 were found multiple clone infections in 8/99, 7/97 and 7/94 samples, respectively. Among the 3 loci include Pv8.504, Pv14.297 and MS1 were the lowest multiple clone infections with 4/100, 3/102 and 2/47 samples, respectively. Multiple of allele sizes at 8 loci in this study are described in Table 6.

The number of alleles (*A*) among the 4 sites was significantly different at $P<0.05$ by ANOVA (Table 7). The overall mean expected heterozygosity (H_E) was 0.77±SE 0.01 (rang 0.75 to 0.79), and the average number of alleles (*A*) was 6.6 alleles±SD 3.07 (range 2.8 to 9.2). Multiple-clone infections were found from 57 (55.3%) samples resulting in MOI of 1.63. Up to 5 different markers were detected from one patient in Ranong province (Fig. 3). Significant LD was observed in every province (Chumphon - $I_A^S=0.0385$, $P<0.000$, Phang Nga - $I_A^S=0.1235$, $P<0.001$, Ranong - $I_A^S=0.2488$, $P<0.0001$ and Surat Thani - $I_A^S=0.4442$, $P<0.0001$) suggesting inbreeding of the parasites.

DISCUSSION

Over the last 4 decades, the prevalence of *P. vivax* in Thailand has been rising from 20% to 50% of all malarial cases [30]. Two main mechanisms have been proposed to explain the phenomena: (i) a competitive suppression between species during co-infection within the human host [31-33]; and (ii) a difference in vector competence and capacity for *P. falciparum* and *P. vivax* by mosquito vector species [34-36]. This study aimed to analyze the genetic diversity of *P. vivax* in the Southern part of Thailand, mainly focusing on the high malaria prevalent provinces such as Chumphon, Ranong, Surat Thani, and Phang Nga. The genetic characterization of the parasite populations was analyzed using the single-copy genes; *PvMSP1* F2, *PvMSP3α*, *PvMSP3β* and 8 polymorphic microsatellite markers. Several previous studies have used this type of genetic markers to study the genetic diversity in endemic malaria transmission [13,15,18,19,23]. The limitation of this study was that there was an insufficient amount of DNA template in the samples owing to a small volume (80 µl) of the collected blood sample. Nevertheless, we successfully amplified about 63% of the samples.

Three distinct sizes of PCR products for *PvMSP3α* were detected for 3 different allelic variants. They were 1.9 kb (Type A), 1.5 kb (Type B) and 1.1 kb (Type C). The results were concordant with allele observed in India, Papua New Guinea, Western Thailand, Afghanistan, and Pakistan [37-40], while the different band size of approximately 0.75 kb or 300 bp was reported in Pakistan [41]. Genotyping *PvMSP3α* marker also showed mixed genotypes. This was concordant with the previous reports from Papua New Guinea, Western of Thailand, Iran, Pakistan, India and French Guiana where a significant

degree of mixed genotype was observed between 2-36% [23,38-40,42,43]. *PvMSP3β* gene in the present study had produced 2 categories, Type A (1.7-2.2 kb) and Type B (1.4-1.5 kb). The finding was similar to those observed along the Thailand–Myanmar border, the Thailand–Cambodia border and area of North-West Frontier Province (NWFP) of Pakistan [44,45], Anhui and Guizhou provinces of Chinese [16], and India [46]. The extra allele type ~0.65 kb (Type C) was previously reported in Mae Sod, Thailand [16]. Our study showed that Type A (1.7-2.2 kb) was the highest frequency of vivax malaria circulating in Southern Thailand. Similarly, the report from Anhui, Hainan, Yunnan, and Myanmar [23] also indicated a higher prevalence of Type A alleles. Type B (1.4-1.5 kb) was found as 60.4% of *P. vivax* populations of Western Thailand and 85% of India [46] whereas from Chinese Bengbu and Guangxi samples, both A and B types were equally prevalent [23]. Mixed infection with *PvMSP3β* was detected in one sample (1.67%) from Ranong which was genotyped by PCR. The PCR/RFLP of *Pst*I analysis further revealed the presence of 16 mixed alleles (29.1%) in type A genotype, isolated from 10 Ranong and 2 Phang Nga provinces. Previously, the mixed infection was reported as 4% in Thailand–Myanmar border and the Thailand–Cambodia border [45], 20.5% in Western Thailand [37] and 5.6% in China [16].

For *PvMSP1* Fragment 2 genotyping, 2 sizes differences were found: Type A (1,150 bp) and Type B (1,090 bp). The result was identical to the previous studies [13,21]. Size polymorphisms could distinguish a total of 17 distinct genotypes after digesting with *Alu*I (Table 3.), and no mixed infection was observed whereas report from *P. vivax* genotyping from endemic regions of Thailand showed 12 patterns and 12.5% with multiple genotypes [21]. This would indicate that the various clones of infection could occur in the same host but one time of mosquito biting would transfer distinct clones into hosts [21]. The overall number of alleles (*A*) for 8 markers was 12.6 in this study, and it was significantly different among the 4 sites, at $P<0.05$ by ANOVA test (Table 5). The result of the number of alleles (*A*) in this study was concordant with the other studies from Asia [18-20,47,48]. High H_E values were observed in *P. vivax* isolates from Southern Thailand (0.87) (Table 4.) whereas the isolates from South Korea was 0.43 [47] indicating the high genetic diversity of *P. vivax* in Southern Thailand. Multiple clone infection was found for 55.3% (57/103) in the 4 study areas, particularly in Surat Thani, Chumphon, Phang Nga and Ranong, respectively. However, this result would reflect sample sizes from these regions (Table 5). The multiplicity of infection of this study was 1.63 which also agreed with the findings from previous reports [18,19,49].

In this study, genotyping the *P. vivax* from 4 malaria-endemic provinces of Southern Thailand using 3 merozoite surface protein genes *PvMSP3α*, *PvMSP3β*, *PvMSP1* F2 genes, and 8 microsatellite markers revealed 14 RFLP patterns of *PvMSP3α*, 20 of *PvMSP3β*, and 17 of *PvMSP1* F2. Mixed genotypes were present in 4.8% of *PvMSP3α* and 29.1% of *PvMSP3β* genes. High H_E values were also observed. 55.3% of samples carried more than one *P. vivax* parasite infection. However, immune selection could interfere with the data interpretation of PCR/RFLP on antigenic marker loci. These results revealed high genetic diversity *P. vivax* isolates from Southern Thailand. The information would help in understanding the epidemiology of *P. vivax* parasites and controlling and elimination of the malaria parasite in Southern Thailand.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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