

Allelic Diversity and Geographical Distribution of the Gene Encoding *Plasmodium falciparum* Merozoite Surface Protein-3 in Thailand

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Abstract: Merozoite surface proteins (MSPs) of malaria parasites play critical roles during the erythrocyte invasion and so are potential candidates for malaria vaccine development. However, because MSPs are often under strong immune selection, they can exhibit extensive genetic diversity. The gene encoding the merozoite surface protein-3 (MSP-3) of *Plasmodium falciparum* displays 2 allelic types, K1 and 3D7. In Thailand, the allelic frequency of the *P. falciparum* *m*sp-3 gene was evaluated in a single *P. falciparum* population in Tak at the Thailand and Myanmar border. However, no study has yet looked at the extent of genetic diversity of the *m*sp-3 gene in *P. falciparum* populations in other localities. Here, we genotyped the *m*sp-3 alleles of 63 *P. falciparum* samples collected from 5 geographical populations along the borders of Thailand with 3 neighboring countries (Myanmar, Laos, and Cambodia). Our study indicated that the K1 and 3D7 alleles co-existed, but at different proportions in different Thai *P. falciparum* populations. K1 was more prevalent in populations at the Thailand-Myanmar and Thailand-Cambodia borders, whilst 3D7 was more prevalent at the Thailand-Laos border. Global analysis of the *m*sp-3 allele frequencies revealed that proportions of K1 and 3D7 alleles of *m*sp-3 also varied in different continents, suggesting the divergence of malaria parasite populations. In conclusion, the variation in the *m*sp-3 allelic patterns of *P. falciparum* in Thailand provides fundamental knowledge for inferring the *P. falciparum* population structure and for the best design of *m*sp-3 based malaria vaccines.

Key words: *Plasmodium falciparum*, genotyping, merozoite surface protein-3, molecular epidemiology

INTRODUCTION

Human malaria is caused by parasitic protozoa in the genus *Plasmodium*. Of the 5 species of malaria parasites that infect humans, *Plasmodium falciparum* is the most deadly and is responsible for severe and lethal cases, thereby causing a major public health concern [1]. Around 2-3 billion people in the tropics and subtropics live in malaria endemic regions and are at risk of exposure to malarial infections. Each year, there are 200 million actual illnesses from malaria and up to 0.6-1 million deaths [2]. With the rapid growth of human populations in regions with high malaria transmission, it has been estimated that, in the absence of effective interventions, the number

of malaria cases will double by 2021 [3]. Within the Southeast Asia region, Thailand and its neighboring countries, such as Myanmar and Cambodia, are major hot spots for anti-malarial drug resistance [4]. The first incidence of artemisinin resistance in *P. falciparum* was reported on the Thailand-Cambodian border in 2008 [5], and the resistance later emerged on the western border of Thailand close to Myanmar and so is now likely to spread to other regions [6,7]. This highlights the importance of deploying effective control measures to delay or prevent the spread of the parasite.

Because the pathology and clinical manifestations of malaria are mainly attributed to the erythrocytic stage development of the *Plasmodium* parasite's life cycle, then vaccines against the erythrocytic stage have been developed to induce protective immunity against these parasites [8,9]. This type of vaccine could prevent invasion of the merozoite into erythrocytes, inhibit the development of the parasite in erythrocytes or speed up the clearance of parasitized erythrocyte. The most common blood stage vaccine candidates are the membrane proteins of

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the merozoites such as merozoite surface protein-1 (MSP-1), merozoite surface protein-2 (MSP-2), and apical membrane antigen-1 (AMA-1) [10]. While these antigens have been extensively studied, there are many other membrane proteins such as merozoite surface protein-3 (MSP-3) that have received much less attention, but could be considered potential candidates for blood stage vaccine development.

MSP-3, also known as secreted polymorphic antigen associated with merozoites (SPAM), is one of the essential proteins associated with the merozoite surface, although it lacks a hydrophobic transmembrane domain or a GPI anchor [11]. MSP-3 was first identified using human hyperimmune serum with monocytes in an antibody-dependent cellular inhibition to screen for antibodies that inhibited the *P. falciparum* erythrocytic stage growth in vitro [12,13]. The antigen responsible was found to be MSP-3, suggesting its involvement in the invasion of erythrocytes. Immunizations with full-length or truncated forms of *P. falciparum* MSP-3 also elicited full or partial protection from a challenge infection with *P. falciparum* in a Saimiri model [14]. Furthermore, evidence from epidemiological and immunological studies showed that the levels of IgG3 antibodies against the allele-specific and conserved epitopes in MSP-3 were strongly associated with protection from clinical malaria [15-18]. Peptides derived from MSP-3 protein of *P. falciparum* have now been incorporated and tested as erythrocytic stage malaria vaccines in field trials [19-22]. *P. falciparum* MSP-3 is a 48 kDa protein and is composed of 3 blocks of (i) 4 heptad repeats at the N-terminal with the hydrophobic amino acid alanine (A) in the 1st and 4th positions (AXXAXXX motif) of each heptad, (ii) a hydrophobic glutamine rich domain, and (iii) a putative leucine zipper domain at the C-terminal [23]. It is encoded by a single-copy *msp-3* gene on chromosome 10 (PF10_0345). The *msp-3* gene exists as the 2 allelic types, K1 and 3D7, that differ in the size variation of the heptad repeat region at the N-terminal [24].

Despite its immunological significance, there is currently very limited data on the extent of the genetic diversity of *P. falciparum* *msp-3* in natural populations. To date, only a few studies have evaluated the natural variation in the *msp-3* sequences of *P. falciparum* field isolates. Analysis of *P. falciparum* samples collected from Peru between 2003 and 2006 revealed the temporal variation of 3D7 and K1 alleles over the 4-year period [25]. The 3D7 and K1 alleles of *msp-3* also appeared to be in equal proportion in parasite populations in Iran [26]. Additionally, the analyses of *msp-3* allele frequencies in *P. falciparum* samples

collected from 6 countries in Central and West Africa between 2007 and 2009 showed a high degree of genetic homogeneity between the countries [27]. Genotyping of *msp-3* from 48 and 50 *P. falciparum* samples from Nigeria and Thailand revealed that the polymorphism of *msp-3* was likely to be maintained under frequency-dependent selection [28]. However, in the latter study, the *msp-3* sequence data from Thailand was derived from only a single population at Tak Province on the Thailand-Myanmar border and, to date, there have been no further reports that elucidate the extent of genetic diversity in other regions of the country or other countries in Southeast Asia.

Here, a cross-sectional survey of the allelic types and distribution patterns of the *msp-3* gene in 5 geographical populations of *P. falciparum* in Thailand, located along the national borders of Thailand and 3 neighboring countries (Myanmar, Cambodia, and Laos), was performed on samples collected between 2002 and 2010. This data will provide a new insight into the genetic structure of natural populations of *P. falciparum* in Thailand and also generate the baseline epidemiological data for further studies on field trials of MSP-3 based vaccines.

MATERIALS AND METHODS

Study locations and parasite collections

A total of 63 blood samples, each from a patient infected with a single *P. falciparum* strain (mono-infection), were collected from patients enrolled in each of the 5 sampling localities between 2002 and 2010 (Table 1). The study sites were Mae Hong Son, Kanchanaburi, and Ranong at the Thailand-Myanmar border. The other study sites were Ubon Ratchathani and Trat at the Thai-Laos and the Thai-Cambodia borders, respectively (Fig. 1). A detailed description of the study locations has been previously described [28]. The procedures of parasite collections and maintenance were performed as previously described [28]. The parasite species were confirmed by microscopic examinations of Giemsa-stained thin blood smears and verified by PCR using primers specific to the *P. falciparum* cytochrome B gene (data not shown). Forty samples collected between 2002 and 2006 were previously genotyped by 12 microsatellite loci and shown to be independent clones [29]. The parasites were grown to a parasitemia level of 5-10% and harvested for genomic DNA preparation. The origins of *P. falciparum* reference clones 3D7 and K1CB1 (K1) were described previously [28].

Table 1. The sample location, year of collection, and genotype of the merozoite surface protein-3 gene of *Plasmodium falciparum*

Origin of Pf isolate	Name of isolate	Year of collection	Genotype of the <i>msp-3</i> gene	Origin of Pf isolate	Name of isolate	Year of collection	Genotype of the <i>msp-3</i> gene
Kanchanaburi	K165*	2005	K1	Ranong	RN19*	2003	K1
Kanchanaburi	K185*	2005	K1	Ranong	RN26*	2003	K1
Kanchanaburi	K195*	2005	3D7	Ranong	RN31*	2003	K1
Kanchanaburi	K205*	2005	K1	Ranong	RN36*	2003	K1
Kanchanaburi	K215*	2005	K1	Ranong	RN63*	2005	Mixed
Kanchanaburi	K386	2008	K1	Ranong	RN66*	2005	K1
Kanchanaburi	K389	2008	3D7	Ranong	RN68*	2005	K1
Kanchanaburi	K391	2008	3D7	Ranong	RN70*	2005	Mixed
Kanchanaburi	K392	2008	K1	Ranong	RN72*	2005	K1
Kanchanaburi	K397	2008	K1	Trat	TD504	2003	3D7
Kanchanaburi	K58*	2002	K1	Trat	TD508*	2003	3D7
Kanchanaburi	K64*	2002	K1	Trat	TD510*	2003	K1
Kanchanaburi	K74*	2002	3D7	Trat	TD515*	2003	K1
Mae Hong Son	MH06	2003	K1	Trat	TD529*	2005	K1
Mae Hong Son	MH07	2003	Mixed	Trat	TD530*	2005	K1
Mae Hong Son	MH09*	2003	Mixed	Trat	TD531*	2005	K1
Mae Hong Son	MH10*	2003	3D7	Trat	TD533	2005	K1
Mae Hong Son	MH11*	2004	3D7	Trat	TD542*	2006	Mixed
Mae Hong Son	MH18*	2005	K1	Trat	TD554	2008	K1
Mae Hong Son	MH20*	2005	K1	Trat	TD556	2008	K1
Mae Hong Son	MH24*	2005	K1	Ubon Ratchathani	UB14*	2003	K1
Mae Hong Son	MH28*	2005	Mixed	Ubon Ratchathani	UB22*	2003	Mixed
Mae Hong Son	MH32*	2005	3D7	Ubon Ratchathani	UB27*	2003	3D7
Mae Hong Son	MH50	2010	K1	Ubon Ratchathani	UB28*	2003	3D7
Mae Hong Son	MH51	2010	K1	Ubon Ratchathani	UB50*	2005	3D7
Mae Hong Son	MH61	2010	K1	Ubon Ratchathani	UB51*	2005	3D7
Mae Hong Son	MH65	2010	K1	Ubon Ratchathani	UB52	2005	3D7
Mae Hong Son	MH66	2010	K1	Ubon Ratchathani	UB58	2005	3D7
Ranong	RN129	2008	K1	Ubon Ratchathani	UB59*	2005	3D7
Ranong	RN130	2008	K1	Ubon Ratchathani	UB7*	2003	3D7
Ranong	RN131	2008	K1	Ubon Ratchathani	UB85	2008	K1
Ranong	RN133	2008	3D7				

* *P. falciparum* samples previously genotyped by microsatellite markers [29].

Extraction of *P. falciparum* genomic DNA

The standard phenol/chloroform DNA extraction method was used to extract genomic DNA from the infected blood. In brief, a total of 200 µl of the packed blood cells was mixed with 0.05% (w/v) saponin solution in PBS (pH 7.4). The parasite pellets were mixed with 400 µl of lysis solution (40 mM Tris-HCl, 80 mM EDTA, 2% w/v sodium dodecyl sulfate, pH 8.0) containing 2 mg/ml proteinase K and incubated at 42°C overnight. The aqueous phase was sequentially extracted with an equal volume of Tris-HCl saturated phenol (pH 8.0), phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v, pH 8.0) and chloroform, harvesting the aqueous phase each time. The DNA was precipitated from the aqueous phase with the addition of a 0.1 × volume of 0.3 M sodium acetate (pH 5.2) and a 1 × vol-

ume of absolute ethanol and centrifugation. The genomic DNA pellets were washed with 70% (v/v) ethanol and later resuspended in standard TE buffer (10 mM Tris-HCl, 0.1 mM Na₂EDTA, pH 8.0) and stored at -20°C prior to PCR genotyping.

Genotyping of the *P. falciparum* merozoite surface protein-3 gene

Primers for genotyping of the *msp-3* gene (PF10_0345) were M3F/O 5'-ATGAAAAGTTTTAT AAATATTACTCTTTC-3' and M3R/O 5'-CATGTTATGAATATAAATTATGTTCA-3, which correspond to nucleotide positions 1404192-1404220 (the start codon at positions 1404192-1404194) and 1405293-1405268 (the stop codon at positions 1405254-1405256) of the chromosome 10 of *P. falciparum* strain 3D7 (NCBI accession no.

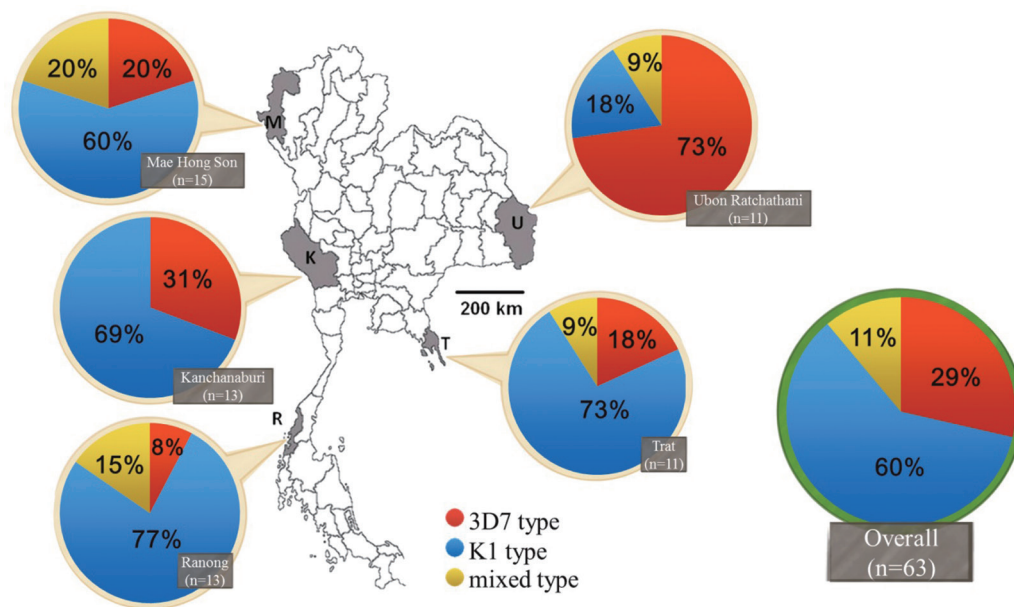


Fig. 1. The distribution of the alleles of merozoites surface protein-3 gene in 5 geographical populations of *P. falciparum* in Thailand. The 5 sampling locations were Mae Hong Son (M), Kanchanaburi (K), and Ranong (R), located at the Thailand-Myanmar border, Ubon Ratchathani (U) located at the Thailand-Laos border, and Trat (T) located at the Thailand-Cambodia border. Numbers (n) in the grey boxes indicate the numbers of parasites from each sampling site or the total number of parasite isolates in Thailand. Numbers in the pie charts represent the percentage of the 3D7-type (red), K1-type (blue) or mixed-type (yellow).

AE014185.2) [30]. The standard PCR reaction was performed in a total volume of 50 µl, containing 200-300 ng of DNA templates, 2 mM of MgCl₂, 200 µM of dNTPs, 0.5 µM of each primer and 2 units of FastStart *Taq* DNA polymerase enzyme in 1 × *Taq* PCR buffer (Roche Diagnostics, Mannheim, Germany). Thermal cycling was performed with an optimized profile of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 40 sec, 56°C for 40 sec, 68°C for 80 sec, and then followed by a final extension at 68°C for 10 min. Subsequently, PCR products were analyzed on 2% (w/v) agarose gel. Electrophoresis condition was at 80 V for 40 min in 1 × TBE buffer. PCR products were visualized and photographed under UV transillumination after ethidium bromide staining. The genotypes of the strains 3D7 and K1 of *P. falciparum* were used as the reference samples (and positive controls). Each sample was genotyped at least 3 times. The allelic types were further confirmed by DNA sequencing of the N-terminal coding region (data not shown).

Population genetics analysis

The data was expressed as the frequencies of the 3D7 and K1 alleles in each sampled parasite population, where any

samples with a mixed genotype were first excluded from the analyses. Population differentiation of the *msp-3* allele frequencies between 2 parasite populations was tested using Wright’s fixation index (F_{st}) in Arlequin suite version 3.5 [31]. Statistical differences were accepted at $P < 0.05$.

RESULTS

Genotypes and distribution patterns of the *msp-3* gene in Thai *P. falciparum* populations

Genotypes of the *msp-3* alleles of 63 *P. falciparum* isolates in Thailand were analyzed by PCR and standard agarose gel electrophoresis as described in Materials and Methods. The genotyping results showed that 89% (56 samples) of the blood samples were comprised of single *msp-3* allelic types of either 3D7 or K1, while 11% (7 samples) had mixed *msp-3* allelic types (Figs. 1, 2). The mixed *msp-3* genotypes were detected in 20%, 15%, 9%, and 9% of *P. falciparum* samples in Mae Hong Son, Ranong, Ubon Ratchathani, and Trat, respectively.

Of the 56 samples with a single *msp-3* allelic type, 29% (18 samples) and 60% (38 samples) were unambiguously classified as 3D7 and K1 subtypes, respectively (Fig. 1). The K1 sub-

type was highly prevalent in 4 *P. falciparum* populations in Trat, Mae Hong Son, Kanchanaburi, and Ranong, representing 73%, 60%, 69%, and 77% of the populations. In contrast, the 3D7 subtype was more prevalent in *P. falciparum* populations in Ubon Ratchatani, representing 73% of the population. Thus, the 3D7 and K1 allelic types of *msp-3* circulated in *P. falciparum* Thai populations, but the allele frequency varied between the different geographic populations.

To further determine whether the parasite populations at the borders of Thailand and the 3 neighboring countries were genetically homogenous, pairwise inter-population compari-



Fig. 2. Genotypes of the merozoite surface protein-3 gene of *P. falciparum* populations in Thailand. Genomic DNA samples obtained from *P. falciparum* parasites in 5 geographical locations, Mae Hong Son (M, n=15), Kanchanaburi (K, n=13), Ranong (R, n=13), Ubon Ratchatani (U, n=11), and Trat (T, n=11), were PCR amplified, resolved, and visualized as described in Materials and Methods. The alleles of the *msp-3* gene were classified into the 3D7, K1, or mixed type.

sons were performed for each parasite population using Wright's fixation index (F_{st}). In this analysis, allele frequency of *msp-3* from a *P. falciparum* population in Tak Province (10 and 40 samples collected in 2000 with single 3D7 and K1 types, respectively), which is located between Mae Hong Son and Kanchanaburi, was also included. Table 2 shows the F_{st} values from the pairs of all 6 parasite populations, where the *P. falciparum* population in Ubon Ratchatani appeared to be genetically distinct from those in the other malaria endemic regions in Thailand ($P < 0.05$). These results suggested that the *P. falciparum* populations in Thailand could be divided according to the *msp-3* alleles into 2 subpopulations. The major group, with the predominance of K1 allele, was comprised of populations at the Thailand-Myanmar border (Mae Hong Son, Tak, Kanchanaburi, and Ranong) and also the population at the Thailand-Cambodia border in Trat. The other minor population, in which the 3D7 *msp-3* allele was predominant, was the parasite population in Ubon Ratchatani at the Thai-Laos border.

Global patterns of allele frequency of *msp-3* in *P. falciparum*

The allele frequencies of *msp-3* from 6 *P. falciparum* populations in Africa (Nigeria, Republic of Congo, Cameroon, Ghana, Burkina Faso, and Senegal), 1 parasite population in the Middle East (Iran), and 1 parasite population in South America (Peru) were obtained through the literature search at the NCBI and Scopus databases (Fig. 3; Tables 3, 4). While the K1 allele was the dominant allelic type in Thailand (data inclusive of the genotypes in Tak [17]), the 3D7 allele was the dominant allelic type in South America, and the K1 and 3D7 allele proportions were close to 1:1 in most Central and West African parasite populations and in Iranian population.

Table 2. Pairwise F_{st} values of the *msp-3* alleles in *P. falciparum* populations in Thailand

	Mae Hong Son	Ubon Ratchathani	Kanchanaburi	Trat	Ranong
Ubon Ratchathani	0.40970* ($P=0.03$)	-			
Kanchanaburi	-0.07817 ($P=0.99$)	0.33065* ($P=0.04$)	-		
Trat	-0.09310 ($P=0.99$)	0.47712* ($P=0.03$)	-0.06450 ($P=0.65$)	-	
Ranong	-0.00301 ($P=0.56$)	0.64391** ($P=0.00$)	0.05359 ($P=0.33$)	-0.05307 ($P=0.62$)	-
Tak ^a	-0.04641 ($P=0.99$)	0.50678** ($P=0.00$)	-0.01586 ($P=0.47$)	-0.06383 ($P=0.99$)	-0.01631 ($P=0.71$)

Grey areas indicate genetic differentiation between *P. falciparum* populations in Ubon Ratchathani and other localities (* $P < 0.05$; ** $P < 0.01$).

^aFrequency of *msp-3* alleles in the *P. falciparum* population from Tak [17].

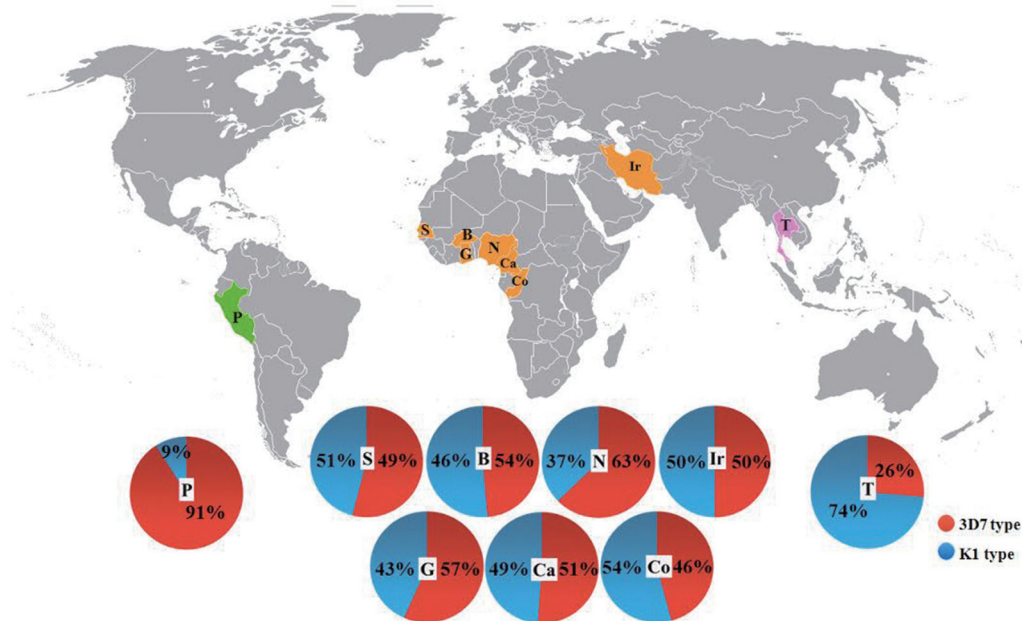


Fig. 3. Global distribution of the merozoite surface protein-3 alleles of *P. falciparum*. Numbers in pie charts represent percentages of the 3D7 (red) and K1 (blue) alleles. Data of the Thai population (T) included the genotyping data from *P. falciparum* population in Tak. Countries from which *msp-3* genotypes were available were: Iran (Ir), Peru (P), Senegal (S), Ghana (G), Burkina Faso (B), Cameroon, (Ca), Republic of Congo (Co), and Nigeria (N).

Table 3. The number of 3D7-type and K1-type alleles of the merozoite surface protein-3 gene in the *Plasmodium falciparum* populations in Thailand, 6 African countries, Iran (the Middle East) and Peru (South America)

Country	Total	3D7 type	K1 type	References
Thailand	106	28	78	Data of the present work and that of [17]
Nigeria	51	32	19	[17]
Republic of Congo	85	39	46	[27]
Cameroon	90	46	44	[27]
Ghana	83	47	36	[27]
Burkina Faso	216	105	111	[27]
Senegal	98	53	45	[27]
Peru	627	570	57	[25]
Total	1,356	920	436	

The *msp-3* allele data of *P. falciparum* populations in Tak Province of Thailand, in Peru, in Iran, and in 6 African countries (Nigeria, Republic of Congo, Cameroon, Ghana, Burkina Faso, and Senegal) had been published previously [17,25-27].

Pair-wise inter-population comparisons, performed for each of the parasite populations using the Wright’s fixation index revealed that the F_{st} values were low and non-significant ($P > 0.05$) between the pairs of the 6 parasite populations within Africa (Table 4). Likewise, low and non-significant F_{st} values ($P > 0.05$) were found when comparing the *msp-3* allele frequency between the parasite populations from Iran and African countries (Table 4). In contrast, significant F_{st} values ($P < 0.05$) were detected between parasite populations from

Thailand and Peru, Thailand and Iran, Iran and Peru, Thailand and the 6 African countries, and between Peru and the 6 African countries. Thus, the overall allele patterns of *msp-3* parasite populations in Thailand, Peru, and the 6 African countries (plus Iran) were different, suggesting that the populations of *P. falciparum* parasites in 3 continents were geographically and genetically isolated or subject to different allele-specific selection processes.

Table 4. Pairwise F_{st} values of the *m*sp-3 alleles in *P. falciparum* populations in Thailand and other malaria endemic regions

<i>P. falciparum</i> populations	Thailand ^a	Nigeria ^b	Republic of Congo ^b	Cameroon ^b	Ghana ^b	Burkina Faso ^b	Senegal ^b	Iran ^b
Nigeria	0.23075** (<i>P</i> =0.00)							
Republic of Congo	0.07000** (<i>P</i> =0.01)	0.04031 (<i>P</i> =0.07)						
Cameroon	0.11270** (<i>P</i> =0.00)	0.01184 (<i>P</i> =0.22)	-0.00605 (<i>P</i> =0.55)					
Ghana	0.16490** (<i>P</i> =0.00)	-0.00825 (<i>P</i> =0.60)	0.01106 (<i>P</i> =0.17)	-0.00554 (<i>P</i> =0.54)				
Burkina Faso	0.08982** (<i>P</i> =0.00)	0.02729 (<i>P</i> =0.09)	-0.00676 (<i>P</i> =0.69)	-0.00667 (<i>P</i> =0.71)	0.00447 (<i>P</i> =0.26)			
Senegal	0.13957** (<i>P</i> =0.00)	0.00031 (<i>P</i> =0.40)	0.0024 (<i>P</i> =0.30)	-0.00899 (<i>P</i> =0.77)	-0.00993 (<i>P</i> =0.76)	-0.00146 (<i>P</i> =0.40)		
Iran	0.10221** (<i>P</i> =0.00)	0.01888 (<i>P</i> =0.12)	-0.00623 (<i>P</i> =0.62)	-0.00907 (<i>P</i> =0.92)	-0.00094 (<i>P</i> =0.36)	-0.000256 (<i>P</i> =0.80)	-0.0055 (<i>P</i> =0.62)	
Peru	0.67676** (<i>P</i> =0.00)	0.29079** (<i>P</i> =0.00)	0.49502** (<i>P</i> =0.00)	0.43042** (<i>P</i> =0.00)	0.36281** (<i>P</i> =0.00)	0.41456** (<i>P</i> =0.00)	0.38946** (<i>P</i> =0.00)	0.42448** (<i>P</i> =0.00)

Grey areas indicate the genetic differentiation between *P. falciparum* populations (***P*<0.01).

^aData from Thailand show the *m*sp-3 allele frequency from 5 localities (Mae Hong Son, Kanchaburi, Ranong, Ubon Ratchatani, and Trat) plus that from Tak province [17].

^bThe *m*sp-3 alleles of 6 *P. falciparum* populations in Africa (Nigeria, Republic of Congo, Cameroon, Ghana, Burkina Faso, and Senegal), 1 population in the Middle East (Iran), and 1 population in South America (Peru) are from the literature [25-27]. See Table 3 for the original allele frequency data.

DISCUSSION

The nature and extent of genetic diversity within and between populations of malaria parasites is essential knowledge for understanding the mechanisms underlying the phenotypic variation and in inferring their population structures. The genetic diversity of the human malaria parasites in Southeast Asia has been a subject of intensive research, which is partly because Thailand and the neighboring countries are major hot spots of drug resistance [4,32]. Several polymorphic markers have been developed and employed for the genotyping of the malaria parasites. These include microsatellite markers and the genes encoding surface antigens such as *m*sp-1, *m*sp-2, and *m*sp-3 [29,33,34]. However, in the case of *m*sp-3 for *P. falciparum* in Thailand, allelic diversity has only been surveyed in a single population in Tak Province, located at the Thailand-Myanmar border [17]. Therefore, the aim of this work was to determine the allelic frequency and distribution patterns for the *m*sp-3 gene in 5 geographical regions of Thailand.

Both K1 and 3D7 alleles of *m*sp-3 were found to co-circulate, but the proportions of individual populations varied according to geographical parasite populations. The K1-type of *m*sp-3 was more prevalent in 3 parasite populations in Mae Hong Son, Kanchanaburi, and Ranong, located along the Thailand and Myanmar borders. This finding was in agreement with the

previous observations in Tak [17], indicating the dominance of K1-allele of *m*sp-3 in the western population of Thailand. Likewise, the dominance of K-1 allele was also observed in a parasite population in Trat, located at the border of Thailand and Cambodia. The similar genetic patterns of the parasite between these 2 regions were detected using polymorphic marker *m*sp-3 α and -3 β of *Plasmodium vivax* [35]. These findings suggest evidence of gene flow between the parasite populations that is due likely to the population movement. In contrast, the dominance of 3D7 allele was detected in a parasite population in Ubon Ratchatani. In this study, the genetic differentiation of *P. falciparum* parasites in Thailand was unambiguously divided into 2 sub-populations. The first was those from the western border of Thailand-Myanmar and also at the Thailand-Cambodia border. The second was a minor population, represented by the population at the Thailand-Laos border. Overall, these results were in agreement with the observations using neutral, genome-wide microsatellite markers that demonstrated the population differentiations between the populations at the eastern and western borders of Thailand [29]. Also, noteworthy is that the population structure based on *m*sp-3 and microsatellite typing was contradictory to that based on the genotypes of the *m*sp-1 gene block 17, which was previously demonstrated that the 5 geographical parasite populations in Thailand were genetically homogenous [28]. This

discrepancy may be due to the *msp-1* gene block 17 being under functional constraints that limited its genetic polymorphism [36,37], or to the relatively small number of samples analyzed.

Extensive human migration in the border areas may have also led to the introduction of new genotypes, resulting in the increase in the genetic diversity [38,39]. A collection of a larger number of samples should be considered for analysis in future studies. Inclusions of *P. falciparum* samples from Myanmar, Laos, and Cambodia would also be of great value to better understand the true epidemiological structure of the malaria parasites in the Indochina region.

In addition, we also conducted population differentiation analysis and included the *msp-3* genotype data from 8 distinct parasite populations in African countries, Iran, and Peru [17, 25-27]. This analysis demonstrated the differences in the allele frequency and patterns of *msp-3* among the parasite populations in different continents. Using the population differentiation statistics, our study suggested that the population structure of *P. falciparum* in Thailand, Africa, and South America were distinct from each other, thereby suggesting that the gene flow between the parasites in these regions was highly unlikely. Our analysis also showed the similar allelic pattern of *msp-3* between parasite populations from Iran and Africa, suggesting the close genetic relationships or gene flow between these populations. Regardless, this information is very crucial for the design of MSP-3 based vaccine. If the full-length MSP-3 molecule was to be developed as a vaccine, it would be preferable to incorporate both the K1 and 3D7 alleles that match the *msp-3* allele frequency for each parasite population, as such a multivalent vaccine would have more long-term usefulness for induction of protective immunity [40].

This study extends our current understanding and knowledge of the variation and prevalence of the *msp-3* alleles in natural populations of *P. falciparum* in Thailand. We showed that the K1 allele was the major variant of the *msp-3* gene in *P. falciparum* populations. The *msp-3* allele frequency varied between different geographical locations, where gene flow may occur in *P. falciparum* populations in Thailand and neighboring countries, but not between other continents. Finally, the present findings provide an overview of the population structure and dynamics of the malaria parasite that is critical for monitoring the population responses to MSP-3 based vaccines in clinical trials.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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