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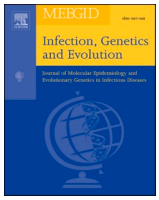

Spatio-temporal analysis of genetic diversity of merozoite surface protein-3 alpha in Myanmar *Plasmodium vivax* isolates

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ABSTRACT

Myanmar aims to eliminate malaria by 2030. However, recent increase of malaria incidence is a great challenge to archive that goal. Increasing prevalence of *Plasmodium vivax* also hinders this endeavor. Monitoring genetic structure of the parasite is necessary to understand genetic nature and evolutionary aspect of *P. vivax* population in Myanmar. Partial fragment flanking blocks I and II of merozoite surface protein-3 alpha of *P. vivax* (*pvmsp-3α*) was amplified from *P. vivax* isolates collected in Pyin Oo Lwin, Mandalay Region, Myanmar in 2013–2015. Sequence analysis of *pvmsp-3α* was performed to determine genetic diversity and natural selection of this gene. Spatio-temporal genetic changes of *pvmsp-3α* in Myanmar *P. vivax* population were also investigated via comparative analysis of gene sequences obtained in this study and previously reported Myanmar *pvmsp-3α* sequences. Genetic diversity of Myanmar *pvmsp-3α* was detected in *P. vivax* isolates analyzed. Size polymorphisms in block I and amino acid changes and recombination events in block II were main factors contributing to the genetic diversity of *pvmsp-3α*. Comparative spatio-temporal analysis with previously reported Myanmar *pvmsp-3α* populations revealed the presence of genetic differences by population with moderate genetic differentiation between populations. Similar pattern of natural selection was also detected in Myanmar *pvmsp-3α* populations. These suggested that enough size of the *P. vivax* population sufficient to generate or maintain the genetic diversity remains in the population. Thus, continuous molecular surveillance of genetic structure of Myanmar *P. vivax* is necessary.

1. Introduction

Myanmar is a country with the highest malaria burden in the Great Mekong Subregion (GMS) (WHO, 2022). It has made significant strides towards malaria elimination in last decades. However, the recent COVID-19 pandemic has caused a reversal of these past achievements. Between 2020 and 2021, Myanmar witnessed an increase of >200,000 malaria cases (WHO, 2022). The mortality rate also increased from 0.2 to 0.74 per 1000 population (WHO, 2022). The increase of anti-malarial drug resistance in the country is also a great concern (Huang et al., 2022; Lê et al., 2022). Although there has been a constant reduction in the prevalence of *Plasmodium falciparum* in the country, *P. vivax* is becoming the predominant species (Kang et al., 2017). Recent studies have reported that the genetic diversity of *P. vivax* population in the country is increasing despite the recent decline of malaria incidences (Naw et al., 2021; Võ et al., 2020), implying that sufficient size of the parasite population is maintained to generate genetic diversity. From this perspective, continuous monitoring of the genetic diversity of Myanmar *P. vivax* population is necessary to understand the genetic polymorphic nature and gene flow in the population.

The merozoite surface protein-3α of *P. vivax* (*pvmsp-3α*) is a member of a multigene family (Jiang et al., 2013). Since *pvmsp-3α* shows genetic

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heterogenicity among the global parasites, it is a reliable polymorphic marker in epidemiological studies (Aresha et al., 2008; Bruce et al., 1999; Cui et al., 2003; Kang et al., 2016; Li et al., 2022; Maneerattanasak et al., 2016; Moon et al., 2009; Mueller et al., 2002; Ord et al., 2005; Ribeiro et al., 2011). Despite genetic heterogenicity of *pvmsp-3α* among global *P. vivax* population, it is regarded as a leading vaccine candidate due to its important biological role and immunogenicity (Bitencourt et al., 2013; Lima-Junior et al., 2011; Stanisic et al., 2013). The polymorphism is mainly found in the central alanine-rich coiled-coil region of the protein, which further divided into two blocks, block I and block II (Rayner et al., 2002). Block I shows great size polymorphism, while block II is relatively conserved. Analysis of genetic polymorphism within this gene can provide valuable information to understand the genetic structure of this gene in *P. vivax* population as well as association between allelic variation and naturally acquired immunologic response.

Previously, two studies on genetic diversity of *pvmsp-3α* in Myanmar *P. vivax* isolates collected from distinct areas have been performed, suggesting substantial level of genetic diversity of this gene in the population (Li et al., 2022; Moon et al., 2009). In this study, we analyzed genetic diversity and natural selection of *pvmsp-3α* in Myanmar *P. vivax* collected from a central region of the country in 2013 to 2015. We also performed comparative analysis of sequences with previous reported *pvmsp-3α* sequences in Myanmar to understand spatio-temporal genetic difference and gene flow of this gene in Myanmar *P. vivax* population.

2. Materials and methods

2.1. Samples

The 86 *P. vivax* DNA samples used in this study were reported previously (Kang et al., 2017; Naw et al., 2021; Võ et al., 2020). The samples were taken from *P. vivax*-infected patients in Pyin Oo Lwin, Mandalay Region, Myanmar, from 2013 to 2015 by finger-prick. The study protocol was reviewed and approved by either the Ethics Committee of the Ministry of Health, Myanmar (97/Ethics 2015) and the Biomedical Research Ethics Review Board of Inha University School of Medicine, Republic of Korea (INHA 15–013).

2.2. Amplification and sequence analysis of Myanmar pvmsp-3α

The *pvmsp-3α* gene was amplified with nested polymerase chain reaction (PCR) using two-pairs of specific primers (Bruce et al., 1999; Moon et al., 2009). Ex Taq DNA polymerase (Takara, Otsu, Japan) with proofreading activity was employed in all PCR procedures to minimize unexpected nucleotide mismatching. Each PCR product was cloned into the T&A cloning vector (Real Biotech Corporation, Banqiao City, Taiwan) and the resulting ligation mixture was transformed into *Escherichia coli* DH5α. Positive clones were selected by colony PCR using nested PCR primers. The nucleotide sequences of the cloned *pvmsp-3α* were analyzed by the Sanger method with M13 forward and reverse primers. To ensure sequence accuracy, plasmids from at least two independent clones from each transformant were analyzed. The nucleotide sequences obtained in this study (Pyin Oo Lwin 2013–2015) have been deposited in GenBank with accession numbers OR225516–OR225611.

2.3. Genetic diversity analysis of Myanmar pvmsp-3α

Sequences acquired in this study were comparatively analyzed with previously reported *pvmsp-3α* sequences from Myanmar (GenBank accession numbers: EU430576–EU430600 from Pyin Oo Lwin 2004, ON314581–ON314674 from Laiza 2012–2015, and ON314675–ON314804 from Myitsone 2012–2015) (Supplement File 1; Fig. S1). These sequences were obtained from *P. vivax* isolates collected from Pyin Oo Lwin in 2004 (Pyin Oo Lwin 2004), Laiza in 2012–2015 (Laiza 2012–2015), and Myitsone in 2012–2015 (Myitsone 2012–2015) (Li et al., 2022; Moon et al., 2009). The *pvmsp-3α* sequence from *P. vivax* Sal I strain (XM_001613154.1) was used as a reference sequence in all analyses. Values for the number of segregating sites (S), haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and the average number of pair-wise nucleotide differences within a population (K) were calculated using DnaSP version 5.10.00 (Librado and Rozas, 2009). The rates of synonymous (dS) and nonsynonymous (dN) substitutions were estimated and were compared using the Z-test ($P < 0.05$) in MEGA6 program (Tamura et al., 2013). The neutral theory of natural selection was evaluated using Tajima's D, Fu and Li's D and F statistics analyses with DnaSP version 5.10.00 (Librado and Rozas, 2009). Linkage disequilibrium (LD) and recombination events (R^2) were examined with the DnaSP program (Librado and Rozas, 2009). Analysis of molecular variance (AMOVA) was performed with an Arlequin ver 3.5.2.2 program (Excoffier and Lischer, 2010) to assess fixation index (Fst) and gene flow (Nm) between and among population. The PopART program (Leigh and Bryant, 2015) and Median Joining algorithm (Bandelt et al., 1999) were used to construct a haplotype network of Myanmar *pvmsp-3α* populations.

3. Results

3.1. Different size polymorphism pattern in Myanmar pvmsp-3α

A total of 96 *pvmsp-3α* sequences were obtained from 86 Pyin Oo Lwin 2013–2015 *P. vivax* isolates by sequencing analyses of cloned genes. Size polymorphisms ranging from approximate 1.1 kbp to 2.0 kbp were detected among sequences. These size polymorphisms were attributed to length polymorphisms in block I. Based on their sizes, *pvmsp-3α* sequences were further categorized into three different variants: A (1.9–2.0 kbp), B (1.4–1.5 kbp), and C (1.1–1.3 kbp). Comparative analysis of variant's composition in *pvmsp-3α* from this study and previous studies revealed differences between and among Myanmar *pvmsp-3α* population (Fig. 1). In Pyin Oo Lwin 2004 population ($n = 141$), frequencies of variants A, B, and C were 46.81%, 18.44%, and 34.75%, respectively. In Pyin Oo Lwin 2013–2015 population ($n = 96$), the frequency of variant A increased to 83.33%, while frequencies of B and C variants dropped to 3.13% and 13.54%, respectively. In Myitsone 2012–2015 population ($n = 129$), variant A was the most prevalent (67.44%), followed by C and B variants with frequencies of 24.80% and 7.75%, respectively. Meanwhile, only two size variants, B (40.43%) and C (59.57%), were detected in Laiza 2013–2015 population ($n = 94$).

3.2. Amino acid polymorphisms in Myanmar pvmsp-3α block II

While size polymorphism in block I was a primary factor generating genetic polymorphism in Myanmar *pvmsp-3α*, amino acid changes in block II were also important factors affecting genetic diversity. The 96 *pvmsp-3α* sequences generated from Pyin Oo Lwin 2013–2015 revealed that block II manifested a total of 20 dimorphic amino acid substitutions compared to Sal I reference sequence (Fig. 2). These 20 substitutions were K445N, D446G, E452K, E484K, D494E, E498G, A527V, S529N, M533L, E535K, K538E, T580K, A581E, N583T, V584A, V585A, D587L, Q617E, K678R, and K685R. Major polymorphic changes were detected in the N-terminal region and motif 1/motif 2 in block II. These amino acid changes caused haplotype diversity of the gene, resulting in 21 distinct haplotypes (H1−H21). Comparative analysis of the sequences with previously reported Myanmar *pvmsp-3α* sequences (Pyin Oo Lwin 2004, Laiza 2012–2015, and Myitsone 2012–2015) revealed that patterns of amino acid changes differed by population (Fig. 3). A total of 41 amino acid changes were detected in Myanmar *pvmsp-3α* population, among which 14 were commonly observed in at least three different populations. Common amino acid changes were mainly detected in two structural motifs, motif 1 and motif 2, albeit their frequencies differed by population. M533L, E535K, and K538E were found in motif 1, while T580K, A581E, N583T, V584A, V585A, and D587L were detected in

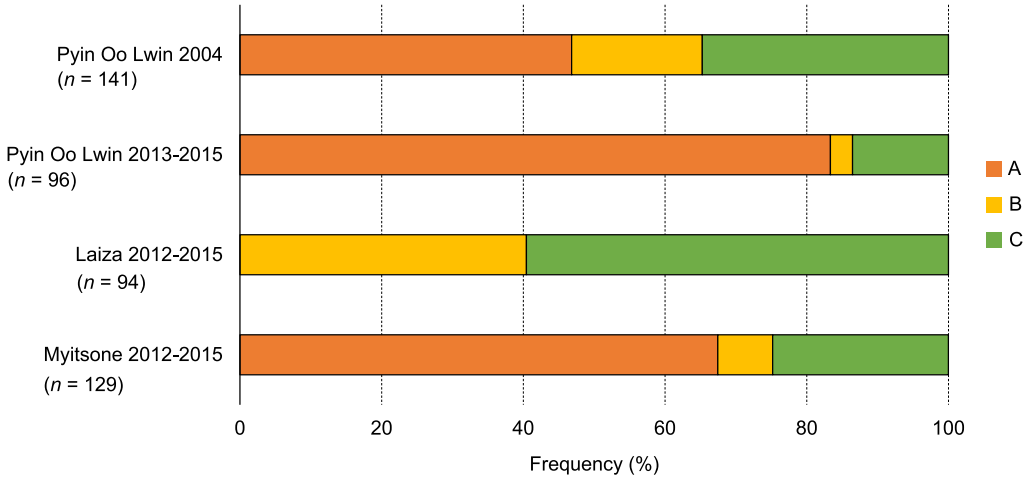

Fig. 1. Spatio-temporal analysis of size polymorphisms in Myanmar *pvmsp-3α*. Three different variant types of *pvmsp-3α* with distinct sizes (A, 1.9–2.0 kbp; B, 1.4–1.5 kbp; C, 1.1–1.3 kbp) were identified in Myanmar *pvmsp-3α* populations. Distribution and proportion of each variant differed by population.

Amino acid position

	445	446	452	484	494	498	527	529	533	535	538	580	581	583	584	585	587	617	678	685	
Sal I	K	D	E	E	D	E	A	S	M	E	K	T	A	N	V	V	D	Q	K	K	*n*
H1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	42
H2	•	•	•	•	•	•	•	•	•	•	•	K	E	T	A	A	L	•	•	•	10
H3	•	•	•	K	•	•	•	•	L	K	E	K	E	T	A	A	L	•	•	•	4
H4	•	•	•	•	•	•	•	•	L	K	E	K	E	T	A	A	L	•	•	•	2
H5	•	•	•	K	•	•	•	•	•	•	•	K	E	T	A	A	L	•	•	•	2
H6	•	•	•	•	•	•	•	•	L	K	E	•	•	•	•	•	•	•	•	•	4
H7	N	•	K	K	•	•	•	•	•	•	•	K	E	T	A	A	L	•	•	•	4
H8	N	•	K	K	•	•	•	•	•	•	•	K	E	T	A	A	L	E	•	•	4
H9	•	•	•	•	•	•	•	N	•	•	•	•	•	•	•	•	•	•	•	•	2
H10	N	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	3
H11	N	•	•	K	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1
H12	•	•	•	K	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1
H13	N	•	K	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	3
H14	N	•	•	K	•	•	•	•	•	•	•	K	E	T	A	A	L	•	•	•	2
H15	N	•	K	K	E	•	•	•	L	K	E	K	E	T	A	A	L	•	•	•	2
H16	•	•	•	•	•	•	•	•	•	•	•	K	E	T	A	A	L	•	•	R	2
H17	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	R	•	2
H18	•	•	•	•	•	G	V	•	•	•	•	K	E	T	A	A	L	•	•	•	3
H19	•	•	K	K	E	•	•	•	L	K	E	K	E	T	A	A	L	•	•	•	1
H20	•	G	•	•	•	•	•	•	L	K	E	•	•	•	•	•	•	•	•	•	1
H21	•	G	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	1

Fig. 2. Amino acid polymorphisms in block II of *pvmsp-3α* in Pyin Oo Lwin 2013–2015. A total of 20 dimorphic amino acid changes were detected in the population. These amino acid changes were scattered in block II, mainly N-terminal part of the region, motif 1 (pink), and motif 2 (sky blue). Dots represent the same amino acid compared to reference strain Sal I (XM_001613154.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

motif 2. Besides these amino acid substitutions, K445N, E452K, and E484K in the N-terminal region of block II were also common amino acid changes found in Myanmar *pvmsp-3α* populations. Analysis of combination patterns of amino acid changes in motif 1/motif 2 generated distinct genetic structures of block II in Myanmar *pvmsp-3α* population. A total of 16 distinct combination types were detected in the population (Supplement File 2; Table S1). Prevalence of each combination type differed by population (Fig. 4). Four combination types, MSELEK/TAANVVKD, MSELEK/KEATAAKL, LSKLEE/TAANVVKD, and LSKLEE/KEATAAKL, were commonly detected in all Myanmar *pvmsp-3α* population, while the other minor 12 combination types (represented as others) were found in either Pyin Oo Lwin 2004 or Myitsone 2013–2015. Significant differences of combination types were also identified between two populations from Pyin Oo Lwin. LSKLEE/KEATAAKL was the most dominant combination type (44.68%) in Pyin Oo Lwin 2004 followed by MSELEK/TAANVVKD (21.28%) and MSELEK/KEATAAKL (13.48%). However, MSELEK/TAANVVKD was the most predominant type (57.29%) and MSELEK/KEATAAKL (28.13%) was the second most

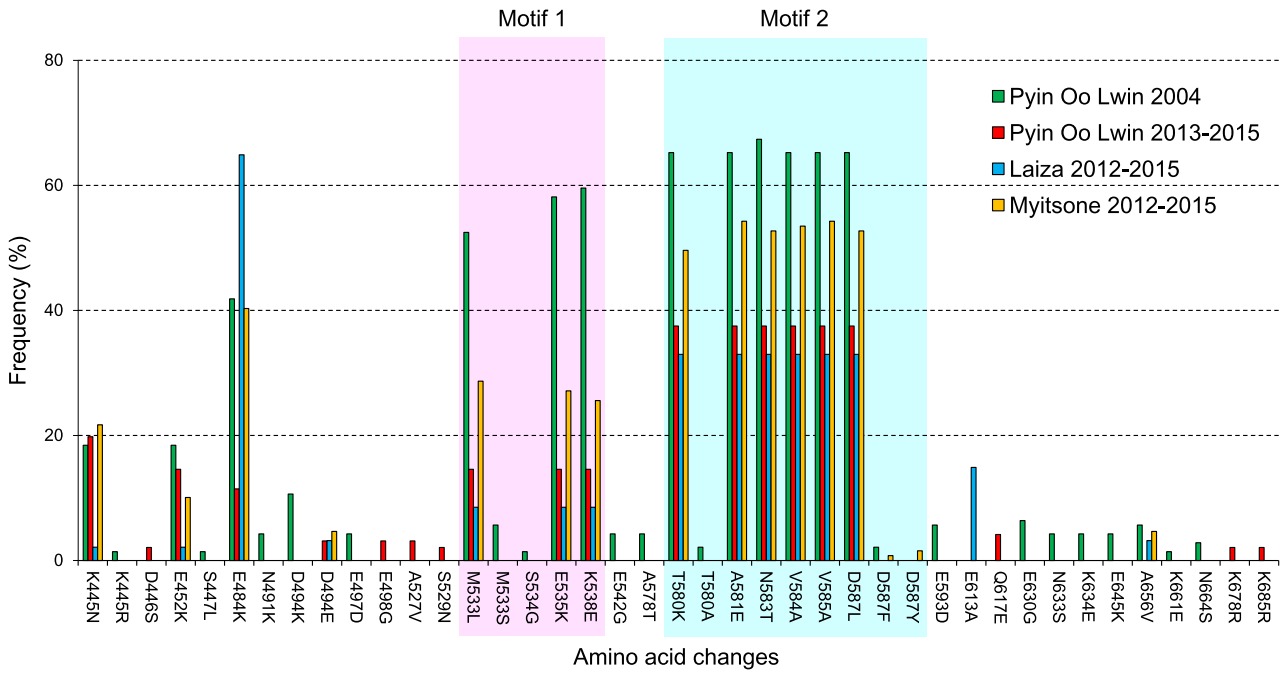

Fig. 3. Spatio-temporal difference in amino acid changes in Myanmar *pvmsp-3α* block II.
A total of 41 amino acid substitutions were observed in Myanmar *pvmsp-3α*, among which 12 were found in all four Myanmar *pvmsp-3α* populations.

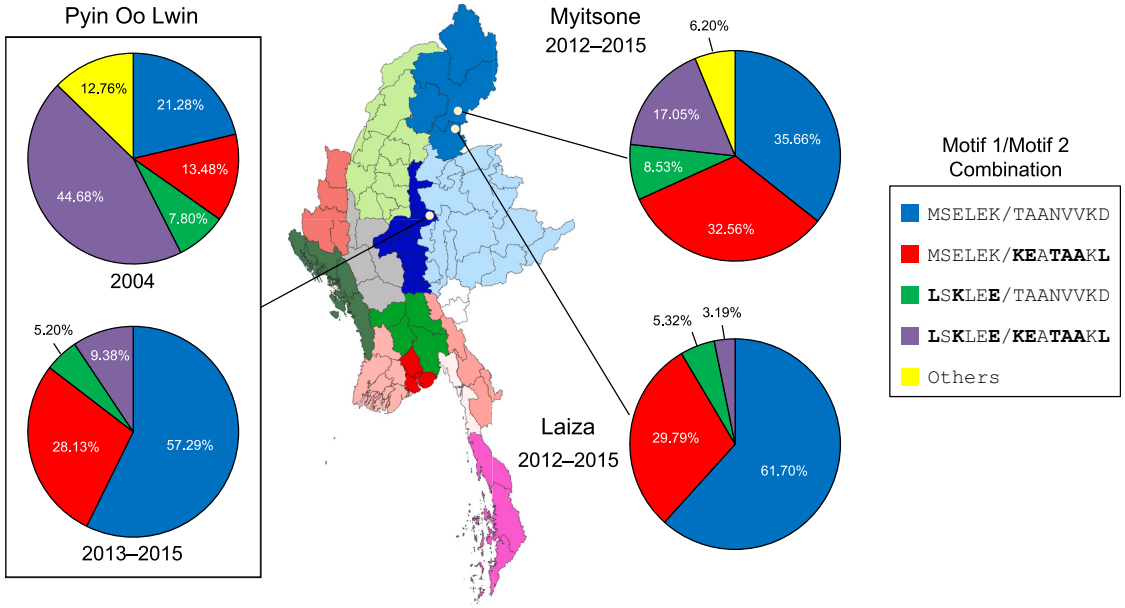

Fig. 4. Spatio-temporal difference in combination types of motif 1/motif 2 in Myanmar *pvmsp-3α* block II. Different combination types of motif 1/motif 2 were detected in Myanmar *pvmsp-3α* populations. Four major combination types were detected in all four Myanmar *pvmsp-3α* populations, although their proportions differed by population. Others mean minor 12 combination types. The frequency of each combination type in each population were presented. The amino acids substituted compared to the reference sequence (XM_001613154.1) were marked with bold letters.

predominant type in Pyin Oo Lwin 2013–2015. Minor types detected in Pyin Oo Lwin 2004 were not observed in Pyin Oo Lwin 2013–2015. Spatial differences of these combination types were also detected in Myanmar *pvmsp-3α* population (Fig. 4). The four major combination types were dominant in Pyin Oo Lwin 2013–2015, Laiza 2012–2015, and Myitsone 2012–2015. In particular, Pyin Oo Lwin 2013–2015 and Laiza 2012–2015 showed similar patterns of combination types. However, Myitsone 2012–2015 revealed different patterns, showing different frequencies of four major types and the presence of other minor types.

3.3. Genetic diversity and test of neutrality of *pvmsp-3α* block II

Analyses of genetic diversity and natural selection in Myanmar *pvmsp-3α* block II revealed that each population showed similar degree of genetic diversity. The average number of nucleotide differences (*K*) ranged from 8.56 (Laiza 2012–2015) to 14.16 (Pyin Oo Lwin 2004). The values of the number of segregating sites (S), total number of mutations (Eta), and number of haplotypes (H) were highest in the Myitsone population and lowest in the Laiza population. Haplotype diversity (Hd) of the population ranged from 0.753 ± 0.032 to 0.936 ± 0.012 (Fig. 5A).

A

Isolates	*n*	*K*	S	Eta	H	Hd ± SD	π ± SD	dN/dS	Tajima's D	Fu & Li's D	Fu & Li's F
Pyin Oo Lwin 2004	141	14.16	61	61	21	0.926 ± 0.009	0.019 ± 0.001	0.325	0.878 (*P* > 0.1)	2.254 (*P* < 0.02)	2.063 (*P* < 0.02)
Pyin Oo Lwin 2013–2015	96	10.65	39	39	29	0.845 ± 0.034	0.014 ± 0.001	0.357	1.261 (*P* > 0.1)	2.060 (*P* < 0.02)	2.089 (*P* < 0.02)
Laiza 2012–2015	94	8.56	31	31	9	0.753 ± 0.032	0.011 ± 0.001	0.429	1.270 (*P* > 0.1)	1.951 (*P* < 0.02)	2.019 (*P* < 0.05)
Myitsone 2012–2015	129	12.46	62	64	46	0.936 ± 0.012	0.016 ± 0.001	0.343	0.182 (*P* > 0.1)	–3.790 (*P* < 0.02)	–2.509 (*P* < 0.05)

n = number of analysed sequences; *K* = average number of nucleotide differences; S = number of segregating sites, Eta = total number of mutations; H = number of haplotypes; Hd = haplotype diversity; π = observed average pairwise nucleotide diversity; SD = standard deviation; dN/dS: rate of nonsynonymous mutation/ synonymous mutation

B

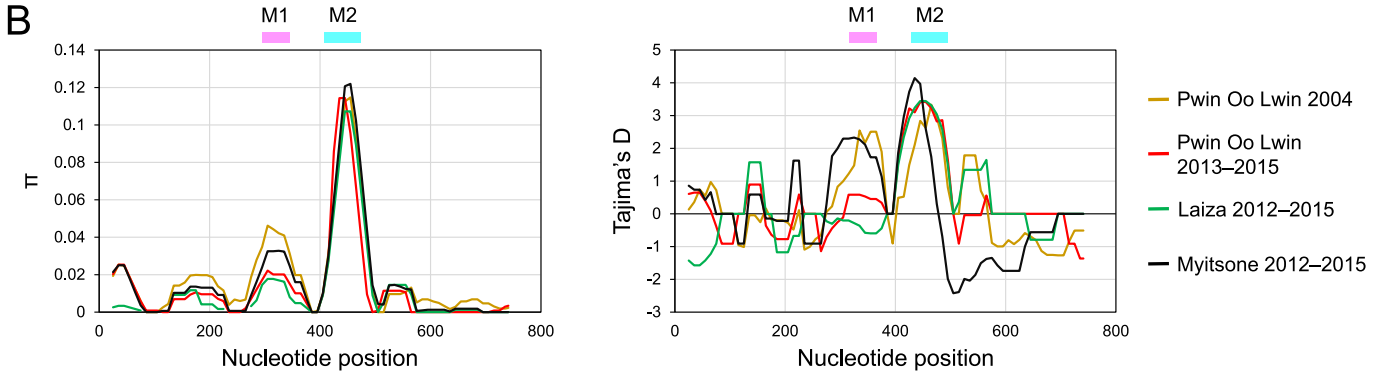

Fig. 5. Nucleotide diversity and natural selection of Myanmar *pvmsp-3α* block II. (A) Estimates of DNA sequence polymorphism and tests of neutrality of Myanmar *pvmsp-3α* block II. (B) Sliding window plot analysis of nucleotide diversity (π) and Tajima's D. A window size of 100 bp and a step size of 25 bp were used. M1, motif 1; M2, motif 2.

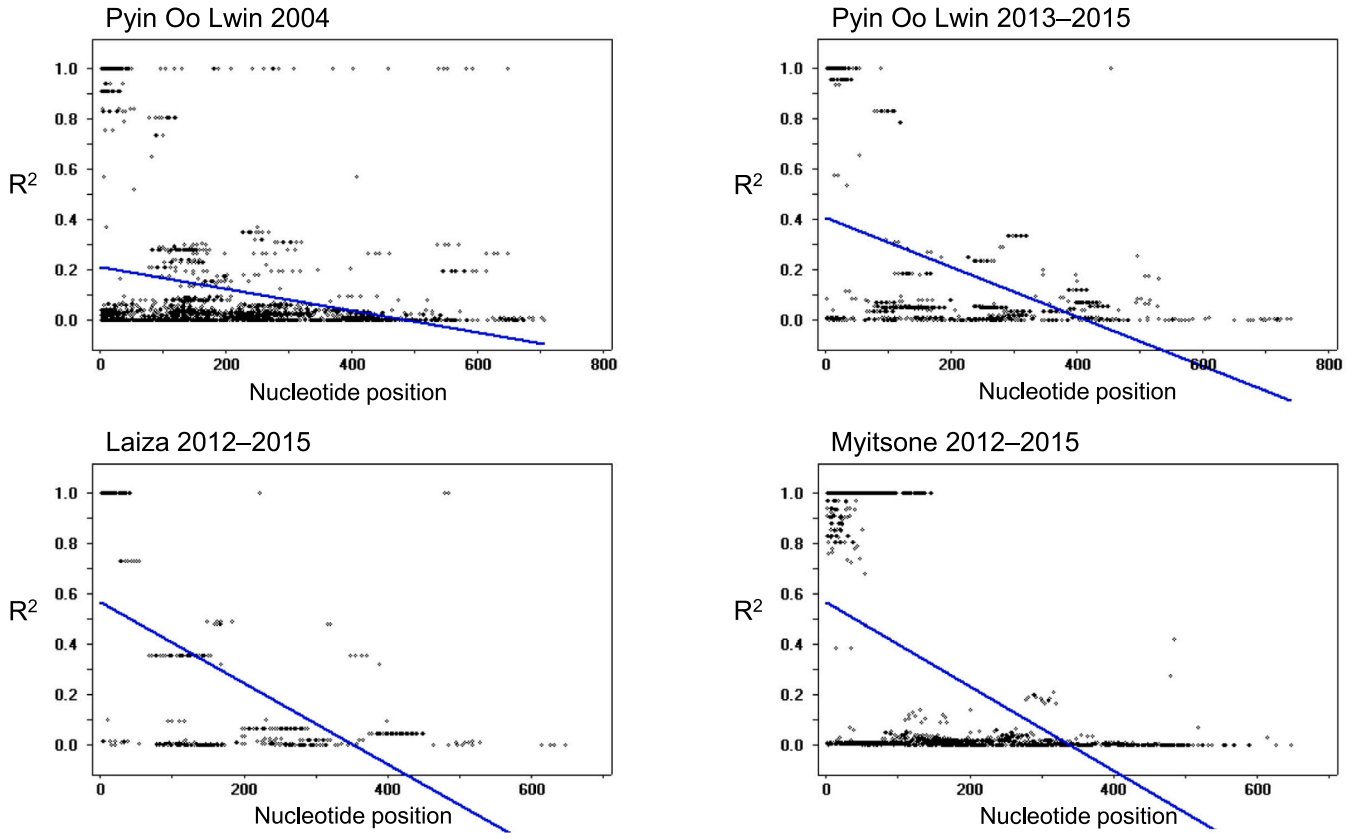

Fig. 6. Recombination event in Myanmar *pvmsp-3α*. Linkage disequilibrium (LD) plot showed non-random associations between nucleotide variants in Myanmar *pvmsp-3α*. R^2 values were plotted against nucleotide distance using a two-tailed Fisher's exact test for statistical significance analysis.

The nucleotide diversity (π) was the greatest in Pyin Oo Lwin 2004 (0.019 ± 0.001). It was the lowest in Laiza 2012–2015 (0.011 ± 0.001). It was found that π values for Pyin Oo Lwin 2013–2015 and Myitsone 2012–2015 were 0.014 ± 0.001 and 0.016 ± 0.001, respectively. These results suggested that Myanmar *pvmsp-3α* block II showed mild genetic diversity among the population. The ratio of dN/dS was lower than 1 in all populations, implying that negative natural selection may occur in the populations. Values of Tajima's D were positive for all populations but not statically significant. Fu & Li's D and Fu & Li's F values were also positive for populations of Pyin Oo Lwin 2004, Pyin Oo Lwin 2013–2015, and Laiza 2012–2015. However, Myitsone 2012–2015 showed negative values of Fu and Li's D and F, indicating that this population was influenced by selective sweep. Overall patterns of π across block II of Myanmar *pvmsp-3α* populations were highly similar (Fig. 5B). Four major peaks of π were detected at the same nucleotide positions in all populations, suggesting these positions were major regions generating nucleotide diversity in Myanmar *pvmsp-3α* block II. Meanwhile, a sliding window plot of Tajima's D values across the region showed slightly different profile by population (Fig. 5B). However, positive values of Tajima's D were detected at regions with corresponding peaks of π.

3.4. Linkage disequilibrium and recombination event

Linkage disequilibrium (LD) in Myanmar *pvmsp-3α* populations was analyzed. The LD index (R^2) exhibited a consistent decrease across analyzed regions in all populations, suggesting that intragenic recombination contributed to the genetic diversity of Myanmar *pvmsp-3α* populations (Fig. 6). The reduction rate of R^2 was greater in Pyin Oo Lwin 2013–2015 than in Pyin Oo Lwin 2004. Similar patterns of R^2 reduction were also predicted for Laiza 2012–2015 and Myitsone 2012–2015. The minimum number of recombination events between adjacent polymorphic sites (Rm) for the Myanmar *pvmsp-3α* was examined. Potential recombination was predicted for all populations. This supports the notion that recombination is a factor generating genetic diversity of Myanmar *pvmsp-3α* (Table 1). Myitsone 2012–2015 showed the highest Rm value.

3.5. Analysis of molecular variance (AMOVA) and genetic differentiation

AMOVA was performed to unveil inter- and intra-population variance in Myanmar *pvmsp-3α* populations, Pyin Oo Lwin 2013–2015, Laiza 2012–2015, and Myitsone 2012–2015. Pyin Oo Lwin 2004 was not included in this comparison as its different collection time. Pairwise fixation index (*Fst*) between Pyin Oo Lwin 2013–2015 and Laiza 2012–2015 or Myitsone 2012–2015 were 0.04574 or 0.04027, respectively (Table 2). Meanwhile, this value between Laiza 2012–2015 and Myitsone 2012–2015 was 0.08991. These results suggest low or moderate genetic differentiation between populations. The pairwise *Nm* values between populations were higher than 1, implying substantial level of gene flows between populations.

3.6. Haplotype network analysis

Haplotype network analysis of Myanmar *pvmsp-3α* showed a dense network with complex relationships (Fig. 7). A total of 83 distinct haplotypes were generated, of which 32 haplotypes (38.6%) were singletons. The most prevalent haplotype was haplotype 6 (H6) with a frequency of 20.6%. This haplotype was the only one shared by all four populations. H7, H8, H17, H25, and H31 were admixed ones by three different populations. Interestingly, Laiza 2012–2015 occupied only 9 haplotypes, suggesting its higher genetic homogeneity and lower haplotype diversity than other populations.

Table 1
Recombination event in Myanmar *pvmsp-3α*.

Population	*n*	Ra	Rb	Rm
Pyin Oo Lwin 2004	141	0.0100	7.6	5
Pyin Oo Lwin 2013–2015	96	0.0022	1.7	6
Laiza 2012–2015	94	0.0005	0.4	4
Myitsone 2012–2015	129	0.0070	5.3	10
Total	460	0.0079	6	14

Table 2
Genetic differentiation and gene flow in Myanmar *pvmsp-3α* populations.

	Pyin Oo Lwin (2013–2015)	Laiza (2012–2015)	Myitsone (2012–2015)
Pyin Oo Lwin (2013–2015)		5.21548	5.95810
Laiza (2012–2015)	0.04574		2.53056
Myitsone (2012–2015)	0.04027	0.08991	

Fst values are shown in the lower left quadrant and *Nm* values are shown in the upper right quadrant.

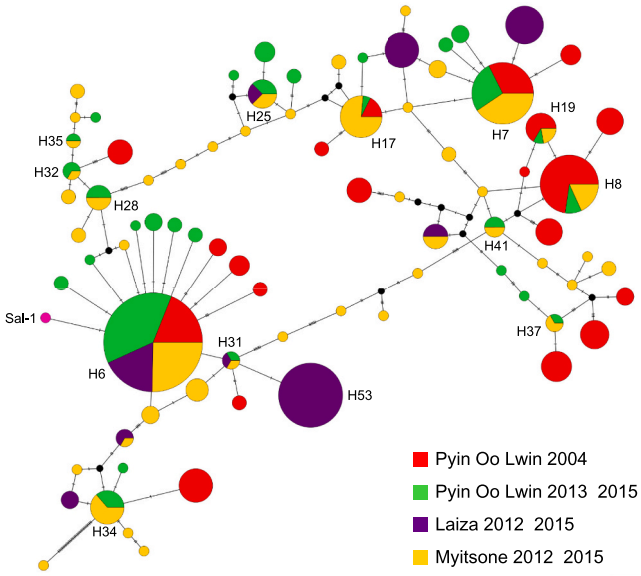

Fig. 7. Haplotype network. Haplotype network was constructed using the PopART program with the Median Joining algorithm. A total of 460 Myanmar *pvmsp-3α* sequences were analyzed. The size of each node reflected the frequency of a particular haplotype. Lengths of lines connecting nodes, measured from their centers, were in proportion to the number of base pair substitutions separating haplotypes. Color of each node indicates different populations.

4. Discussion

Genetic diversity of *P. vivax* is one of the important factors influencing transmission and host immunity (Arnott et al., 2012). Genetic polymorphisms of vaccine candidate antigens in the parasite could generate antigenic variations contributing to host immune evasion and abrogation of host immune recognition (Aresha et al., 2008; Cui et al., 2003; Putaporntip et al., 2002), which further hinder effective vaccine development. In this perspective, understanding population genetic structure of the parasite is necessary to determine the epidemiology, diversity, distribution, and dynamics of the natural population of the parasite.

Here, we analyzed genetic diversity and natural selection of *pvmsp-3α* in *P. vivax* isolates collected in Pyin Oo Lwin, Myanmar in 2013–2015. As reported in previous studies, Myanmar *pvmsp-3α* (Pyin Oo Lwin 2004, Laiza 2012–2015, and Myitsone 2012–2015) revealed

substantial degree of genetic diversity in blocks I and II (Li et al., 2022; Moon et al., 2009). Similar polymorphic characters were also identified in Pyin Oo Lwin 2013–2015. Size polymorphism was the main cause generating genetic polymorphisms of the gene. These variations at the central alanine-rich domain were mainly induced by insertion or deletion resulting in noticeable size variations (Moon et al., 2009). Three variants (A, B, and C) with different lengths were detected in four Myanmar *pvmsp-3α* populations. However, the proportion of each variant differed by population. In particular, only two variants (B and C) were detected in the Laiza 2013–2015 population. Thus, block I of Myanmar *pvmsp-3α* population showed genetic differences among the populations.

Block II of Myanmar *pvmsp-3α* population also revealed genetic heterogeneity, which was mainly caused by nucleotide substitutions resulting in amino acid changes. Amino acid changes in block II were mainly detected in motifs 1 and 2. Most amino acid changes observed in motifs 1 and 2 were commonly identified in 4 Myanmar *pvmsp-3α* populations, but the frequencies of the amino acid changes were highest in Pyin Oo Lwin 2004. Combination patterns of these amino acid changes in motif 1/motif 2 generated distinct genetic structures of block II in Myanmar *pvmsp-3α* populations. Among 16 distinct combination types detected, four major combination types were commonly identified in all Myanmar *pvmsp-3α* populations, although the frequency of each combination type differed by population. Meanwhile, 12 minor combination types were observed in only Pyin Oo Lwin 2004 or Myitsone 2013–2015, incurring the greater genetic diversity of the 2 populations compared to the other 2 populations. Interestingly, these minor combination types were not shared between Pyin Oo Lwin 2004 and Myitsone 2013–2015, but specific for each population. These results suggest that motifs 1 and 2 are major region contributing to the genetic diversity of block II in Myanmar *pvmsp-3α* populations, which could further generate antigenic variations. Besides the commonly identified amino acid changes in all Myanmar *pvmsp-3α* populations, amino acid changes not shared by all populations were also detected. The distributions and frequencies of these amino acids differed by population, generating population-specific amino acid changes.

The *K*, Hd, and π values in Pyin Oo Lwin 2004 were greater than those in Pyin Oo Lwin 2013–2015, suggesting that genetic diversity of *pvmsp-3α* in the parasite population of the area declined consistent with a decrease of transmission intensity. Meanwhile, Myitsone 2012–2015 showed higher values of *K*, Hd, and π than Pyin Oo Lwin 2013–2015 and Laiza 2012–2015. Interestingly, these values were much lower in Laiza 2012–2015 than in Pyin Oo Lwin 2013–2015 and Myitsone 2012–2015, although Laiza is geographically close to Myitsone. These differences between the two populations from geographically close regions could be explained by transmission intensity and mobility of the human population in these two regions. Parasite samples in Myitsone were mainly collected from migrant workers who frequently move across the border, thereby increasing their susceptibility to heightened levels of transmission intensity (Li et al., 2022). Meanwhile, parasite samples in Laiza were collected from stable residents in areas under intensive malaria control measures and low transmission intensity (Li et al., 2022). Nevertheless, overall patterns of genetic diversity and natural selection in block II of all Myanmar *pvmsp-3α* populations were similar. Slide window plots for π and Tajima's D revealed that major nucleotide diversity and natural selection in Myanmar *pvmsp-3α* were detected at the same positions in all four populations, indicating these positions were under natural selection. Non-neglected levels of Rm predicted in Myanmar *pvmsp-3α* populations implied that recombination was also a driving force facilitating genetic diversity of the gene. The highest recombination event in Myitsone 2012–2015 suggested that the genetic diversity observed in this population was also attributed to recombination.

*F*st is one of the most useful measures for analyzing the genetic differentiation among populations. *F*st values at each locus are considered as no differentiation (0), low genetic differentiation (0–0.05), moderate differentiation (0.05–0.15), or high differentiation (0.15–0.25) (Balloux and Lugon-Moulin, 2002). The *F*st values between and among 3 Myanmar *pvmsp-3α* populations (Pyin Oo Lwin 2013–2015, Laiza 2012–2015, and Myitsone 2012–2015) suggested low or moderate genetic differentiation between populations. Interestingly, the *F*st value between Laiza 2012–2015 and Myitsone 2012–2015 was greater than that between Pyin Oo Lwin 2013–2015 and Laiza 2012–2015 or Myitsone 2012–2015. It could be due to transmission intensity and mobility of the human population in these regions (Li et al., 2022) as discussed above. High Hd and π values in Myitsone 2012–2015 and low Hd and π values in Laiza 2012–2015 also supported this notion. Different human population and socio-environmental conditions in the two closely located region in the Myanmar-China border might have caused different genetic diversity in the two populations. Meanwhile, substantial levels of *Nm* between and among Myanmar *pvmsp-3α* populations implied that gene flow occurred among Myanmar *pvmsp-3α* populations.

Our results suggest that a substantial level of genetic diversity is still maintained in Myanmar *pvmsp-3α* populations, albeit spatio-temporal differences are observed. Similar phenomenon has also been identified in Myanmar *pvmsp-1* population (Naw et al., 2021). Although the overall genetic diversity of *pvmsp-3α* in Pyin Oo Lwin 2013–2015 declined compared to Pyin Oo Lwin 2004, a non-neglectable degree of genetic diversity of the gene was identified. A similar or higher level of genetic diversity was also observed in Laiza 2012–2015 and Myitsone 2012–2015. These results suggest that enough size of *P. vivax* population is still maintained to produce genetic diversity and transmission in the country. In particular, genetic diversity of *pvmsp-3α* in Myitsone 2012–2015 emphasized the importance of malaria control in border areas. However, this study had a limitation in that only small numbers of *pvmsp-3α* sequences from *P. vivax* populations in limited areas of Myanmar were applied due to the lack of sequence information of the gene from *P. vivax* isolates in other areas of Myanmar. Moreover, considering that samples analyzed in this study were collected 10 or 20 years ago, a study using recently collected isolates would be necessary to gain in-depth understanding for genetic diversity and evolutionary aspects of *pvmsp-3α* in Myanmar *P. vivax* population.

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Institutional review board statement

The study was approved by the Ethics Committee of the Ministry of Health, Myanmar (97/Ethics 2015) and the Biomedical Research Ethics Review Board of Inha University School of Medicine, Republic of Korea (INHA 15–013).

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CRediT authorship contribution statement

Tuấn Cường Võ: Writing – original draft, Methodology, Formal analysis, Data curation. **Jung-Mi Kang:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Data curation. **Hương Giang Lê:** Writing – review & editing, Validation, Formal analysis. **Haung Naw:** Writing – review & editing, Validation, Formal analysis. **Tong-Soo Kim:** Writing – review & editing, Formal analysis. **Ho-Joon Shin:** Writing – review & editing, Formal analysis. **Moe Kyaw Myint:** Writing – review & editing, Resources, Methodology. **Zaw Than Htun:** Writing – review & editing, Resources, Methodology. **Byoung-Kuk Na:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this article are provided within the article. The original datasets analyzed in this study are available from the corresponding author upon request.

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