



Genetic diversity of *Plasmodium falciparum* erythrocyte membrane protein 1 in field isolates from central Myanmar



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Abstract

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Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1), encoded by the polymorphic *var* multigene family, is a highly polymorphic antigen that plays a crucial role in the pathology of malaria. The contribution of the genetic diversity of *var* toward the immune escape of *P. falciparum* has not yet been fully elucidated. This study aimed to characterize the diversity of *var* repertoires by screening *P. falciparum* Duffy-binding-like α domain (PfDBL α) among field isolates from central Myanmar. Genetic analysis revealed that the D-H segments of *var* in Myanmar populations have an extensive polymorphic repertoire, with high numbers of unique sequence types in each individual. However, *var* genes from the global population, including Myanmar, shared close genetic lineages regardless of their geographic origins, indicating that they have not undergone rapid evolutionary changes.

Keywords: *Plasmodium falciparum*, erythrocyte membrane protein 1, genetic diversity, Myanmar

Introduction

Malaria, one of the most serious vector-borne infections, is caused by *Plasmodium* parasites. In 2020, 241 million people were diagnosed with malaria, of which 627,000 died. Half of the world's population is at risk of contracting the disease [1]. Malaria parasites exploit the diversity of their surface antigens to evade the host's immune system, which is a major impediment to the development of effective malaria vaccines [2].

Several *Plasmodium* species contain multigene families encoding variant surface antigens involved in cell binding. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the polymorphic *var* multigene family, is a well-characterized variant antigen expressed on the surface of infected erythrocytes and implicated in the pathology of malaria [3]. Although PfEMP1 is highly variable among parasite isolates, only one type is expressed on the surface of infected red blood cells. It is either stably inherited through successive cell cycles or switched off by the expression of a different gene during infection [4,5].

PfEMP1 comprises domain assemblies that vary in their composition and sizes and is arranged in intra- and extracellular regions. Each member of the multicopy *var* family con-

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Conflict of interest

The authors declare no conflict of interest related to this study.

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tains 2 exons (Fig. 1). Exon 2, approximately 1.5-kb long, is highly conserved and encodes a putative intracellular region called the acidic terminal segment, whereas exon 1, approximately 4-10-kb long, is highly variable and encodes a putative extracellular region and a transmembrane domain [6]. The extracellular region predominantly comprises an N-terminal segment, a duplicated arrangement of Duffy-binding-like (DBL) domains, tandem cysteine-rich interdomain regions (CIDRs), and C2 domains. The DBL and CIDR domains form 5 (α , β , γ , δ , and ϵ) and 3 (α , β , and γ) types, respectively, in which sequences have unique specific consensus motifs that can be used to characterize distinct DBL-CIDR domains. The prototypical head structure of most PfEMP1 members includes a DBL1 α -CIDR1 α semi-conserved tandem, followed by DBL δ -CIDR β [6-10].

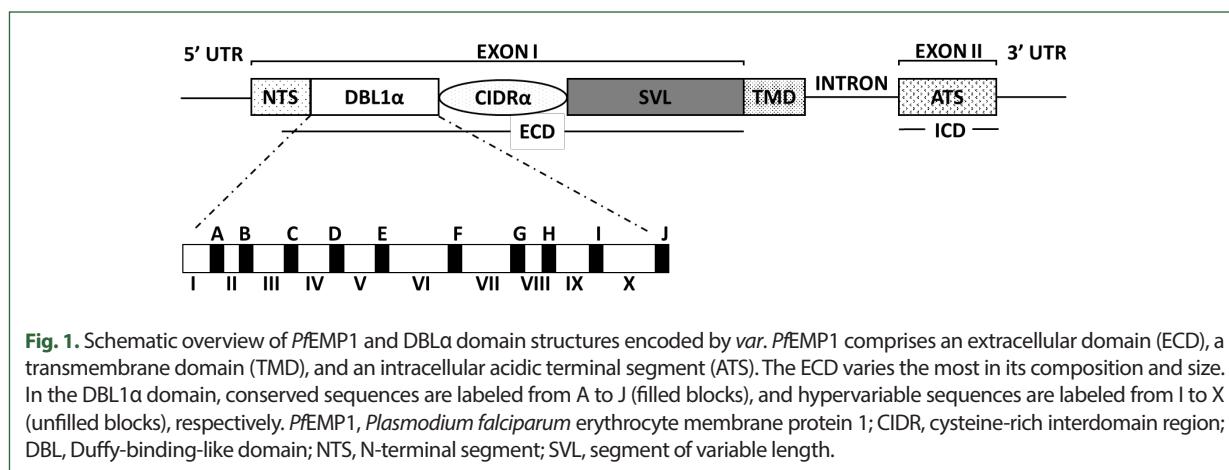
Malaria distribution in Myanmar is heterogeneous and is influenced by several factors, such as the environment, population geospatial repartition, and international and internal migration [11-13]. Some molecular epidemiological studies have been conducted on *P. falciparum* isolates collected from the border areas of Myanmar [14,15]; however, information on the genetic diversity of PfEMP1 among isolates from other regions of Myanmar is lacking.

This study aimed to characterize the genetic diversity of *var* repertoires. We screened PfDBL α in *P. falciparum* isolates collected from central Myanmar. Understanding the nationwide diversity of PfEMP1 can provide insights into the genetic structure of the parasite and the root of persistent disease transmission, ultimately aiding in the successful elimination of the parasite.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of the Ministry of Health, Myanmar (97/Ethics 2015) and Kyungpook National University (2017-0010). Written informed consent was obtained from each participant. For individuals aged < 18 years, consent was obtained from their parents.



Study sites and sample collection

Samples were collected from the central areas of Myanmar, including Mandalay, Naung Cho, Pyin Oo Lwin, and Tha Beik Kyin, from patients diagnosed with *P. falciparum* infections between August 2013 and December 2015. *Falciparum* malaria was diagnosed using a rapid diagnostic test and a subsequent microscopic examination followed by polymerase chain reaction (PCR) for confirmation [16]. A total of 48 blood samples from *P. falciparum*-infected patients (42 men and 6 women, age 3-50 years) were analyzed in this study (Supplementary Table S1). Genomic DNA (gDNA) was purified from the samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

PCR amplification of *PfDBLα*

PfDBLα was amplified from the gDNA of each isolate using previously used degenerate primer sets BF (forward: 5'-GCMTGYGCDCCRTWYMGAMG-3') and BH (reverse: 5'-CK-GCCCATTCYTCRAACCA-3') targeting the semi-conserved blocks B and H of *DBLα* [3,17]. Briefly, PCR was performed with the following conditions: 94°C for 2 min (initial denaturation); 35 cycles at 94°C for 5 sec, 50°C for 20 sec, and 60°C for 45 sec; and 60°C for 2 min (final extension) [18]. The PCR results were confirmed by electrophoresis on a 1.5% agarose gel, followed by staining with ethidium bromide and visualization under ultraviolet light.

Cloning and sequencing

For each isolate, amplicons scattered in the 450-700-bp region were gel-purified, ligated into the pGEM-T Easy Cloning Vector Kit (Promega, Madison, WI, USA), and transformed into *Escherichia coli* DH5α competent cells according to the manufacturer's instructions. Twenty to forty colonies per each cloned amplicon were selected and sequenced using Sanger's method (Macrogen Inc., Daejeon, Korea). The obtained sequences were aligned with global sequences from GenBank and analyzed using CLC Main Workbench 6.

Sequence analysis

Raw sequence data were obtained by excluding all nonspecific and low-quality sequences as well as human or vector contamination of each clone sequence. The *PfEMP1*-encoding sequences were identified from the high-quality sequences using BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Clones acquired from the same isolate and with a *DBLα* nucleotide sequence identity of at least 96.0% were classified into the same sequence type (ST) of *var*. DNA sequences were translated using EMBL-EBI (https://www.ebi.ac.uk/Tools/st/emboss_transeq/), and amino acid sequences were aligned against those hosted on the 3D7 genome database of the PlasmoDB interface (<https://plasmodb.org/plasmo/>) using BLAST to categorize their homology groups.

Var sequence data from the global population

The global *var* sequence data used for the comparison study were downloaded from GenBank and corresponded to 3 regions: Southeast Asia, Sub-Saharan Africa, and South America. In total, 73 sequences were obtained from Papua New Guinea, Thailand, and Indonesia

(Asian population); Gabon, Kenya, Mali, Malawi, Sudan, Tanzania, and Senegal (African population); and Brazil, Venezuela, and Honduras (South American population). Additionally, 38 *var* sequences from the 3D7 strain were obtained from PlasmoDB. Accession numbers for the downloaded sequences are listed in Supplementary Table S2.

Phylogenetic analysis

The phylogenetic tree was constructed using MEGA X (<https://www.megasoftware.net/>) with the minimum-evolution method and the maximum composite likelihood model. Evolutionary analyses were performed on the Myanmar *var* sequences using the 3D7 strain and global populations as references.

Results

*Pf*DBL α sequence types

Of the 48 Myanmar *P. falciparum* isolates, 42 successfully yielded DBL α reads. After applying the exclusion criteria, 1033 individual BF/BH inserts were successfully sequenced, resulting in 82 distinct DBL STs (Table 1). The *PfEMP1*-encoding sequences obtained have been deposited in GenBank (Accession Nos. ON991795–ON991950). Most isolates had multiple STs with unique sequences that occurred only once in an isolate (singletons). The number of distinct DBL *var* sequences detected per isolate varied from 1 to 20. The distribution of STs in the study population is shown in Fig. 2.

Distribution and frequency of *Pf*DBL α groups

The DBL α sequence from each isolate was aligned against the sequences hosted on the 3D7 genome, and the sequence with the highest alignment score was assigned the name of the gene as identified from the 3D7 genome (Table 2). Thirty-nine different 3D7 homologs containing *Pf*DBL α -encoding sequences were identified. Transcripts from the DBL α groups A, B, C, B/A, and B/C were detected in isolates, and those from groups B and C were mostly shared. The highly dominant DBL α sequences were confirmed to belong to *var* B, including the transcripts PFA0005W (33.3%), PFD0005W (26.2%), and PFB0010W (21.4%), followed by *var* C, including the transcripts PFD0630C (23.8%) and PFD0615C (21.4%; Table 2).

Table 1. Summary of the sequencing results for Myanmar isolates

Parameter	Value	Values after trimming and exclusion
Total sequence reads from the Myanmar isolates	1,121	1,033
Total no. of isolates with sequences reads	44	42
No. of isolates with DBL α sequence reads	42	42
Mean no. of isolates with DBL α sequence reads per isolate	4	3
Median no. of isolates with DBL α sequence reads per isolate	5	6
No. of DBL α sequence types	106	82
Median no. of DBL α sequence types reads per isolate	4	2
No. of unique DBL α types or singletons in the sample	68	50

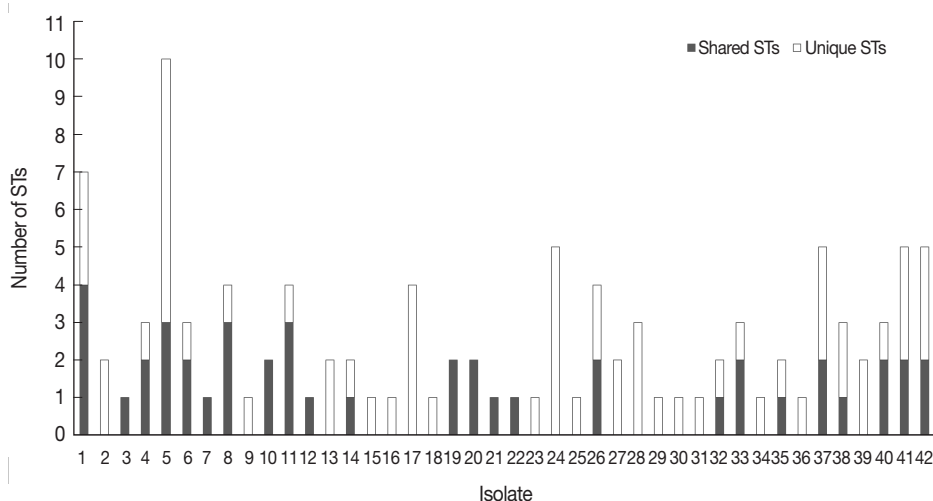


Fig. 2. Distribution of *PfDBLA* variants in 42 Myanmar isolates. *PfDBLA* nucleotide sequences with at least 96.0% identity were classified into the same sequence type (ST) of *var*. Shared STs, sequences shared between isolates; Unique STs, sequences occurring only once within isolates.

Table 2. Summary of predominant DBLA sequences

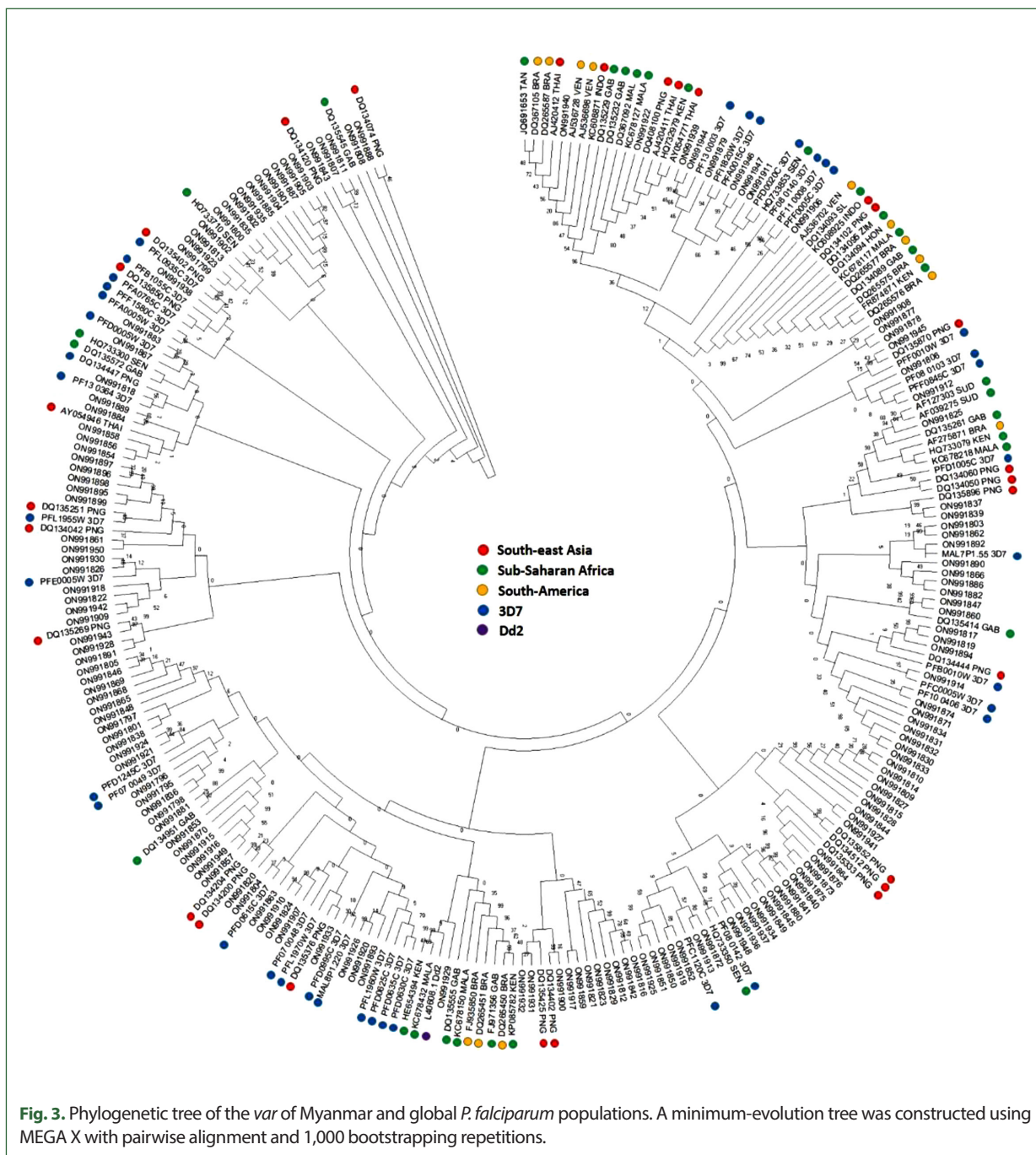
Transcript blasted (Var group)	No. of isolates (% among studied isolates)	No. of sequences
PFA0005W (Var B)	14 (33.3)	20
PFD0005W (Var B)	11 (26.2)	20
PFD0630C (Var C)	10 (23.8)	12
PFB0010W (Var B)	9 (21.4)	18
PFD0615C (Var C)	9 (21.4)	15
PFB1055C (Var B)	7 (16.7)	9

Genetic diversity and phylogenetic analysis of *PfDBLA* in Myanmar and global populations

The genetic diversity of the studied population was compared by aligning against the D-H segment of the *P. falciparum* 3D7 *var* genes. All individuals with DBLA exhibited conserved cysteine-rich motifs at all positions (Supplementary Fig. S1). *Var* sequences from the Myanmar population were highly diverse and clustered with the global *var* genes used as a reference by sharing the bootstrap root from the Asian, European, African, and American *var* sequences (Fig. 3). In addition, the D-H segment sequences of *var* genes from all continents (Asia, Europe, Africa, and America) and the 3D7 strain were distributed in all branches without any region-specific cluster.

Discussion

Myanmar bears the high burden of malaria in the Greater Mekong Subregion. Although the recent incidence of malaria in the country has decreased, the genetic diversity of malaria parasites remains high, making it an important public health problem. Asymptomatic infections and the spread of antimalarial drug resistance also are major concerns [13,15].



In addition, the level of malaria transmission in Myanmar varies by region, making the regional persistence of the disease heterogeneous [19-22]. Therefore, understanding the genetic diversity of malaria parasites represents a major step toward disease eradication because it can provide insights into effective vaccine development.

In this study, we investigated the genetic diversity of *var* genes within a natural *P. falciparum* population in central Myanmar by analyzing their DBL α domain-encoding sequenc-

es. This particular sequence motif was chosen because DBL α domains are used as polymorphic markers in gene diversity and gene expression studies. Moreover, hyperendemic regions, such as DBL α domains, directly help the parasite evade the immune response [17,23, 24]. A high degree of *var* recombination increases the genetic diversity between isolates in neighboring and distant geographic locations [6,25]. Therefore, examining the genetic diversity of DBL α -encoding sequences can provide valuable information about the molecular structure of the *P. falciparum* population in Myanmar. An extensive range of *var* repertoires was found among the studied population. After applying a 96.0% cutoff to define the DBL α types with a minimum sequence overlap, 106 STs were identified, and singletons occurred at a high frequency in the population (Fig. 3). The mean number of shared ST sequences among isolates was not significant, suggesting a constant event in the *var* recombination repertoire and extensive diversity.

The *var*-encoded hypervariable PfEMP1 family of proteins mediates the adhesion of infected erythrocytes to various host cells during the blood stage of malaria infection. Most PfEMP1-encoding *var* genes are located in the subtelomeric regions, whereas others are located centrally in chromosomes [26]. Based on their sequence similarity and analysis of the intron and 5' and 3' untranslated regions, *var* genes can be categorized into 3 major subgroups (*var* groups A, B, and C) and 2 intermediate groups (B/A and B/C), which represent transitions between the 3 main groups [27]. Cham et al. reported that children progressively acquired a broader repertoire of anti-PfEMP1 antibodies in the background of high exposure to malaria infection, but as their age increased, the identified antibodies acquired against particular DBL-like domains belonged to groups other than A or B/A [28]. Therefore, in individuals frequently exposed to malaria, parasites express higher levels of groups B, C, or B/C PfEMP1, which correlates with their reduced fitness and pathogenicity during subsequent infections [28]. The population examined in this study was within the working age (93.0%), and *var* group B predominated, followed by *var* group C (Table 2). The expression of a restricted and antigenically semi-conserved subset of PfEMP1 suggests that this population has acquired a minimum level of immunity against malaria infection because of being constantly exposed to the disease.

In this study, within individual patients, several DBL α sequences were found more frequently than others but they were also shared between individuals. The DBL α domain contains 16-18 conserved cysteine residues, which likely play an important role in its folding and structure [29]. The number of cysteine residues determines the classification of the DBL α domain and the severity of the disease [6,30]. Polymorphism within the hypervariable region induced via gene recombination and/or duplication results in length and sequence variability among parasites between and within hosts in malaria-endemic areas [31]. The DBL α domain has an average of 4 cysteine residues, and the number can vary from 2 to 9. Variants with unusual numbers of residues may exhibit altered antigenic and adhesive properties. Among the Myanmar population studied, the cysteine at the CRC motif was almost conserved in all the *var* genes and was highly conserved in the homology blocks VWKAfTC and FRkTC. The semi-conservation of cysteine residues within the Myanmar sequences suggests that cysteine contributes toward parasite maintenance in these low-endemic areas. Even with the low number of patients included in this study, the targeted DBL1 α domain showed a global distribution of *var* within the central regions of Myanmar. On ac-

count of sharing borders with China, India, and Thailand, we expect to find predominant geographical *var* types related to any or all of these countries. However, these results highlight the constancy of population movement in and out of different countries that share borders with Myanmar [32]. Consistent with the conserved cysteine residues among populations, phylogenetic analysis of *var* genes from Myanmar and global isolates revealed that the D-H segment was not distributed in region-specific clusters, although the gene showed genetic diversity within isolates.

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