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Original Article

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

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Abstract

Plasmodium falciparum erythrocyte membrane protein 1 (*Pf*EMP1), 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 (PfDBLα) 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 (*Pf*EMP1), 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 *Pf*EMP1 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].

*Pf*EMP1 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-

Author contributions
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Data curation: Dinzouna-Boutamba SD, Lee S, Goo YK
Formal analysis: Dinzouna-Boutamba SD, Lee S, Moon Z
Funding acquisition: Goo YK
Investigation: Dinzouna-Boutamba SD, Lee S, Na BK
Resources: Chung DI, Myint MK, Naw H, Na BK
Validation: Naw H, Visualization: Lee S
Writing – original draft: Dinzouna-Boutamba SD, Lee S, Goo YK
Writing – review & editing: Na BK, Goo YK

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 *Pf*EMP1 members includes a DBL1α-CIDR1α semi-conserved tandem, followed by DBLδ-CIDRβ [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 *Pf*EMP1 among isolates from other regions of Myanmar is lacking.

This study aimed to characterize the genetic diversity of *var* repertoires. We screened *Pf*DBLα in *P. falciparum* isolates collected from central Myanmar. Understanding the nationwide diversity of *Pf*EMP1 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.

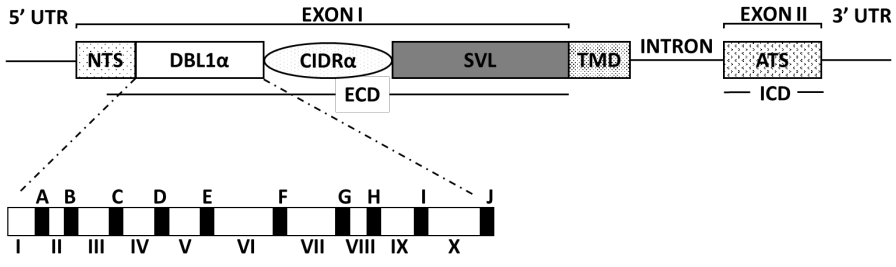

Fig. 1. Schematic overview of *Pf*EMP1 and DBLα domain structures encoded by *var*. *Pf*EMP1 comprises an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular acidic terminal segment (ATS). The ECD varies the most in its composition and size. In the DBL1α domain, conserved sequences are labeled from A to J (filled blocks), and hypervariable sequences are labeled from I to X (unfilled blocks), respectively. *Pf*EMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; CIDR, cysteine-rich interdomain region; DBL, Duffy-binding-like domain; NTS, N-terminal segment; SVL, segment of variable length.

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 *Pf*DBLα

*Pf*DBLα was amplified from the gDNA of each isolate using previously used degenerate primer sets BF (forward: 5′-GCMTGYGCDCCRTWYMGAMG-3′) and BH (reverse: 5′-CKGCCCATTCYTCRAACCA-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 *Pf*EMP1-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*DBLα sequence types

Of the 48 Myanmar *P. falciparum* isolates, 42 successfully yielded DBLα reads. After applying the exclusion criteria, 1033 individual BF/BH inserts were successfully sequenced, resulting in 82 distinct DBL STs (Table 1). The *Pf*EMP1-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*DBLα groups

The DBLα 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*DBLα-encoding sequences were identified. Transcripts from the DBLα 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 DBLα 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 DBLα sequence reads	42	42
Mean no. of isolates with DBLα sequence reads per isolate	4	3
Median no. of isolates with DBLα sequence reads per isolate	5	6
No. of DBLα sequence types	106	82
Median no. of DBLα sequence types reads per isolate	4	2
No. of unique DBLα types or singletons in the sample	68	50

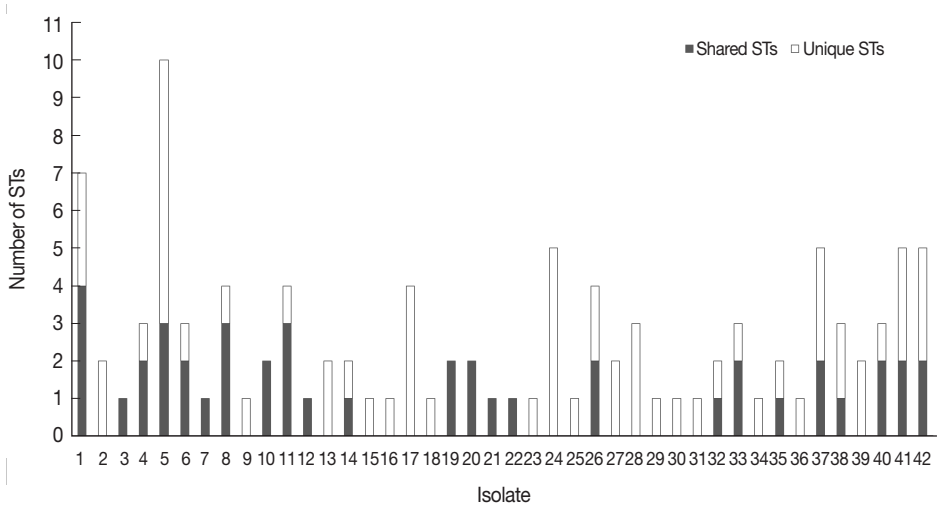

Fig. 2. Distribution of *Pf*DBLα variants in 42 Myanmar isolates. *Pf*DBLα nucleotide sequences with at least 96.0% identity were classified into the same sequence type (ST) of *var*. Shared STs, sequences shared between isolates; Unique STs, sequences occurring only once within isolates.

Table 2. Summary of predominant DBLα 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 *Pf*DBLα 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 DBLα 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].

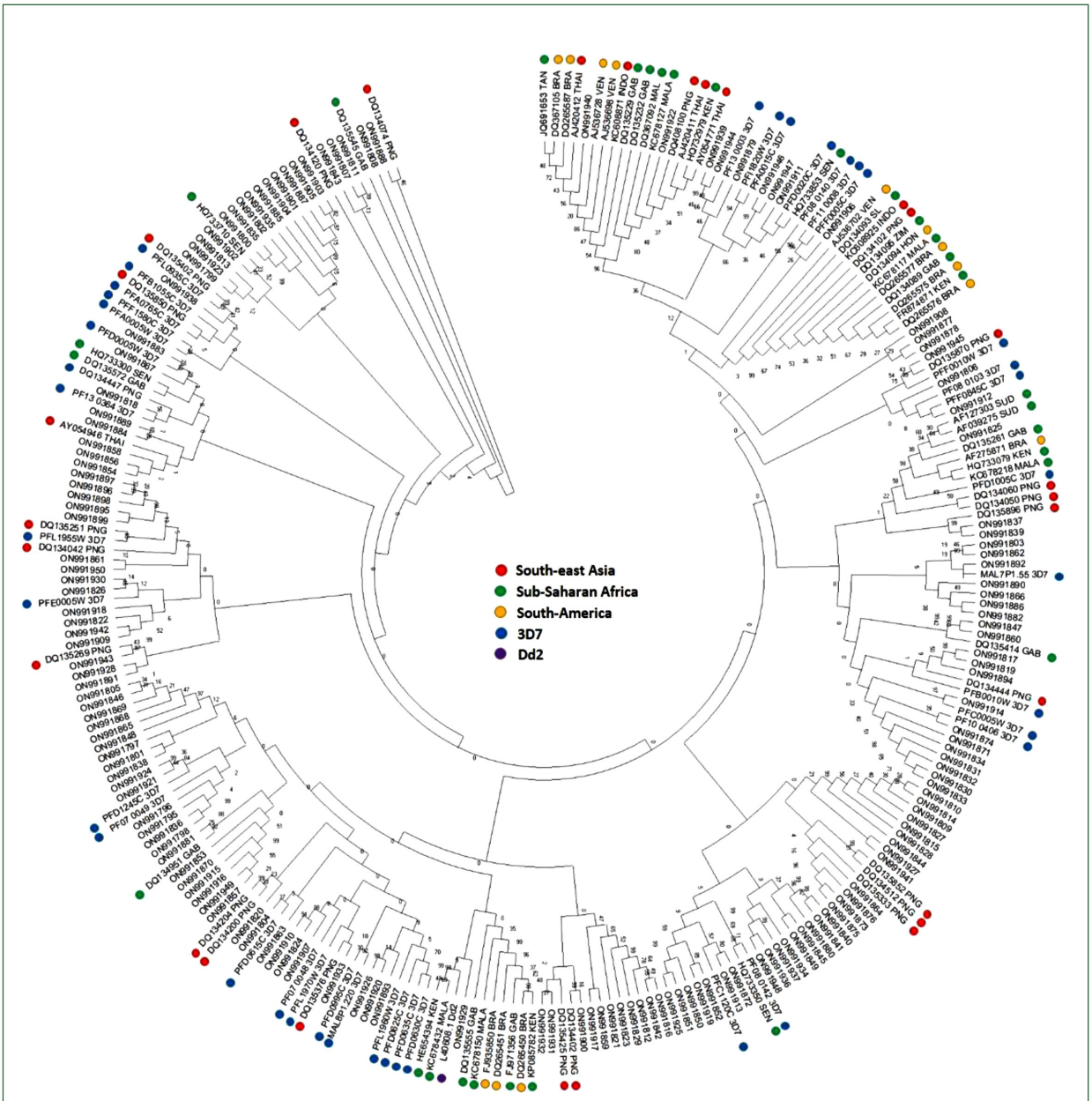

Fig. 3. Phylogenetic tree of the *var* of Myanmar and global *P. falciparum* populations. A minimum-evolution tree was constructed using MEGA X with pairwise alignment and 1,000 bootstrapping repetitions.

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 DBLα domain-encoding sequenc-

es. This particular sequence motif was chosen because DBLα domains are used as polymorphic markers in gene diversity and gene expression studies. Moreover, hyperendemic regions, such as DBLα 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 DBLα-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 DBLα 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 *Pf*EMP1 family of proteins mediates the adhesion of infected erythrocytes to various host cells during the blood stage of malaria infection. Most *Pf*EMP1-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-*Pf*EMP1 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 *Pf*EMP1, 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 *Pf*EMP1 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 DBLα sequences were found more frequently than others but they were also shared between individuals. The DBLα 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 DBLα 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 DBLα 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 VWKA*i*TC and FR*k*TC. 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 DBL1α 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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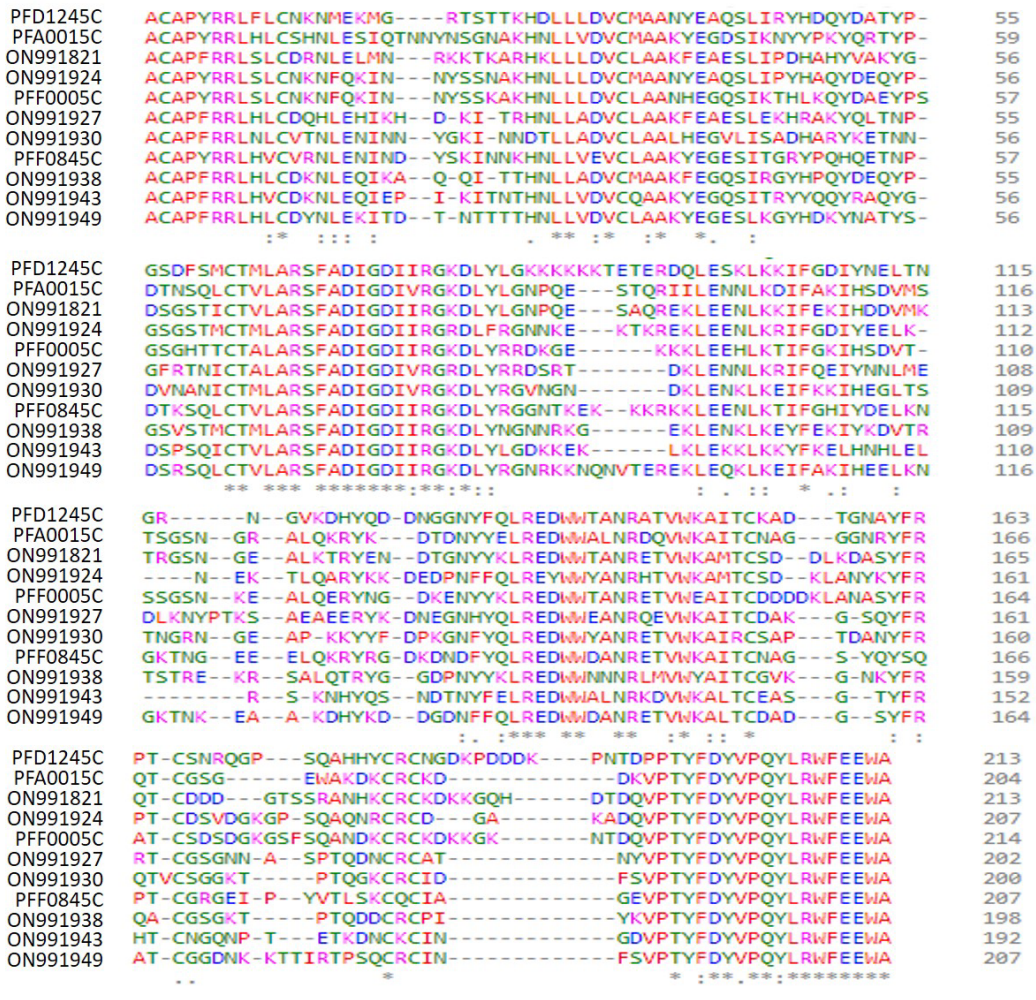
Supplementary Table S1. Characteristics of the studied population

Parameters	No. of samples	Percentage (%)
Year of collection		
2013	29	60.4
2014	3	6.3
2015	16	33.3
Total	48	100
Age		
Age range (yr)	3 to 50	-
Median age	26	-
Sex		
Male	42	87.5
Female	6	12.5
Malaria infection by PCR		
Plasmodium falciparum	48	100

Supplementary Table S2. List of sequence data used for phylogenetic analysis

South-East Asia	Sub-Saharan Africa	South America	3D7
DQ134042 (PNG)	DQ135555 (GAB)	DQ265587 (BRA)	PFA0005W
DQ134074 (PNG)	DQ135572 (GAB)	DQ367105 (BRA)	PFD0005W
DQ134100 (PNG)	DQ135232 (GAB)	DQ265450 (BRA)	PFD0630C
DQ134200 (PNG)	DQ135229 (GAB)	DQ265451 (BRA)	PFB0010W
DQ134204 (PNG)	DQ135414 (GAB)	FJ935850 (BRA)	PFD0615C
DQ134120 (PNG)	FJ971356 (GAB)	DQ265577 (BRA)	PFB1055C
DQ134060 (PNG)	DQ134089 (GAB)	DQ265576 (BRA)	PFC1120C
DQ135269 (PNG)	DQ134951 (GAB)	DQ265575 (BRA)	PFD1005C
DQ135850 (PNG)	DQ135545 (GAB)	AF275871 (BRA)	PFD0625C
DQ134447 (PNG)	DQ135261 (GAB)	AJ536728 (VEN)	PF08_0142
DQ135852 (PNG)	HQ732979 (KEN)	AJ536698 (VEN)	PF07_0049
DQ134102 (PNG)	KP085782 (KEN)	AJ536702 (VEN)	PFC0005W
DQ134512 (PNG)	FR874871 (KEN)	DQ134094 (HON)	PFD1245C
DQ135870 (PNG)	HE654394 (KEN)		PFD0995C
DQ134050 (PNG)	HQ733079 (KEN)		PF13_0364
DQ134402 (PNG)	DQ367092 (MAL)		PF07_0048
DQ135333 (PNG)	KC678127 (MALA)		PFF1580C
DQ134444 (PNG)	KC678150 (MALA)		PFL1970W
DQ135425 (PNG)	KC678117 (MALA)		PF08_0140
DQ135402 (PNG)	KC678432 (MALA)		PF11820W
AJ420411 (THAI)	KC678218 (MALA)	3D7	PFD0020C
AY054771 (THAI)	DQ134093 (SL)	PFL0935C	PF08_0103
AJ420412 (THAI)	DQ134095 (ZIM)	MAL8P1.220	PFE0005W
AY054946 (THAI)	AF127303 (SUD)	PFD0635C	MAL7P1.55
KC608871 (INDO)	AF039275 (SUD)	PF11_0008	PFF0845C
KC608925 (INDO)	JQ691653 (TAN)	PFA0015C	PF11830C
DQ408100 (PNG)	HQ733853 (SEN)	PFL1955W	PFL1960W
DQ135376 (PNG)	HQ733710 (SEN)	PFF0010W	PFA0765C
DQ135251 (PNG)	HQ733300 (SEN)	PFF0005C	PF10_0406
DQ135896 (PNG)	HQ733350 (SEN)	PFE0060W	PF13_0003

*Countries of origin and abbreviations: Brazil (BRA), Gabon (GAB), Honduras (HON) Indonesia (INDO), Kenya (KEN), Malawi (MALA), Mali (MAL), Papua New Guinea (PNG), Senegal (SEN), Sierra Leone (SI), Sudan (SUD), Tanzania (TAN), Thailand (THAI), Venezuela (VEN), Zimbabwe (ZIM).



Supplementary Fig. S1. Alignment of the DBLα sequences of Myanmar population and 3D7 strain.