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Evidence of triple mutant *Pf*dhps IS*GNG*A haplotype in *Plasmodium falciparum* isolates from North-east India: An analysis of sulfadoxine resistant haplotype selection

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ABSTRACT

Background: North-east region of India has consistent role in the spread of multi drug resistant *Plasmodium (P.) falciparum* to other parts of Southeast Asia. After rapid clinical treatment failure of Artemisinin based combination therapy–Sulphadoxine/Pyrimethamine (ACT-SP) chemoprophylaxis, Artemether-Lumefantrine (ACT-AL) combination therapy was introduced in the year 2012 in this region for the treatment of uncomplicated *P. falciparum* malaria. In a DNA sequencing based polymorphism analysis, seven codons of *P. falciparum* dihydropteroate synthetase (*Pf*dhps) gene were screened in a total of 127 *P. falciparum* isolates collected from Assam, Arunachal Pradesh and Tripura of North-east India during the year 2014 and 2015 to document current sulfadoxine resistant haplotypes.

Materials and methods: Sequences were analyzed to rearrange both nucleotide and protein haplotypes. Molecular diversity indices were analyzed in DNA Sequence Polymorphism software (DnaSP) on the basis of *Pf*dhps gene sequences. Disappearance from selective neutrality was assessed based on the ratio of non-synonomous to synonomous nucleotide substitutions [dN/dS ratio]. Moreover, two-tailed Z test was performed in search of the significance for probability of rejecting null hypothesis of strict neutrality [dN = dS]. Presence of mutant *P. falciparum* multidrug resistance protein1 (*Pf*mdr1) was also checked in those isolates that were present with new *Pf*dhps haplotypes. Phylogenetic relationship based on *Pf*dhps gene was reconstructed in Molecular Evolutionary Genetics Analysis (MEGA).

Results: Among eight different sulfadoxine resistant haplotypes found, IS***GNG***A haplotype was documented in a total of five isolates from Tripura with association of a new mutant M538***R*** allele. Sequence analysis of *Pf*mdr1 gene in these five isolates came to notice that not all but only one isolate was mutant at codon 86 (N86***Y***; ***Y***YSND) in the multidrug resistance protein. Molecular diversity based on *Pf*dhps haplotypes revealed that *P. falciparum* populations in Assam and Tripura were under balancing selection for sulfadoxine resistant haplotypes but population from Arunachal Pradesh was under positive selection with comparatively high haplotype diversity ($h = 0.870$). In reconstructed phylogenetic analysis, isolates having IS***GNG***A haplotype were grouped into two separate sub-clusters from the other isolates based on their genetic distances and diversities.

Conclusion: This study suggests that sulfadoxine resistant isolates are still migrating from its epicenter to the other parts of Southeast Asia and hence control and elimination of the drug resistant isolates have become impedimental. Moreover, *P. falciparum* populations in different areas may undergo selection of particular sulfadoxine resistant haplotypes either in the presence of drug or after its removal to maintain their plasticity.

1. Introduction

In Southeast Asia (SEA), North-east region of India is one of the most drug resistant *P. falciparum* foci due to its geographic location near to the multi drug resistant (MDR) epicenter *i.e.,* Thai-Cambodia border. Early evidences suggest that this region is acting as a gateway in spreading drug resistant *P. falciparum* isolates from the other parts of SEA [1–5]. In 2012, ACT-SP was switched over to ACT-AL therapy for the treatment of uncomplicated *P. falciparum* malaria cases in this region due to observation of increased resistance against SP partner drug leading to clinical treatment failure [6]. Therefore, this study aimed to screen sulfadoxine resistant haplotypes in this part of the country that are either under positive selection or balancing selection with emerged resistant isolates.

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2. Results

2.1. *Pfdhps* mutant alleles and haplotypes

711 bp partial coding sequence of *Pf*dhps gene (codon 411 to 646) was sequenced from a total of 127 isolates; 45 from Arunachal Pradesh, 51 from Assam and 31 from Tripura.

All isolates were found with wild alleles at codon 431 and 436 *i.e.,* I431 and A436 respectively. All polymorphic codons, except 540, were found to contain both wild as well as mutant alleles with different frequencies in the studied sample set. However, two different alleles were seen affecting the codon 540 (K540***E*** and K540***N***) along with wild allele. Interestingly, rare K540***N*** allele was observed in five isolates (3.9%; 5/127) exclusively from Tripura (Fig. 1). Moreover, A437***G*** allele was found prevalent among isolates (74.8%; 95/127).

Our samples were set into sixteen different *Pf*dhps nucleotide haplotypes (Hap1 to Hap16) by following the numbering of constituent polymorphisms (Fig. 2). Total 8 segregating sites were determined in this population of which seven were nonsynonymous changes. Wild haplotype Hap_1 was documented in isolates from Assam and Arunachal Pradesh only. Mutant haplotypes in association with synonymous and nonsynonymous polymorphisms found in this study were classified as: single mutant [Hap7 (A437***G***) and Hap9 (P511)], double mutant [Hap3 (A437***G***, A581***G***); Hap10 (A437***G***, K540***E***); Hap11 (S436***A***, A437***G***)], triple mutant [Hap4 (A437***G***, K540***E***, A581***G***); Hap5 (S436***A***, A437***G***, K540***E***); Hap8 (A437***G***, P511, A581***G***); Hap13 (A437***G***, L547, A581***G***); Hap14 (A437***G***, K540***N***, A581***G***); and Hap16 (S436***A***, A437***G***, L547)] and quadruple mutant [Hap2 (S436***A***, A437***G***, P511, K540***E***); Hap6 (A437***G***, P511, K540***E***, A581***G***); Hap12 (A437***G***, M538***R***, K540***N***, A581***G***) and Hap15 (S436***A***, A437***G***, K540***E***, L547)].

2.2. *Pfdhps* protein haplotypes

Eight different mutant *Pf*dhps protein haplotypes were arranged on the basis of the type of nucleotide changes in the dihydropteroate synthetase gene. Apart from the nucleotide haplotypes, a single mutant, three double mutants, three triple mutants and a quadruple mutant *Pf*dhps protein were observed to be associated with sulfadoxine resistance (Fig. 3). Wildtype ISAKAA and triple mutant I***AGE***AA both were present equally in the total *P. falciparum* population (25.2%; 32/127). Double mutant IS***G***K***G***A, in overall, was found moderately high in numbers (19.7%; 25/127), but the prevalence of the same was high in Tripura isolates (42%; 13/31).

2.3. Mutant ISGNGA protein haplotype with new M538R allele

A total of five isolates from Tripura (16.1%; 5/31) were seen affected by K540***N*** allele at codon 540. As a result, introduction of triple mutant IS***GNG***A protein haplotype was evidenced exclusively in Tripura and

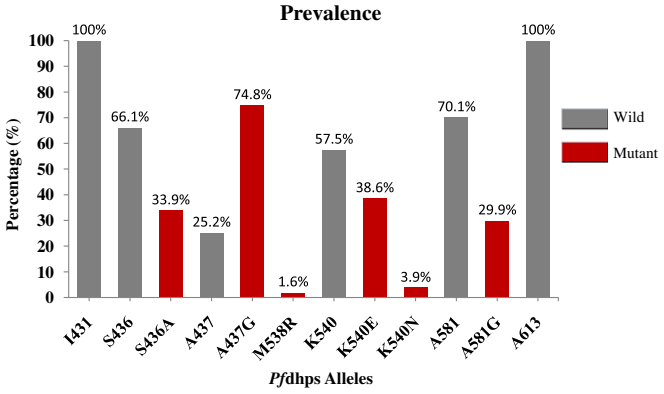

Fig. 1. Different alleles of *Pf*dhps gene and their prevalence.

K540***N*** allele was observed as a constituent allele only in those isolates which had this new haplotype. Prior to this new finding, it was first observed in Cambodian isolates. K540***N*** mutant allele was observed earlier in isolates from Andaman and Nicobar, India but not from any state of North-east India. Among these five isolates, one new nonsynonymous mutation M538***R*** (substitution of methionine by arginine) was found associated with IS***GNG***A haplotype in two isolates leading to introduction of a new quadruple mutant (*i.e.,* I***SGRNG***A) *Pf*dhps protein. However, overall prevalence of IS***GNG***A and I***SGRNG***A mutant protein were observed to be 2.4% and 1.6% respectively (Fig. 3).

2.4. Synonymous mutations in *P. falciparum* population

More than half of the total isolates from Tripura (51.6% prevalence within the state and 12.6% overall prevalence; Haplotype Hap13 and Hap15–16) were observed with a point mutation at nucleotide site 1641; substitution of adenine by guanine (CTA to CT***G*** in codon 547). Similarly, total 11% isolates particularly from Assam and Arunachal Pradesh were also found to contain another mutation in nucleotide position 1533 (CCA to CC***C*** in codon 511; Haplotype Hap2, Hap6 and Hap8–9). Translation analysis showed that these mutations had no role in changing overall protein amino acid sequence and considered to be synonymous mutations *i.e.,* leucine at codon 547 and phenylalanine at codon 511.

2.5. *Pfmdr1* mutation present with ISGNGA haplotype

Further, we analyzed polymorphism in *Pf*mdr1 gene of those five isolates which were found with IS***GNG***A and I***SGRNG***A mutant protein haplotype. This involved screening of two separate fragments of *Pf*mdr1 gene *i.e.,* fragment-I covering codon 86 & 184 and fragment-II covering codon1034, 1042 &1246. Sequence analysis revealed that one isolate contained mutant N86***Y*** allele and wild Y184 allele in fragment-I which gives the clue of association of multidrug resistance in that isolate. Rests of these isolates were found to contain wild allele in codon 86 and 184 of the *Pf*mdr1 gene. Moreover, fragment-II of all isolates was present with wild type alleles indicating no polymorphism at codon 1034, 1042 and 1246 in isolate populations.

2.6. Molecular diversity based on *Pfdhps* gene

In case *Pf*dhps gene, the overall nucleotide diversity and the mean haplotype diversity in studied populations were found to be 0.00318 and 0.884 respectively (Table 1). Nucleotide diversity were found significantly higher among Tripura sequences than other two states ($\pi = 0.00335$, $P = 0.001$) and haplotype diversity was found significantly higher among Arunachal Pradesh sequences (Hd = 0.870, $P = 0.005$). But, haplotype diversity was found comparatively low and almost equal in sequences from Tripura and Assam isolates (0.764 and 0.766 respectively).

Analyses of Tajima's D value [dN/dS] showed a ratio >1 (1.35) in Arunachal Pradesh isolates, indicating positive selection of *Pf*dhps haplotypes while isolates from Assam and Tripura showed a positive value greater than zero (0.7 and 0.8 respectively) suggesting balancing selection of *Pf*dhps haplotypes. However, the probability of rejecting the null hypothesis of strict neutrality (dN = dS) was found to be non-significant in all three populations.

2.7. Phylogeny evaluation

Phylogenetic relationship among 127 isolates bearing different *Pf*dhps haplotypes was reconstructed. Neighbour-Joining tree based on *Pf*dhps partial coding sequence (711 bp) showed two major subclusters within one lineage, one having thirty (30) isolates from Arunachal Pradesh and Assam; other having twenty two (22) isolates

Polymorphism in *Pf*dhps gene	Amino acid codon & alleles	436 S/*A*	437 A/*G*	511# P	538* M/*R*	540 K/*E*	540 K/*N*	547# L	581 A/*G*	Prevalence of Haplotype
	corresponding nucleotide	TCT *G*CT	GCT GG*T*	CCA CCC	ATG AG*G*	AAA *G*AA	AAA AA*T*	CTA CT*G*	GCG G*G*G	
		1306	1310	1533	1613	1618	1620	1641	1742	
Location of number of samples from	Haplotype									
ASM (21), AP (9)	Hap1									23.60%
ASM (1), AP (8)	Hap2	■	■	■		■				7.00%
ASM (7), AP (4), TRP (1)	Hap3		■						■	9.50%
AP (6)	Hap4		■			■			■	4.70%
ASM (4), AP (9), TRP (9)	Hap5	■	■			■				17.30%
AP (2)	Hap6		■	■		■			■	1.60%
ASM (1), AP (4)	Hap7		■							3.90%
AP (1)	Hap8		■	■					■	0.80%
AP (2)	Hap9			■						1.60%
ASM (9)	Hap10		■			■				7.00%
ASM (8)	Hap11	■	■							6.30%
TRP (2)	Hap12		■		■		■		■	1.60%
TRP (12)	Hap13		■					■	■	9.50%
TRP (3)	Hap14		■				■		■	2.40%
TRP (1)	Hap15	■	■			■		■		0.80%
TRP (3)	Hap16	■	■					■		2.40%
Prevalence of mutation		33.9%	74%	11%	1.6%	38.6%	3.9%	12.6%	29.9%	

Fig. 2. Representation of *Pf*dhps polymorphisms in 127 Northeastern isolates from Assam, Arunachal Pradesh and Tripura. Nucleotide sequences and respective amino acid codons with alleles are given according to *P. falciparum* 3D7 isolate sequence (GenBank accession No. XM_001349382). Sample location (numbers are given in parentheses) are abbreviated as ASM-Assam; AP-Arunachal Pradesh; TRP-Tripura. * New allele found in Tripura and #Synonymous mutation found in Tripura.

from all the three states indicating close proximity and genetic relatedness.

Interestingly, it was observed that all isolates bearing the new *Pf*dhps haplotypes placed into another lineage from the origin and grouped into two separate sub-clusters. In this reconstructed tree, three isolates **KT315563**, **KT315565** and **KT315567** were seen grouped together but two isolates **KP998537** and **KP998538** were placed distantly (Fig. 4). Other isolates having same genetic distances were observed to be distributed into separate clusters within the tree.

3. Discussion

Over the past time, *P. falciparum* population groups of this region were exposed to several sorts of antimalarial drugs starting from mono-therapy to artemisinin based combination therapy [15]. The evolution of mutant isolates, from drug resistance point of view, is a continuous process which is continuing in this part of the country. Specific as well as non-specific mutations in the key metabolic genes are of importance because they may actually contribute to the impaired function of metabolic enzymes against the drugs.

Mutation in *Pf*dhps gene is progressive, although 437 codon is the primary site to acquire the resistance [16]. Evidence of IS***GNG***A mutant haplotype with moderate frequency (16%) in Tripura is one of the key findings as it was not reported earlier from any other part of this country. However this mutant haplotype was reported earlier in Western Cambodia explaining that IS***GKG***A protein haplotype is the background for evolution of this resistant haplotype [17]. However, one of the contributing alleles of this newly reported sulfadoxine resistant haplotype was described earlier in India [18]. We also found larger number of isolates, particularly in Tripura, were with IS***GKG***A mutant haplotype (42% in Tripuran isolates). Additionally, overall prevalence of IS***GKG***A haplotype was noticed to be higher (19.7%) in this region than earlier finding [5]. So evidence of IS***GNG***A could be due to the abundance of its background haplotype that might have evolved and it may be justified that introduction of this sulfadoxine resistant can be due to the absence of the drug pressure. Also, declined in the number of sulfadoxine resistant haplotypes is under support of earlier evidences that resistant parasites are less fit to that of ancestral wild type in the absence of drug pressure [19–22]. Moreover, the presence of other mutations and their unequal distribution in parasite population may be due to the disequilibrium in allele linkage. In this regard, it can also be inferred that introduction of this resistant parasite is due to the population

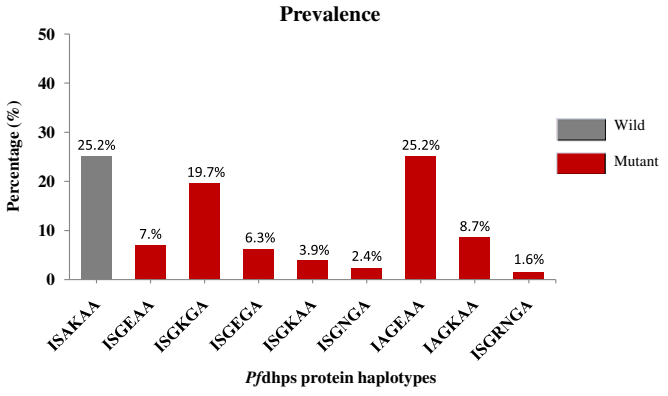

Fig. 3. *Pf*dhps wild and mutant protein haplotypes and their prevalence.

Table 1
Nucleotide polymorphisms observed in 711bp dhps gene of 127 P.*falciparum* isolates from Arunachal Pradesh, Assam and Tripura of N.E. India

Parameters and characteristics	Value of.			
	Arunachal Pradesh	Assam	Tripura	All Sites
Sample size	45	51	31	127
Total nucleotide sites observed	711	711	711	711
Monomorphic	706	706	705	703
Polymorphic	5	5	6	8
No. of segregating sites	5	5	6	8
No. of changes				
Synonomous	1	1	1	1
Non-Synonomous	4	4	5	7
No. haplotypes	9	7	7	16
Nucleotide diversity, n (SD, 0)	0.00310 (0.00161)	0.00221 (0.00156)	0.00335 (0.00211)	0.00318 (0.00208)
Haplotype diversity (Hd)	0.870 (0.020)	0.764 (0.040)	0.766 (0.051)	0.884 (0.014)
Tajima's D [dS/dN] ratio	1.347	0.747	0.865	0.569

Fig. 4. Reconstructed Neighbour-Joining tree based on *Pf*dhps sequences of parasite population. Each sequence was represented after 1000 replication (bootstrap value = 1000). GenBank accession numbers of submitted sequences are given after the parasite ID number.

migration from its origin despite geographical barriers as resistance in parasite populations spread to the other Southeast Asian countries through Indian subcontinent [23,24]. Higher prevalence of A437***G*** allele in this region is also consistent with statement that this mutation is presently worldwide as seen in 3D7 isolate of Southeast Asia [25–27]. In a total of eight different sulfadoxine resistant haplotypes, most of the isolates were highlighted harboring A437***G*** allele and also with remarkable strong selection of I431, A581 and A613 wild alleles.

Interestingly, finding of new M538***R*** allele near codon 540 of *Pf*dhps gene and its association with IS***GNG***A resistant haplotype gives a clue for further investigations. This mutation was not evident in any other part of the country, but emergence of unusual I588***F*** together with K540***T*** mutant allele in *Pf*dhps gene was described recently as a reason of inadequate and low response to Sulfadoxine-Pyrimethamine [28]. Apart from its unknown function, we also assume that this new mutation may have a role in changing conformational dynamicity of *Pf*dhps protein against sulfadoxine and lifting silent resistance against the drug. Moreover, results obtained this study did not tell us about the presence of any full box mutant haplotypes (*i.e.,* ***F/AGEGS/T***) and other sulfadoxine resistance haplotypes (*i.e.,* ***F***AKAA and ***FG***KAA) as described earlier [3,5].

N86***Y*** allele of *Pf*mdr1 gene alone has role in modulating levels of drug resistance [29]. Polymorphism in this gene is scanty from North-eastern *P. falciparum* isolates. But in this study, we screened polymorphisms of *Pf*mdr1 gene in only those isolate which were found with IS***GNG***A and IS***GRNG***A haplotypes. No specific association of *Pf*mdr1 polymorphism was observed except one isolate that was found to be resistant against other antimalarials with the presence of N86***Y*** allele.

Comparing molecular diversity indices of *Pf*dhps sequences in these three states, we observed greater haplotype diversity among Arunachal Pradesh isolates and this could be explained due to the presence of greater number of haplotypes in this state. Comparatively greater nucleotide diversity in Tripura isolates can also be illustrated due to non-synonymous mutation and higher number of polymorphic nucleotide sites. Analysis of dN/dS ratio marked an indication that isolates in Arunachal Pradesh had undergone positive selection for nine different haplotypes. Contrary to this, *P. falciparum* population from other two states still under the process of balancing sulfadoxine resistant haplotype selection in the absence of the drug pressure.

Tracking phylogenetic relationship on the basis of *Pf*dhps gene sequences brings the better picture about relatedness of isolates from different origin. In the reconstructed Neighbour-Joining phylogenetic tree it was observed that five isolates bearing IS***GNG***A haplotype have clustered together into two separate sub-clusters. This was due to the acquisition of M538***R*** allele in two isolates that made them genetically diverged from other three. Though these five isolates were clustered together, but remarkable genetic distances and diversities were observed among them. But actual relationship could be precisely determined if there was availability of *Pf*dhps nucleotide sequences with IS***GNG***A haplotype from its origin *i.e.,* Western Cambodia.

4. Conclusion

From this sequence based study it can be concluded that emergence of IS***GNG***A triple mutant haplotype in North-east Region of India, most particularly in Tripura, gives the clue of either sweep of this haplotype from its multidrug resistant origin or might have evolved from IS***GKG***A mutant haplotype. Though this mutant is suspected to be entered through the nearest country and presently low in frequency, but further molecular analysis will imperatively provide information about its spread to other malarious areas. Presence of M538***R*** mutation particularly with IS***GNG***A may also have role in resistance manifestation. Moreover, mapping of this *Pf*dhps mutant haplotype in this region confirms the evolution and spread of sulfadoxine resistant isolates which is still continuing from its origin and therefore, this region is consistently considered to be the gateway of multi drug resistant *P. falciparum* to other parts of Southeast Asia.

5. Materials and methods

5.1. Ethics

The study was reviewed and ethical approval was obtained from Institutional Ethics Committee (IEC) Regional Medical Research Centre, North-east region (ICMR), Dibrugarh, Assam. A structured questionnaire was used to obtain required information and written consent was obtained from subjects or from guardians prior to include in this study.

5.2. Study site and source of P. falciparum isolates

P. falciparum samples were collected from three states of North-east India *i.e.,* Arunachal Pradesh, Assam and Tripura during the year 2014 and 2015 (Fig. 5). As a source of isolates, venipunctured human blood samples were collected from individuals with symptomatic and uncomplicated malaria. Individuals below 3 years of age, adults with low haemoglobin and other complications were excluded.

Symptomatic subjects were initially screened with rapid diagnostic test kit (RDT, SD ALERE Ag Kit) from their finger pricked blood to check presence of *P. falciparum* infection. 3 ml venous blood was collected in K_3-EDTA vacutainer from subjects who were found positive in RDT. Plasma & whole blood were separated, initially transferred into cool condition (−20°C) during sample collection and later kept in −40°C until further molecular analysis was done.

5.3. DNA extraction and species determination

100 μl (final volume) whole genomic DNA (gDNA) was isolated from each sample (200 μl whole blood) by Qiamp DNA mini kit (QIAGEN, Germany) according to manufacturer's standard protocol. *P. falciparum* mono-infection and mix infection with other *Plasmodium* species was screened by a highly sensitive nested polymerase chain reaction (PCR) protocol [7]. Both primary and nested amplification was performed in 20 μl final reaction volume. All DNA samples were preserved at −80°C for genotyping study of drug resistant molecular markers.

5.4. PCR amplification of Pfdhps and Pfmdr1 target gene

One partial fragment of *Pf*dhps gene (711 bp) and two partial fragments of *Pf*mdr1 gene (534 bp and 864 bp) including all polymorphic and monomorphic sites were amplified isolate wise in Veriti 96 well plate thermal cycler (Applied Biosystem) using nested PCR protocols described earlier with slight changes [8,9]. PCR reaction mixtures for both primary and nested amplification contained 10 μmol final concentrations of forward and reverse primer (synthesized at Integrated DNA Technologies, Singapore), 1.5 mM $MgCl_2$ (Promega, USA), 250 μM of each deoxynucleotide triphosphate (Promega, USA), and 1U Taq DNA polymerase (Promega, USA). All primary amplifications were carried

Fig. 5. Demographic map representing three states of North-east India and number of isolates collected from each sites.

out in 25 μl (final volume) by adding 1 μl of template DNA. For nested amplification, PCR was performed in a total 50 μl (final volume) by adding 2 μl of amplified primary PCR product. Two distinct no template controls (NTC) were used in each PCR batch for experimental validation- one in laminar air flow hood where PCR master mix were prepared and another in the hood where DNA templates were added. In NTCs, nuclease free water was added as template.

5.5. Sequencing, sequence analysis and sequence alignment

Amplified PCR amplicons were gel extracted, purified using QIAEX II Gel extraction kit (QIAGEN, Germany) after verifying in Agarose gel (1.5%) electrophoresis (Bio-Rad) followed by gel documentation (*E*-gel Imager system, Life Technologies) with a known 100 bp DNA ladder (Promega, USA). Purified PCR products were outsourced to 1st Base DNA sequencing service, Malaysia for bidirectional capillary sequencing.

Consensus sequences for targeted genes were prepared isolate wise in BioEdit Sequence Alignment Editor (Version 6.0.7) [10]. Sequences thus obtained were aligned with *P. falciparum* 3D7 reference sequences (Accession no. XM_001349382 for *Pf*dhps gene and XM_001351751 for *Pf*mdr1 gene) using ClustalW algorithm and amino acid sequences were compared in Molecular Evolutionary Genetics Analysis version 6.0 (MEGA 6.0) [11]. Six polymorphic codons (431-436-437-540-581-613; wild type alleles: ISAKAA) of *Pf*dhps gene and five polymorphic codons (86-184-1034-1042-1246; Wild type alleles: NYSND) of *Pf*mdr1 gene were analyzed to construct nucleotide and protein haplotypes.

5.6. Phylogenetic tree reconstruction

Neighbour-Joining Phylogenetic relationship was reconstructed for *Pf*dhps gene in MEGA version 6.0 [11]. For phylogenetic tree reconstruction, each sequence was represented after 1000 replication (bootstrap value = 1000) and each cluster was considered to be significant on the basis of presence of >50% of the permuted trees.

5.7. Statistical analysis

Data were input into Microsoft Excel 2010 software for analyses of allele and haplotype frequencies. DnaSP software version 5.10 was used to perform sequence polymorphism analysis of monomorphic & polymorphic sites, synonomous & non-synonomous mutations, nucleotide diversity (π), and haplotype diversity (Hd) [12]. Disappearance from selective neutrality was assessed by the ratio of non-synonomous to synonomous nucleotide substitution [dN/dS ratio]. Significance for probability of rejecting null hypothesis of strict neutrality [dN = dS] was determined using the two-tailed Z test in MEGA 6.0 (Nei-Gojobori method, bootstrap value 10,000 replication) [11,13]. *P* value of <0.05 was considered to be statistically significant at 5% level for all performed tests and haplotypes were presented as described earlier by Tanomsing et al. [14].

5.8. Nucleotide sequences and accession numbers

Nucleotide sequences for new haplotypes in *Pf*dhps gene sequences in this study were deposited in GenBank under accession numbers **KP998537, KP998538, KT315563, KT315565,** and **KT315567**. *Pf*mdr1 gene sequences in this study were submitted under GenBank under accession numbers **KP998532** and **KP998534**.

Competing interests

The authors declare that they have no competing interests.

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