Sequence analysis of coding DNA fragments of *pfcrt* and *pfmdr-1* genes in *Plasmodium falciparum* isolates from Odisha, India

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The global emergence and spread of malaria parasites resistant to antimalarial drugs is the major problem in malaria control. The genetic basis of the parasite's resistance to the antimalarial drug chloroquine (CQ) is well-documented, allowing for the analysis of field isolates of malaria parasites to address evolutionary questions concerning the origin and spread of CQ-resistance. Here, we present DNA sequence analyses of both the second exon of the Plasmodium falciparum *CQ-resistance transporter (*pfcrt*) gene and the 5' end of the* P. falciparum *multidrug-resistance 1 (*pfmdr-1*) gene in 40* P. falciparum *field isolates collected from eight different localities of Odisha, India. First, we genotyped the samples for the* pfcrt *K76T and* pfmdr-1 *N86Y mutations in these two genes, which are the mutations primarily implicated in CQ-resistance. We further analyzed amino acid changes in codons 72-76 of the* pfcrt *haplotypes. Interestingly, both the K76T and N86Y mutations were found to co-exist in 32 out of the total 40 isolates, which were of either the CVIET or SVMNT haplotype, while the remaining eight isolates were of the CVMNK haplotype. In total, eight nonsynonymous single nucleotide polymorphisms (SNPs) were observed, six in the* pfcrt *gene and two in the* pfmdr-1 *gene. One poorly studied SNP in the* pfcrt *gene (A97T) was found at a high frequency in many* P. falciparum *samples. Using population genetics to analyze these two gene fragments, we revealed comparatively higher nucleotide diversity in the* pfcrt *gene than in the* pfmdr-1 *gene. Furthermore, linkage disequilibrium was found to be tight between closely spaced SNPs of the* pfcrt *gene. Finally, both the* pfcrt *and the* pfmdr-*1 genes were found to evolve under the standard neutral model of molecular evolution.*

Key words: antimalarial resistance - *Plasmodium falciparum* - *pfcrt* - *pfmdr-1* - Odisha - India

Malaria is a highly infectious disease, resulting in approximately 300 million new cases and causing approximately 0.8 million deaths every year across the globe (WHO 2009). Most malaria-related deaths are caused by *Plasmodium falciparum*, a protozoan parasite that completes its sexual life cycle in mosquito and its asexual life cycle in humans. While chloroquine (CQ) was once considered to be the gold standard drug used to treat malaria, the emergence and spread of CQ-resistance in *P. falciparum* over the last 50 years has significantly exacerbated global malaria control programs (Wongsrichanalai et al. 2002). Both the mechanisms of CQ action and the genetic basis of CQ-resistance have been well-studied in *P. falciparum*. The *P. falciparum* multidrug-resistance 1 (*pfmdr-1*) gene located on chromosome 5 of *P. falciparum* was initially implicated in CQ-resistance (Wilson et al. 1989, Foote et al. 1990). However, the high correlation of a mutation in a transporter gene located on chromosome 7, called *P. falciparum* CQ-resistance transporter (*pfcrt*) and the corresponding CQ response (Fidock et al. 2000, Sidhu et al. 2002) has led to a better understanding of the genetic basis of CQ-resistance in malaria parasites. Specifically, an amino acid mutation in codon 76 of the *pfcrt* gene is strongly correlated with CQ-resistance from both in vitro and in vivo studies (Fidock et al. 2000, Sidhu et al. 2002) and has thus become the genetic marker to determine CQ-resistance in *P. falciparum* isolates. Similar studies of the *pfmdr-1* gene, which encodes a P-glycoprotein, have uncovered interesting details; however a direct association between mutations in this gene and CQ-resistance has not been established (Basco & Ringwald 1998, Reed et al. 2000, Le Bras & Durand 2003). In many cases, the N86Y mutation in the *pfmdr-1* gene was found to be either compensating for mutations in the *pfcrt* gene, or to have a role in CQ-resistance by itself (Adagu & Warhurst 2001, Babiker et al. 2001, Djimdé et al. 2001). Hence, both *pfcrt* K76T and *pfmdr-1* N86Y mutations are now used as genetic markers to determine CQ-resistance in *P. falciparum* isolates.

While much genetic information is known about these two genes, the estimation of DNA sequence variation in the nucleotide regions flanking the 76th and 86th amino acid positions of the *pfcrt* and *pfmdr-1* genes, respectively, is generally uncharacterized. Haplotype profiles of populations based on mutations in the five amino acids adjacent to the known K76T mutation site (codons 72-76 in the *pfcrt* gene) have been analyzed to study the origin and migration history of CQ-resistance in *P. falciparum*. India is one of the malaria-endemic countries that have a relatively high incidence of CQ-resistant *P. falciparum* cases (Vathsala et al. 2004, Mittra et al. 2006, Singh et al. 2009, Mixson-Hayden et al. 2010). Several studies have reported haplotypic data on the *pfcrt* gene

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related to five amino acid changes (72nd-76th positions) and the frequency of the N86Y mutation in the *pfmdr-1* gene (Fidock et al. 2000, Djimdé et al. 2001, Vathsala et al. 2004). Additional observations on microsatellite variations in and around the *pfcrt* gene and the evolutionary history of this gene in India have also been reported (Das & Dash 2007, Mixson-Hayden et al. 2010). However, evolutionary analyses of the DNA sequences of these two genes have not been performed. To understand the effect of additional mutations in these genes and the evolutionary interactions among these different mutations, we carried out studies in Odisha (earlier known as Orissa), a southeastern state of India (Fig. 1), that has the highest number of *P. falciparum* malaria cases in India, and where CQ-resistance among *P. falciparum* field isolates is highly prevalent (Singh et al. 2009). Therapeutic efficacy studies of *P. falciparum* field isolates in different localities of Odisha revealed that the state is under a strong belt of CQ-resistant malaria (Ranjit et al. 2009). This study is an initiative to map *pfcrt* haplotypes and to identify new mutations in the *pfcrt* and the *pfmdr-1* genes, in order to infer the evolutionary history of these two genes in Odisha.

SUBJECTS, MATERIALS AND METHODS

Forty field-isolates collected from eight different locations of Odisha (Fig. 1) constitute the samples for this study. The total number of samples collected from each location is depicted in Fig. 1. The samples were collected from adult patients with malarial symptoms attending the local primary health centres (PHCs) in the eight locations. The Ethical Committee of the Regional Medical Research Center, Bhubaneswar, Odisha, has approved the study protocols used here and informed consents were obtained from all the patients prior to sample collection. To diagnose malaria, both thin and thick smears from finger-prick blood samples of malaria-symptomatic patients attending the PHCs were Giemsa-stained and examined microscopically. Approximately 1 mL of venous blood was added to a tube 1.8 mg/mL after containing ethylenediamine tetraacetic acid (EDTA) from the patients who were confirmed to be positive for the presence of *P. falciparum* parasites following microscopic examination. Genomic DNA of *P. falciparum* was isolated from 100 µL of venous blood through lysis of erythrocytes in lysis buffer (10 mM Tris-HCl, 0.1 M EDTA pH 8.0, 20 µg/mL RNAase A, 0.5% sodium dodecyl sulfate and 100 µg/mL proteinase K) at 55ºC for 16 h followed by phenol-chloroform extraction and ethanol precipitation (Sambrook & Russell 2001).

Because mutations in the 76th and 86th positions in the *pfcrt* and *pfmdr-1* genes, respectively, are considered to be the determinants of CQ-sensitivity or resistance, we genotyped all 40 isolates for the well-characterized *pfcrt* K76T and *pfmdr-1* N86Y mutations. To analyze the *pfcrt* gene, we amplified a 264-bp region of the second exon (Fig. 2) that contains the K76T polymorphism using the primer set 5'-GGCTCACGTTTAGGTGGA-3' and 5'-TGAATTTCCCTTTTTATTTCCAAA-3,' as described by Vathsala et al. (2004). To establish the presence of the K76T mutation, this polymerase chain reaction (PCR) product from a *P. falciparum* isolate was enzymatically digested with *Apo*I. If restriction digestion took place and divided the PCR product into two fragments (128 and 136 bp), the isolate was considered CQ-sensitive (Vathsala et al. 2004). Similarly for the *pfmdr-1* gene, the primer pair 5'-ATGGGTAAAGAGCAGAAAGA-3' and 5'-AACGCAAGTAATACATAAAGTCA-3' was used to amplify a 603-bp fragment at the extreme 5' end of the gene (Vathsala et al. 2004) (Fig. 2) that contains the N86Y mutation. This PCR product subsequently underwent restriction digestion with *Afl*III. The amplified PCR product of a *P. falciparum* isolate digested into two fragments (253 and 350 bp) indicated the presence of the

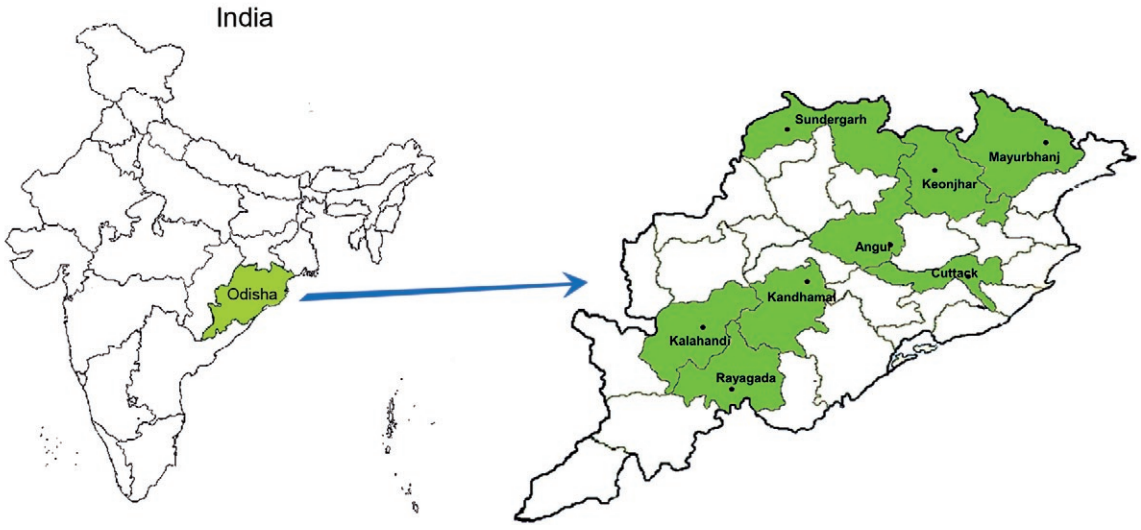

Fig. 1: map of India indicating Odisha and location of the eight places from where samples of *Plasmodium falciparum* isolates have been collected. The sample size from each location is: Sundergarh, 7; Mayurbhanj, 6; Keonjhar, 6; Angul, 5; Kandhamal, 5; Rayagada, 5; Kalahandi, 2; Cuttack, 4.

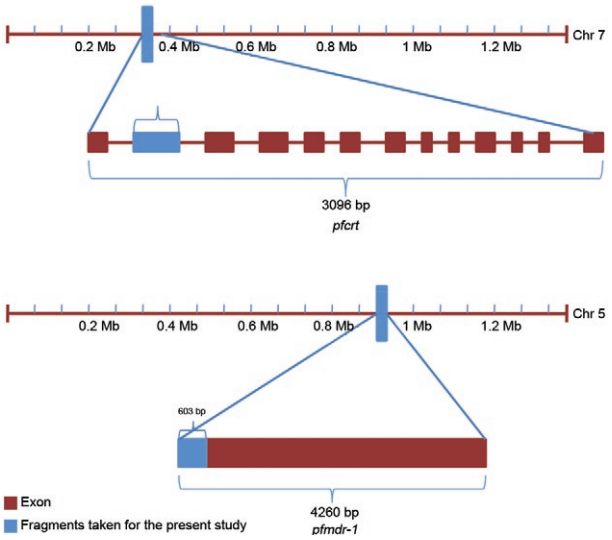

Fig. 2: locations of *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) and *P. falciparum* multidrug-resistance 1 (*pfmdr-1*) genes on their respective chromosomes and of the genetic regions taken for sequencing.

N to Y mutation in the 86th codon position of *pfmdr-1*, whereas PCR products from CQ-sensitive isolates were resistant to digestion (Vathsala et al. 2004).

To determine the CQ-resistant haplotypes in the *pfcrt* gene based on mutations in codons 72-76 and to uncover novel single nucleotide polymorphisms (SNPs), we sequenced the PCR products of *pfcrt* and *pfmdr-1* genes (as amplified). Undigested amplified products of all 40 isolates were purified with Exo-SAP (Fermentas Life Sciences) at 37ºC for 60 min and at 85ºC for an additional 15 min. For each sequencing reaction, we used 2-4 µL of the purified PCR product and sequencing was performed by the cycle sequencing method using Big Dye Terminator chemistry on an ABI 3730XL automated DNA sequencer at the National Institute of Malaria Research, New Delhi. Each fragment was sequenced in both the forward and reverse directions (2X coverage) and assembled and edited using the SeqMan program (DNASTAR, Madison, WI, USA). Homologous DNA fragments were aligned using the program MegAlign of DNASTAR, with the ClustalW algorithm. Haplotypes were inferred based on the SNPs present in these DNA fragments from multiple sequence alignments of both gene fragments. The sequences are deposited in GenBank with accessions HQ287007-HQ287086. Estimations of diversity parameters, such as haplotype diversity (Hd) (Nei 1987) and two measures of nucleotide diversities, θ_w and π (Watterson 1975, Tajima 1983), were calculated using the program DnaSP, version 5.00.02 (Rozas et al. 2003). While estimation of π is based on the mean number of pair-wise nucleotide differences in a sample (Tajima 1983), estimation of θ_w is based on the number of segregating mutations (Watterson 1975). To test whether the observed allele frequency spectrum was in accordance with the expectations from the neutral model of molecular evolution in each DNA fragment, Tajima's *D* was calculated, which is based on the normalized discrepancy between π and θ_w (Tajima 1989). We also calculated other measures of neutrality, such as Fu and Li's *D** (FLD) and Fu and Li's *F** (FLF) (Fu & Li 1993). The FLD and FLF (Fu & Li 1993) rely on the differences between the number of polymorphic sites in external branches (polymorphisms unique to an existing sequence) and number of polymorphic sites in internal phylogenetic branches (polymorphisms shared by existing sequences) (Zhang & Ge 2007). For both tests, negative values indicate an excess of low-frequency polymorphisms, while positive values indicate an excess of intermediate polymorphisms (Tajima 1989, Fu & Li 1993, Fu 1997). Furthermore, the number of nonsynonymous substitutions per nonsynonymous site (dN) and the number of synonymous substitutions per synonymous site (Nei & Gojobori 1986) were estimated and a z-test was performed to estimate the p value using the program MEGA, version 4 (Tamura et al. 2007). To determine whether any of the eight total SNPs (present in both the genes) are associated with each other, both inter and intragenic linkage disequilibrium (LD) were estimated with the r^2 values, using the program Haploview (Barrett et al. 2005).

RESULTS

A total of 40 *P. falciparum* isolates of unknown CQ-sensitivity were analyzed for the presence of the K76T and N86Y mutations in the *pfcrt* and *pfmdr-1* genes, respectively. For the *pfcrt* gene, only eight PCR samples produced the expected 128 and 136 bp amplicon when digested with *ApoI*, indicating the presence of the wild-type K76 codon, while the remaining 32 isolates resisted enzymatic digestion, indicating the presence of the K76T mutation in these samples (Table I). Similarly, restriction digestion of the 603 bp DNA fragment of *pfmdr-1* with *AflIII* produced two fragments (253 and 350 bp) in only those 32 isolates that also possessed the K76T mutation, indicating the presence of the N86Y mutation in these isolates (Table I).

The presence of these two mutations was further confirmed in all 40 isolates through DNA sequencing of the amplified fragments from these two genes. In our analysis of both the *pfcrt* and *pfmdr-1* genes, we sequenced 867 nucleotides in each of the 40 *P. falciparum* isolates from Odisha, totaling 34,680 nucleotide bp sequenced with 2X coverage. Separate sequence alignment for both genes revealed six SNPs in the *pfcrt* gene and two SNPs in the *pfmdr-1* gene. The number of haplotypes identified varied between the *pfcrt* and *pfmdr-1* genes, with four in the *pfcrt* gene and three in the *pfmdr-1* gene. Sequenced fragments of both genes were translated into amino-acid sequences. Five point mutations were observed in codons 72, 74, 75, 76 and 97 of the *pfcrt* amino acid sequence and two point mutations were observed in codons 86 and 184 position of *pfmdr-1* (Table I). No synonymous (or silent) substitutions were observed, indicating that all the eight SNPs detected in both the genes were nonsynonymous (amino acid-changing nucleotide substitutions). The N86Y mutation and wildtype Y184 codon were found together in 32 *P. falciparum* isolates,

while the Y184F mutation was present in two isolates that had the N86Y mutation in the *pfmdr-1* gene. Although several haplotypes have been reported to exist due to the presence of different amino acids in codons 72-76 of the *pfcrt* gene, only three haplotypes (CVIET, SVMNT and CVMNK) were found in the presently studied Odisha samples. We identified the CVIET haplotype the most frequently (67.5%), followed by the CVMNK (20%) and SVMNT (12.5%) haplotypes.

The two estimates of nucleotide diversity, as measured by π and θ_w, were found to be variable for both the genes examined (*pfcrt* and *pfmdr-1*) (Table II). In general, the *pfcrt* gene showed higher genetic diversity than the *pfmdr-1* gene (Table II). It is clear from Table II that the π values in both the genes are slightly higher than the θ_w values, indicating a greater number of intermediate frequency mutations. We also conducted three tests of neutrality and none of them showed statistically significant results to indicate any departure from neutrality (Table II). However, in general, the data signifies that both genes are evolving under the standard neutral model of molecular evolution in the population sample of Odisha. All the mutations detected in this study were nonsynonymous; therefore, only dN values, 0.011 ± 0.006 for *pfcrt* and 0.001 ± 0.001 for *pfmdr-1* genes, could be estimated and these were found to be statistically significantly different from the silent polymorphisms (not detected in the study) in the case of the

TABLE I

Nucleotide sequences and amino acid residues of codons 72-76 of the *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) and 86 and 184 codons of *P. falciparum* multidrug-resistance 1 (*pfmdr-1*) genes

	pfcrt gene fragment					*pfmdr-1* gene fragment	
Position of SNP	72	74	75	76	97	86	184
Wild genotype	**T**GT Cys	AT**G** Met	**A**A**T** Asn	A**A**A Lys	C**A**C His	**A**AT Asn	T**A**T Tyr
Isolates with wild genotype (n)	35	13	13	8	30	8	38
Mutant genotype	**A**GT Ser	AT**T** Ile	**G**A**A** Glu	A**C**A Thr	C**T**C Leu	**T**AT Tyr	T**T**T Phe
Isolates with mutations (n)	5	27	27	32	10	32	2

the single nucleotide polymorphisms (SNP) in each codon has been indicated as bold and underlined nucleotide.

TABLE II

Details of *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) and *P. falciparum* multidrug-resistance 1 (*pfmdr-1*) genes fragments and population genetic parameters in *P. falciparum* isolates of Odisha, India

Genes		*pfcrt* (308526-311620) 3096 bp	*pfmdr-1* (957885-962144) 4260 bp
Isolates (n)		40	40
Size of the fragment (bp)		264	603
Nucleotide positions		308619-308883	957885-958487
SNPs (n)		6	2
Haplotypes (n)		4	3
Haplotype diversity		0.719	0.344
Nucleotide diversity	θ	0.00541	0.00078
	π	0.00866	0.00071
Tests of neutrality			
Tajima's *D*		1.6583	-0.1789
Fu and Li's *D**		1.1919	0.77124
Fu and Li's *F**		1.5621	0.57422

SNP: single nucleotide polymorphisms.

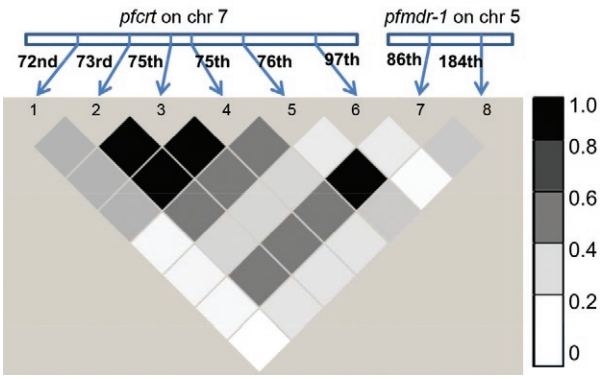

Fig. 3: linkage disequilibrium (LD) plot (r^2) between 28 possible pairs of single nucleotide polymorphisms (SNPs) in *Plasmodium falciparum* chloroquine-resistance transporter (*pfcrt*) and *P. falciparum* multidrug-resistance 1 (*pfmdr-1*) genes in *P. falciparum*. The strength of statistical significance of LD between a pair of SNPs is represented with the extent of darkness of the boxes (increasing intensity of black color depicts strong LD and the fading grey and white color show week and no LD, respectively).

pfmdr-1 gene. However, in the case of the *pfcrt* gene, no statistically significant difference was obtained (data not shown). Furthermore, LD estimates between all possible pairs of SNPs of both the *pfcrt* and *pfmdr-1* genes were determined (Fig. 3). It is evident from Fig. 3 that there are three statistically significant intragenic associations between three SNPs (2 SNPs in codon 74 and 1 SNP in codon 75) in the *pfcrt* gene and one intergenic association between a SNP of the *pfcrt* gene (K76T) and a SNP of the *pfmdr-1* gene (N86Y).

DISCUSSION

Although the genetic basis of CQ-resistance has been attributed to both the *pfcrt* and *pfmdr-1* genes, the *pfcrt* gene is primarily and directly implicated in CQ-resistance in *P. falciparum*. This first-ever *pfcrt* and *pfmdr-1* DNA fragment sequencing and sequence analysis in India has provided several interesting findings. Specifically, 32 of the 40 *P. falciparum* isolates possessed both the K76T and the N86Y mutations. These 32 isolates had also acquired either the CVIET or SVMNT, the haplotypes associated with CQ-resistance (Fidock et al. 2000, Vathsala et al. 2004), suggesting that the K76T and the N86Y mutations are the hallmarks of CQ-resistant *P. falciparum* isolates. This is further evidenced from the analyses. It is widely known that both the CVIET and SVMNT haplotypes and the K76T mutation are associated with CQ-resistance in *P. falciparum* (Fidock et al. 2000, Vathsala et al. 2004). The dominance of the CVIET over the SVMNT haplotype in Odisha contradicts earlier reports of SVMNT prevalence in India (Vathsala et al. 2004, Mixson-Hayden et al. 2010), as well as in Odisha (Pati et al. 2007). However, earlier work (Pati et al. 2007) examined samples from only one particular geographical region of Odisha, whereas in this study we collected data from eight different localities of the state (Fig. 1), providing a much broader analysis of the genetic pattern of CQ-resistance in *P. falciparum* in Odisha. Furthermore, in India, a predominance of the SVMNT haplotype (Mixson-Hayden et al. 2010) and a progressive reduction in the number of cases with the rising CVIET haplotype were observed when evaluated for two years (Mixson-Hayden et al. 2010). The present observation of the dominance of the CVIET haplotype might be due to a high proportion of CQ-resistance in Odisha (Ranjit et al. 2009), as the CVIET haplotype is known to be associated with a higher level of CQ-resistance as compared to other haplotypes (Mittra et al. 2006).

The strong association between the occurrence of the K76T and N86Y mutations is one of the interesting findings of this study. Although the involvement of the *pfmdr-1* mutation is still under debate, the N86Y mutation has been reported to modulate levels of CQ-resistance to a higher degree when present with the *pfcrt* K76T mutation (Foote et al. 1990, Djimdé et al. 2001, Mita et al. 2006). Some previous studies have confirmed the association between the N86Y mutation in the *pfmdr-1* gene and the CQ response (Póvoa et al. 1998, Volkman & Wirth 1998). However, many other studies have reported a negative correlation between the N86Y mutation and the CQ response (Wellems et al. 1990, Basco et al. 1996, Basco & Ringwald 1997, McCutcheon et al. 1999). The high prevalence of both the mutations K76T and N86Y in Odisha might be due to the high selection pressure of CQ-resistance, which is a matter of great concern to public health. Further confirmation of the association between the N86Y mutations with CQ-resistance using both in vitro and in vivo assays are needed to initiate appropriate measures to restrict the increasing frequency of these mutations and their spread across the country.

The finding that all new SNPs discovered in this study were nonsynonymous and that the dN value was significantly higher in the *pfmdr-1* gene fragment than the *pfcrt* gene fragment indicate the influence of positive natural selection on the mutations. Besides the well-characterized mutations at codons 72-76 in the *pfcrt* gene and codon 86 in the *pfmdr-1* gene, two additional mutations (H97L in *pfcrt* and Y184F in *pfmdr-1*) were also observed in an appreciable frequency. The H97L has already been reported in the *pfcrt* gene of CQ-resistant *P. falciparum* isolates (Fidock et al. 2000, Durrand et al. 2004, Chen et al. 2005) and this amino acid mutation in the *pfcrt* gene is frequently found in *P. falciparum* isolates in Odisha, with one-fourth of the presently analyzed isolates found to contain this mutation. Interestingly, this mutation was found in all four isolates from Angul (central Odisha) (Fig. 1) containing the K76T and N86Y mutations and has also been found in the adjoining areas of Angul (Keonjhar, Sundergarh, Cuttack, Kandhamal). The H79L mutation in the *pfcrt* gene has also reached Rayagada, but at a very low frequency. Although we did not find any strong association of this mutation with any other mutations in the *pfcrt* and *pfmdr-1* genes, the occurrence of this mutation with K76T and N86Y requires further investigation at both the population and functional level. Two isolates with the wildtype K76 codon and the CVMNK haplotype had the Y184F

mutation in the *pfmdr-1* gene. The Y184F mutation in the *pfmdr-1* gene has also been reported to be associated with CQ-resistance in laboratory isolates (Foote et al. 1990), but was unrelated to CQ-resistance in cases of field isolates (Ojurongbe et al. 2007).

Estimation of both the nucleotide diversity parameters (θ_w and π) and Hd were generally higher in the *pfcrt* gene than the corresponding values in the *pfmdr-1* gene. In the analysis of a *pfcrt* gene fragment of only 264 bp, six SNPs were observed, which is comparatively higher than that observed in the *pfmdr-1* gene, which had only two SNPs in a 603 bp gene fragment. This is in contrast to the reported low allelic diversity reported within the *pfcrt* gene based on microsatellite diversity (Wootton et al. 2002, Mixson-Hayden et al. 2010). High microsatellite diversity in the gene has been previously reported (Vinayak et al. 2006). Although the general observation of selective neutrality was reported for both the genes analyzed in this study, these results are not comprehensive, as we have only sequenced a small DNA fragment within each of these two genes. Sequence analysis of the entire *pfcrt* and *pfmdr-1* genes would provide more detailed information on whether these two genes are under the influence of natural selection. Likewise, the LD observed in this study is explained by the physical distance between the SNPs, as the three strongest associations observed were present in the *pfcrt* gene itself. A broad-scale LD study with full sequence information of both the *pfcrt* and *pfmdr-1* genes might provide additional meaningful associations.

In conclusion, this DNA sequence dataset of two different gene fragments attributed to CQ-resistance in *P. falciparum* isolates of Odisha indicates that in a highly malaria-endemic region, sufficient evolutionary changes at the molecular level are quite extensive. This is primarily because in a haploid genome with a short generation time (as in case of the malaria parasites), reconstruction of genetic material is fairly easy. This rapid reconstruction not only generates new mutations and high genetic variation, but also opens up the genetic system for natural selection to operate on. This study, although limited to a few isolates and to a limited number of DNA sequences from Odisha, provides preliminary evidence to test the above hypotheses in India, as well as in other malaria-endemic countries.

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