Differential genetic hitchhiking around mutant pfcrt alleles in the Indian Plasmodium falciparum population

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Objectives: To study the origin and spread of the chloroquine-resistant Plasmodium falciparum population in the Indian subcontinent.

Methods: Fourteen microsatellites spanning a ~120 kb region, flanking the P. falciparum chloroquine resistance transporter (pfcrt) gene, were analysed in 185 parasite isolates.

Results: The Indian P. falciparum population exhibited a selective valley of reduced genetic variation in the flanking microsatellites of the mutant pfcrt alleles (up to +29 kb) as compared with the wild-type allele. This valley is much narrower than the +200 kb valley reported from African and South-East Asian countries. The majority of the isolates showed asymmetry in the selective valley, where upstream microsatellites showed less genetic variation than the downstream microsatellites. Regional variation in the width and symmetry of the selective valley was noticed, which seems to be related to the number of pfcrt alleles present in the parasite population of a region. Forty-six different microsatellite haplotypes were observed among the P. falciparum isolates containing mutant pfcrt alleles. Parasite populations from different regions of mainland India shared microsatellite haplotypes between them, but they shared none with the isolates from the Andaman and Nicobar Islands, and vice versa. Indian isolates shared microsatellite haplotypes with the isolates from Papua New Guinea and Thailand.

Conclusions: With regard to chloroquine there is regional variation in the selection pressure on the P. falciparum population in India. These findings will help the regional implementation of drug policy in India’s malaria control programme.

Keywords: malaria, chloroquine resistance, microsatellite markers, population genetics

Introduction

The Plasmodium falciparum parasite has developed resistance to the most commonly used antimalarial drug, chloroquine. This resistance was first observed in the late 1950s in South-East Asia (Thai–Cambodian border) and South America (Venezuela), and then spread throughout Asia and Africa.1–2 In India, chloroquine resistance (CQR) was first reported from Assam (north-east region) during 1973 and then spread to different parts of the country.3–5 The prevalence of the drug-resistant parasite, however, varies from region to region in India.6 Parasite populations from hyperendemic (north-east and east), mesoendemic (central India) and low endemic (northern India) regions of this country show high, moderate and low levels of CQR (www.nvbdcp.gov.in).6–8 Although artemisinin-based combination therapy has replaced chloroquine in high CQR areas, chloroquine is still used in low CQR regions against P. falciparum in India. The origin and spread of CQR in India largely remains elusive, although a study on the parasite population from the mesoendemic region of central India showed that it shared a genetic background with Papua New Guinea (PNG) isolates.9

Certain point mutations in the P. falciparum CQR transporter (Pfcrt) protein have been shown to be associated with CQR10,11 Under selection, the resistant alleles spread through the population, causing recombination in the regions flanking the coding sequence of the mutant gene.12 This results in reduced genetic diversity in the flanking nucleotide
sequences, both upstream and downstream of the coding region of the resistance gene. Selective sweep and hitchhiking are used to track the emergence and spread of drug-resistant allele in the parasite population by using various molecular markers, such as microsatellites.\textsuperscript{13,14} The dramatic reduction in the genetic variation in microsatellites around mutant \textit{pfcrt} alleles has been reported from parasite populations of different countries.\textsuperscript{15–17} Variations in the extent of hitchhiking of these microsatellites in parasite populations exposed to different levels of selection pressure have also been reported.\textsuperscript{16} Data from population genetic surveys suggest that CQR has emerged independently at a limited number of sites: two in South America,\textsuperscript{17,18} one in PNG\textsuperscript{19} and one in the Philippines.\textsuperscript{20} These data have shown the similarities of the parasites from Asian and African origins, but these differ from those parasites from South America and PNG, supporting the hypothesis that parasite migration must have played a critical role in the spread of CQR.\textsuperscript{21}

Several mutant \textit{pfcrt} alleles from CQR parasite populations from different regions of the country have been reported in the literature.\textsuperscript{2,10,17,19} We and others have reported various types of \textit{pfcrt} alleles having mutations at amino acid positions 72–76 of this transporter protein (CVMNT, SVMNT, CVIET and CVIIDI, mutated amino acids are underlined) in the Indian \textit{P. falciparum} population.\textsuperscript{3,4,9,22–25} A parasite population with the SVMNT allele was found in almost all regions of India, but it was highly predominant in low and mesoendemic regions.\textsuperscript{4} We noticed a high frequency of the CVIET allele in the parasite population from hyperendemic regions with a higher level of CQR and none so far from low-endemic regions of northern India where the level of CQR has been very low.\textsuperscript{3,4,22} Recently, Awasthi et al. have also described similar findings, detecting a predominance of the SVMNT allele in Rajasthan (north-west India) and the CVIET allele in Mizoram (north-east India).\textsuperscript{26} In order to investigate the origin and spread of CQR \textit{pfcrt} alleles in the Indian subcontinent, we describe here the analysis of microsatellites flanking the \textit{pfcrt} gene from Indian \textit{P. falciparum} populations.

**Materials and methods**

**Sample source**

As described previously,\textsuperscript{26} \textit{P. falciparum} genomic DNA was isolated from dried blood spots on filter paper using the Qiagen DNA mini kit according to the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany). Briefly, these blood spots were made from finger pricks of malaria-infected patients who had fever and tested positive microscopically for the presence of \textit{P. falciparum}. According to their oral history, they had not received any antimalarial drug during the past 1 month. Blood samples were collected during the malaria transmission period in 2008–09. These patients were from Aligarh in Uttar Pradesh (UP), Kamrup in Assam, Car Nicobar in the Andaman and Nicobar (A & N) Islands, Ganjam in Orissa, and Ranchi in Jharkhand. These regions represent different geographical areas of the country (Figure 1) that have varying endemicity and chloroquine usage; UP has a lower level of CQR and low malaria transmission rates, while Assam, Orissa, the A & N Islands and Jharkhand have high levels of CQR and high malaria transmission rates.\textsuperscript{6} The Ethics Committee of the All India Institute of Medical Sciences approved the use of leftover parasite DNA for this study.

**PCR amplification**

The primary and nested PCR strategy was used to amplify the microsatellites from previously isolated parasite DNA. The details of the primers for these microsatellites along with the cycling conditions are given in Table S1 (available as Supplementary data at JAC Online). A total of 14 microsatellite loci flanking the \textit{pfcrt} gene on chromosome 7 were used. Out of these, seven microsatellites were located upstream (2E10, 3E7, B5M77, CH7 304928, CH7 278053, –40 and –60) and seven were located downstream (9B12, P5590, 2H4, PE14F, CH7 334596, CH7 321409 and CH7 350596) of the gene. These loci were the same as described previously,\textsuperscript{6,17} except loci –40 and –60, which were designed in the present study. These microsatellite markers span across the ~120 kb region flanking the \textit{pfcrt} gene (at –60, –40, –29.4, –18.2, –10.9, –4.8, –2.9, +1.5, +6, +10.4, +18.8, +23.6, +40.5 and +57.1, where the negative and positive numbers refer to the positions of the markers either upstream or downstream of the gene, respectively). PCR amplification and sequencing of part of the \textit{pfcrt} gene covering codons 72–76 was carried out as described previously.\textsuperscript{4}

**Microsatellite analysis**

The aliquots of PCR products were checked on 1.5% agarose gel. The remaining PCR products were then PCR purified using an Accuprep purification kit from Bioneer, according to the manufacturer’s protocol (Bioneer Corporation, South Korea). The purified products of the microsatellite loci were diluted in a ratio of 1:10, and then separated using an ABI 3130xl Genetic Analyzer and analysed using GeneMapper software v3.7 (Applied Biosystems, Foster City, CA, USA).

**Statistical analysis**

Genetic variation in these microsatellite loci was estimated by calculating expected heterozygosity (\(H_e\)) values for each locus using the formula \(n/(n-1)\) \([1-\Sigma p_i^2]\), where \(n\) is the number of samples and \(p_i\) is the frequency of the \(i\)th allele. The \(P\) values to test the significance between the groups were calculated using the Kruskal–Wallis test and the Stata 9 package. These \(H_e\) values were then plotted against the distance from \textit{pfcrt} (in kb) in GraphPad Prism 4 to measure the extent of the selective sweep. The Microsatellite Excel Toolkit was used for calculating \(H_e\) values and allele frequencies.\textsuperscript{27}

**Results**

**Genetic variation at microsatellite loci**

A total of 235 isolates were collected from the above-mentioned regions (Figure 1). Out of these 235 isolates, 31 showed multiplicity of infection by merozoite surface protein 1 (MSPI) analysis (data not shown) and were excluded. The remaining 204 isolates were then subjected to microsatellite analysis, where 19 of them did not produce data for all the required microsatellite loci. Therefore, a total of 185 isolates were analysed in the present study. The distribution of these 185 isolates was as follows: the A & N Islands (\(n=48\)), Jharkhand (\(n=35\)), Orissa (\(n=26\)), Assam (\(n=40\)) and UP (\(n=36\)). Three different \textit{pfcrt} mutant alleles at the 72–76 amino acid positions (CVIETH, \(n=1\); SVMNT, \(n=62\); and CVIET, \(n=112\)) besides the wild-type allele SVMNK (\(n=10\)) were observed among the isolates. The genetic variation, in terms of \(H_e\), was calculated for each microsatellite locus flanking the \textit{pfcrt} gene. The mean \(H_e\) value for microsatellites flanking the wild-type \textit{pfcrt} allele SVMNK was significantly lower than the \(H_e\) values for the other microsatellite loci.
higher (mean $H_e = 0.77 \pm 0.25$) than the overall mean $H_e$ value for microsatellites flanking the mutant $pfcrt$ alleles ($H_e = 0.62 \pm 0.23$; $P = 0.0025$). The $H_e$ value for microsatellites flanking the wild-type allele was also significantly higher than the $H_e$ values of these microsatellites for the isolates bearing CVIET ($H_e = 0.57 \pm 0.24$; $P = 0.0081$) or SVMNT ($H_e = 0.47 \pm 0.26$; $P = 0.0016$) alleles (Figure 2). However, the mean $H_e$ value of these microsatellites flanking the double mutant $pfcrt$ allele SVMNT ($H_e = 0.47 \pm 0.26$) was insignificantly lower than the mean $H_e$ value for microsatellites flanking the triple mutant allele CVIET ($H_e = 0.57 \pm 0.24$; $P = 0.33$). The difference between the $H_e$ values for these two types of mutant alleles also remained statistically insignificant ($P = 0.52$) when we considered the core microsatellites (up to $+10$ kb) for this calculation (Figure 2, inset). Considering the core microsatellites, the mean $H_e$ value for the wild-type allele was also significantly higher than the $H_e$ values for SVMNT ($P = 0.0278$) or CVIET ($P = 0.0208$) mutant alleles (Figure 2, inset). Thus, there was a selective valley of reduced genetic variation in the flanking microsatellites of the mutant $pfcrt$ alleles. This selective valley, however, was asymmetric for both the resistance alleles where the upstream microsatellite loci showed comparatively less genetic variation than the downstream microsatellite loci (data not shown).

**Regional variation in selective sweeps around $pfcrt$**

The genetic variation was computed according to the geographical region of origin (Table S2, available as Supplementary data at JAC Online). The minimum $H_e$ value for flanking microsatellites around the $pfcrt$ alleles was observed for the UP isolates (mean $H_e = 0.29 \pm 0.24$) followed by those from the A & N Islands (mean $H_e = 0.44 \pm 0.25$); the $H_e$ value was maximum for Orissa isolates (mean $H_e = 0.63 \pm 0.23$). Isolates with the SVMNT allele from UP and Assam showed different hitchhiking patterns (Figure 3a). There was symmetry in the selective valley of reduced genetic variation for the UP isolates, but it was asymmetric for the Assam isolates. Unlike the UP isolates, isolates from Assam did not show much reduction in genetic variation in microsatellites of the downstream region (Figure 3a). This hitchhiking pattern shown by UP isolates was

![Figure 1](https://academic.oup.com/jac/article-abstract/67/3/600/797766) Map of India showing the parasite collection sites. The name of each place and the state (in brackets) are given. The number of isolates collected from each site is also indicated.
also different from that of isolates from other regions. For the same SVMNT allele, there was a significant difference (P < 0.05) in the He values between isolates from UP (He = 0.29 ± 0.24) and Assam (He = 0.50 ± 0.28) or the other regions (He = 0.52 ± 0.30). The P values remained similar when we considered only core microsatellite loci for this analysis (Figure 3a, inset). The selective valley of reduced genetic variation in the microsatellites flanking the mutant pfcrt allele CVIET was also visible among the isolates, but the hitchhiking was more prominent in the upstream microsatellites than in those of the downstream region (Figure 3b). Isolates from the A & N Islands, however, showed a selective valley of reduced genetic variation in both upstream and downstream regions as compared with others. Upstream microsatellites of the CVIET allele in Assam isolates showed the least genetic variation when compared with isolates from other regions (Figure 3b). Nevertheless, the He values for microsatellites flanking this resistance allele were not significantly different among isolates from different regions. The P values remained similar when we considered only core microsatellite loci for this analysis (Figure 3b, inset).

**Microsatellite haplotypes among mutant pfcrt alleles**

Five microsatellite loci (present at −10.9, −4.8, −2.9, +1.5 and +10.4 kb relative to the pfcrt gene) were considered to construct the haplotypes around the mutant pfcrt alleles. This is because the above-mentioned results have already revealed that the microsatellite loci near to the coding region (up to +10 kb) of the mutant pfcrt allele show the least genetic variation, while the farthest microsatellites show a higher level of genetic variation. Since microsatellites beyond ±10 kb showed a higher rate of genetic variation they were unsuitable for haplotype analysis. A total of 46 haplotypes were observed amongst the mutant pfcrt alleles (Table 1). Amongst these, 37 haplotypes were observed for the CVIET allele, 16 for the SVMNT allele and 1 for the CVMNT allele. Eight haplotypes (H1, H16, H26, H28, H30, H31, H35 and H37) were shared between the SVMNT and...
CVIET alleles. The most predominant haplotypes (H2 and H12) among isolates with the CVIET allele were not shared with the SVMNT allele. However, the converse was not true, as the most predominant haplotypes (H1 and H26) of the SVMNT allele were shared with the CVIET allele. The haplotype for the CVMNT allele was unique and not shared by any of the other alleles.

The frequency of these haplotypes was found to vary from region to region (Figure 4). For the CVIET allele, haplotype H12 was most prevalent among A & N Islands isolates, whereas haplotypes H2, H5 and H10 were predominant in Assam, Orissa and Jharkhand isolates, respectively. Among isolates with the SVMNT allele, haplotype H26 was prevalent in UP, whereas H1 followed by H26 haplotypes were prevalent in Assam. Numbers of isolates with the SVMNT allele were fewer in the A & N Islands, Orissa and Jharkhand. It is interesting to note that none of the haplotypes of the A & N Islands is shared by the isolates from mainland India, and vice versa, although different regions of mainland India shared haplotypes between them (Figure 4). We also analysed Thailand isolates (provided by Dr T. J. C. Anderson, South-west Foundation of Biomedical Research, San Antonio, TX, USA) with the CVIET allele and Indian isolates with the SVMNT allele (used in our earlier studies where they shared the haplotypes with PNG isolates) for the amplification and genotyping of the microsatellites. Haplotype H26 was present in PNG isolates, and H5 and H7 were found in Thai isolates (Table 1).

Discussion
The aim of this study was to investigate the origin and spread of chloroquine-resistant P. falciparum parasite in the Indian subcontinent, where levels of CQR and malaria transmission rates vary.

![Figure 3. Regional variation in genetic hitchhiking of microsatellites around mutant pfcrt alleles. (a) Differences in $H_e$ values for microsatellites flanking the SVMNT allele between Assam and UP isolates. (b) Differences in $H_e$ values for microsatellites flanking the CVIET allele between isolates from different regions. The number of isolates is given in brackets. Inset shows the $H_e$ values for core microsatellites (up to ±10 kb).](https://academic.oup.com/jac/article-abstract/67/3/600/797766)
Table 1. Microsatellite haplotypes among different \textit{pfcr}t alleles in the Indian \textit{P. falciparum} population

<table>
<thead>
<tr>
<th>\textit{pfcr}t alleles$^a$</th>
<th>Haplotypes</th>
<th>Location of microsatellite loci (in kb) with respect to the \textit{pfcr}t gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVIET ($n = 112$)</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H14</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H18</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H22</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H13</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H12</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>H2</td>
<td>164</td>
<td>233</td>
</tr>
<tr>
<td>CVIET ($n = 62$)</td>
<td>157</td>
<td>233</td>
</tr>
<tr>
<td>H1</td>
<td>157</td>
<td>233</td>
</tr>
<tr>
<td>H15</td>
<td>157</td>
<td>233</td>
</tr>
<tr>
<td>H17</td>
<td>157</td>
<td>233</td>
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<td>H26</td>
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<td>H8</td>
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<td>H9</td>
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<td>H31</td>
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<td>223</td>
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<td>H35</td>
<td>157</td>
<td>223</td>
</tr>
<tr>
<td>H37</td>
<td>157</td>
<td>223</td>
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</table>

Continued...
from region to region. Recently, Mixson-Hayden et al. described evidence of selective sweeps in \textit{pf} among \textit{P. falciparum} isolates from Madhya Pradesh (MP), which is in central India, where levels of CQR and malaria transmission rates are at a moderate level. These authors observed only the SVMN\textit{T} mutant \textit{pf} allele among isolates (95%, $n = 104$). In the present study, we analysed \textit{P. falciparum} isolates from other regions of the country where levels of CQR and malaria transmission rates differ from those of MP. Similar to MP, the majority of isolates (97.22%, $n = 36$) from UP, where levels of CQR and malaria transmission rates are lower, were also found to contain only the SVMN\textit{T} mutant \textit{pf} allele. However, isolates from other regions with high levels of CQR and high malaria transmission rates were found to contain both the SVMN and CVIET alleles; the single isolate with the CVMN\textit{T} allele was from Orissa.

Table 1. Continued

<table>
<thead>
<tr>
<th>\textit{pf} alleles$^a$</th>
<th>Haplotypes</th>
<th>\text{-10.9}</th>
<th>\text{-4.8}</th>
<th>\text{-2.9}</th>
<th>\text{1.5}</th>
<th>\text{10.44}</th>
<th>$p_i$</th>
<th>no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVMN\textit{T}</td>
<td>H35</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SVMN\textit{T}</td>
<td>H28</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
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<td></td>
</tr>
<tr>
<td>SVMN\textit{T}</td>
<td>H41</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
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<tr>
<td>SVMN\textit{T}</td>
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<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
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<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
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</tr>
<tr>
<td>SVMN\textit{T}</td>
<td>H38</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CVIET\textit{(n=1)}</td>
<td>H46</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PNG isolates (n=11), SVMN\textit{T}</td>
<td>H26</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thai isolates (n=5), CVIET\textit{I}</td>
<td>H7</td>
<td>1.174</td>
<td>1.174</td>
<td>1.121</td>
<td>1.177</td>
<td>0.016</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Predominant haplotypes are shaded medium grey. Haplotype sharing is indicated by light and dark grey shading.

$^a$Amino acid positions 72–76 for each \textit{pf} allele. The mutated amino acids are underlined.

Figure 4. Geographical distribution of microsatellite haplotypes around mutant \textit{pf} alleles. The number of isolates is given in brackets.

Figure 4. Geographical distribution of microsatellite haplotypes around mutant \textit{pf} alleles. The number of isolates is given in brackets.
the width of the selective valley of reduced genetic variation around this resistant allele among the two populations. Surprisingly, a much wider valley was observed for UP isolates (± 29 kb) than the valley reported by Mixson-Hayden et al.\textsuperscript{9} for MP isolates (up to ± 10 kb). A difference in the hitchhiking patterns between UP and MP isolates is expected, as the selection pressures and the transmission intensities of these two parasite populations are different.\textsuperscript{6,8}

A selection valley of reduced genetic variation around the \textit{pfcrt} gene with the CV\textsubscript{IET} allele was also observed among these Indian \textit{P. falciparum} isolates (Figure 3b). However, the reduction in genetic variation in microsatellites around the CV\textsubscript{IET} allele was lower than that of the microsatellites around the SVM\textsubscript{NT} allele. This was also evident from the haplotype data,\textsuperscript{31} where we observed a greater number of haplotypes for the CV\textsubscript{IET} allele than for the SVM\textsubscript{NT} allele (Table 1). It is possible that the CV\textsubscript{IET} allele was introduced earlier than SVM\textsubscript{NT} in India and, hence,\textsuperscript{11} this triple mutant allele may have evolved multiple times due to recombination events with the ancestral type to give rise to new alleles with time.

It is known that longer and stronger selective pressure on \textit{pfcrt} leads to a wider selection valley of reduced genetic variation in its flanking sequences. The selection valley in the Indian isolates was narrower (± 29 kb) in comparison with the wider valley (± 200 kb) reported from Africa and South-East Asia.\textsuperscript{16,17} This could be because there has been considerable time for recombination in the parasite populations of India at more distant regions around \textit{pfcrt} as compared with African and South-East Asian isolates.

There was a regional difference in the width and symmetry of the selective valley of reduced genetic variation around the \textit{pfcrt} gene among Indian isolates. The observed difference in the symmetry of selective sweeps in different geographical regions (Figure 3) seems to be related to the frequency and diversity of mutant \textit{pfcrt} alleles. For example, isolates from A & N Islands and UP contain only two types of \textit{pfcrt} alleles; CV\textsubscript{IET} is predominant in the A & N Islands and SVM\textsubscript{NT} is predominant in UP. Coincidently, they also show selective sweeps in both downstream and upstream regions, and symmetry in the width of the selective valleys. On the other hand, isolates from Orissa and Jharkhand were found to contain more \textit{pfcrt} alleles and showed higher genetic variation in the microsatellites as well as asymmetry in the selective valley. Asymmetry in the selective valleys around \textit{pfcrt} had also been reported in isolates from Thailand and Laos.\textsuperscript{16,22} This asymmetry could arise if recombination occurred at different rates in the upstream and downstream regions of the gene.\textsuperscript{29}

The selective valley of reduced genetic variation around \textit{pfcrt} (up to ± 29 kb) observed here is wider than the reported width of the valley around \textit{pfmdfr} (up to ± 20 kb) and \textit{pfmdhs} mutant alleles among these Indian isolates.\textsuperscript{22,30,31} The enzymes encoded by the \textit{pfmdfr} and \textit{pfmdhs} genes are the targets for pyrimethamine and sulfadoxine, respectively. This suggests that stronger selective events have occurred around \textit{pfcrt} than the \textit{pfmdfr} and \textit{pfmdhs} genes in the same parasite population. The reason could be that for a long time chloroquine has been used extensively as a first-line antimalarial drug to treat \textit{P. falciparum} malaria, as compared with the use of sulfadoxine and pyrimethamine. Nevertheless, the patterns of genetic hitchhiking around the \textit{pfcrt} and \textit{pfmdfr} genes among parasite isolates were similar to each other (Figure 3).\textsuperscript{31}

Analysis of the predominant haplotypes around \textit{pfcrt} revealed that the mutant alleles evolved differently from the same progenitor. Haplotype H26 was most predominant in the SVM\textsubscript{NT} allele followed by H1 (Table 1). Haplotype H26 was also observed among the SVM\textsubscript{NT} alleles from PNG, indicating a shared origin.\textsuperscript{28} Our results were consistent with the previous report on isolates from central India, which also revealed the similarity in the haplotypes between PNG and Indian isolates with the SVM\textsubscript{NT} allele.\textsuperscript{9}

Haplotype H12 was predominant among A & N Islands isolates with the CV\textsubscript{IET} allele, whereas H2, H5, H7 and H10 were predominant and specific for this allele among isolates from mainland India. Indeed, none of the haplotypes present in the A & N Islands was shared with any of the isolates from mainland India, and vice versa. Isolates from Thailand (provided by Dr T. J. C. Anderson) showed the presence of H7 and H5 haplotypes from Thailand to the A & N Islands, due to their close proximity, and recombination could have led to the evolution of H12. However, in mainland India the alleles have been driven, as such, and, hence, are sweeping the whole country. Some of the haplotypes, such as H1 and H37, were common to both the SVM\textsubscript{NT} and CV\textsubscript{IET} alleles, indicating that SVM\textsubscript{NT} haplotypes may have arisen from recombining with the CV\textsubscript{IET} ancestral type.

In conclusion, there was a selective sweep in \textit{pfcrt} in the Indian parasite population, but the selective valley of reduced genetic variation around this gene showed regional variation and was asymmetric for most of the regions studied. This valley was much narrower than that observed for isolates from Africa and South-East Asia. Haplotype H26, which was most prevalent among Indian isolates, was the same as the parasite population of PNG, suggesting that there is an invasion of the South-East Asian type of SVM\textsubscript{NT} allele from PNG in India.\textsuperscript{28} Like in Africa, the CV\textsubscript{IET} allele in India shared microsatellite haplotypes with the South-East Asian CV\textsubscript{IET} type.\textsuperscript{16,32} It seems that there is a gene flow of resistant \textit{pfcrt} alleles from neighbouring countries.

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Transparency declarations
None to declare.
Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


8. Singh N, Dash AP, Thimasarn K. Fighting malaria in Madhya Pradesh 2011; Infect Dis in India: changes over time and space.


12. Singh N, Dash AP, Thimasarn K. Fighting malaria in Madhya Pradesh (Central India): are we losing the battle? Malar J 2009; 8: 93.


