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Selective sweep and phylogenetic models for the emergence and spread of pyrimethamine resistance mutations in *Plasmodium vivax*

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ABSTRACT

Pyrimethamine resistance is a major concern for the control of human haemoprotozoa, especially *Plasmodium* species. Currently, there is little understanding of how pyrimethamine resistance developed in *Plasmodium vivax* in the natural field conditions. Here, we present for the first time evidence of positive selection pressure on a dihydrofolate reductase locus and its consequences on the emergence and the spread of pyrimethamine resistance in *P. vivax* in the Punjab province of Pakistan. First, we examined the dihydrofolate reductase locus in 38 *P. vivax* isolates to look for evidence of positive selection pressure in human patients. The S58R (AGA)/S117N (AAC) double mutation was most common, being detected in 10/38 isolates. Single mutation S117N (AAC), I173L (CTT) and S58R (AGA) SNPs were detected in 8/38, 2/38 and 1/38 isolates, respectively. The F57L/I (TTA/ATA) and T61M (ATG) SNPs were not detected in any isolates examined. Although both soft and hard selective sweeps have occurred with striking differences between isolates, there was a predominance of hard sweeps. A single resistance haplotype was present at high frequency in 9/14 isolates, providing a strong evidence for single emergence of resistance by the single mutation, characteristics of hard selective sweeps. In contrast, 5/14 isolates carried multiple resistance haplotypes at high frequencies, providing an evidence of the emergence of resistance by recurrent mutations, characteristics of soft selective sweeps. Our phylogenetic relationship analysis suggests that S58R (AGA)/S117N (AAC) and S117N (AAC) mutations arose multiple times from a single origin and spread to multiple different cities in the Punjab province through gene flow. Interestingly, the I173L (CTT) mutation was present on a single haplotype, suggesting that it arises rarely and has not spread between cities. Our work shows the need for responsible use of existing and new antimicrobial drugs and their combinations, control the movement of infected patients and mosquito vector control strategies.

1. Introduction

Malaria is a significant source of morbidity and mortality especially in pregnant women and young children (Petersen et al., 2011). Despite causing probably 72 to 80 million-malaria cases annually, *Plasmodium vivax* usually produces less severe symptoms and has not received as much attention as *P. falciparum*. Nevertheless, *P. vivax* leads to a disabling disease that can be fatal and exacts a similar economic burden to *P. falciparum*. Furthermore, the severity of disease caused by *P. vivax* is increasing in south Asia (Conway, 2007; Cui et al., 2012). Majority of malaria cases in Pakistan is caused by *P. vivax*, trends in the past few decades, reported by World Health Organisation. In the recent years, uptick in malaria can partially attributed to floods in Pakistan that effected approximately 20 million people in over 60 districts. Despite a well-established malaria programme, 0.5 million infections occur each year with approximately 37% of cases estimated to occur in regions along the borders with Afghanistan and Iran (Khattak et al., 2013).

Practically all knowledge of the genetics of pyrimethamine drug resistance has been based on candidate gene studies of dihydrofolate reductase. Functional analysis has demonstrated that *P.vivax* dihydrofolate reductase resistant mutant strains do not allow the uptake of pyrimethamine in *Saccharomyces cerevisiae* and *Escherichia coli* models, providing the mechanism by which dihydrofolate reductase confers resistance to the pyrimethamine drug class (Hastings et al., 2005; Hastings et al., 2004; Hastings and Sibley, 2002). The *P. vivax* dihydrofolate reductase locus has been analysed in susceptible and resistant

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isolates and single nucleotide polymorphisms (SNPs) have been observed at codons F57L/I (TTA/ATA), S58R (AGA), T61M (ATG), S117N/T (AAC/ACC) and I173L/F (CTT/TTT) (de Pecoulas et al., 1998; Lee et al., 2010). This provides strong evidence that these mutations in the dihydrofolate reductase locus are important determinants of pyrimethamine resistance in *P. vivax*. (Auliff et al., 2006; Huang et al., 2011). Despite the public health importance, *P. vivax* has not been well documented with respect to pyrimethamine resistance in Pakistan. There are only a few published studies in its prevalence, but this information suggest that resistance may not yet be widespread in this country. The sequencing of dihydrofolate reductase gene fragment from *P. vivax* collected from the Punjab and North West Frontier provinces revealed just a 23% frequency of the S58R (AGA) mutation (Khattak et al., 2013) and 15.2% frequency of S117N/T (AAC) mutation (Zakeri et al., 2011).

Although, some initial progress has been made in elucidating the molecular genetics of pyrimethamine resistance in *P. vivax*, there are very few published reports investigating the genetics of the pyrimethamine resistance mutations (Alam et al., 2007; Hawkins et al., 2008), and it is still unclear how resistance develops and spreads in the field. Hypothetically the development of resistance depends on multiple factors including: (a) the intensity of positive selection pressures on the candidate locus due to treatment compliance, or fitness costs associated with the resistance mutations; and (b) emergence of resistance alleles due to high rates of mutations followed by the spread by gene flow (Chaudhry, 2015; Petersen et al., 2011).

Selective sweep models could potentially answer how antimicrobial resistance mutations emerge in parasite; whereby use of antimicrobial drugs provides a positive selection pressure for adaptive mutations in the resistance candidate loci of the parasite, resulting in selective sweeps at the loci under selection. There are two different types of selective sweep models. A ‘hard’ selective sweep is characterised by a single resistance haplotype rising at high frequency in each parasite population from a single mutation. This allows little time for recombination to break up the initial haplotype on which the resistance mutation appeared, therefore, the genetic footprint of selection is expected to involve a reduction in polymorphism around the locus after selection. A ‘soft’ selective sweep is characterised by the presence of multiple resistance haplotypes at high frequency in each population derived from either recurrent mutation appearing after the onset of selection, or pre-existing mutations before the onset of selection. The genetic footprint of selection would not be expected to include a marked reduction in polymorphism around the locus under selection (Chaudhry, 2015).

Understanding of adaptive mutations in response to selection can help to show their spread. There are two phylogenetic models in which adaptive mutations can spread in the parasite that are under selection. A new resistance mutation could arise from a single origin, become fixed by selection and then spread through parasite by migration, likely a consequence of the flow of drug resistance alleles through human movement. Alternatively, resistance mutations could repeatedly arise from multiple origins, become fixed by selection and migrate between parasite as a result of animal movement (Chaudhry, 2015).

Previously most targeted studies characterising pyrimethamine resistance have been undertaken in *Plasmodium falciparum* (Petersen et al., 2011). There is currently little understanding of how pyrimethamine resistance developed, emerged and spread in *P. vivax* (Cubides et al., 2018; Jiang et al., 2013). In the present study, we have identified a single pyrimethamine resistance haplotype in nine isolates at high frequency, with an evidence of the emergence of resistance by single mutation. In contrast, five isolates carried multiple resistance haplotypes at high frequencies, with an evidence of the emergence of resistance by recurrent mutations. Our analysis shows that pyrimethamine resistance mutations were spread to multiple different cities in the Punjab province through human travelling, except I173L (CTT) mutation was not spread thus far. These findings helps to explain why pyrimethamine resistance is so common in *Plasmodium* parasites and suggests that the emergence and the spread of pyrimethamine resistance is likely when large number of parasites are exposed to antimicrobial drugs.

2. Materials and methods

2.1. Parasite material, genomic DNA isolation and identification of Plasmodium

Malaria patients referred to the Chughtai diagnostic laboratory in the Punjab province were invited to participate in this study. The procedures involved in sample collection by venipuncture are minimal. Discussions have been held with key administrative and community leaders to raise awareness of the study. Samples were taken by trained paramed workers under the supervision of local collaborator and veterinary staff. This rigorous approach has been continued throughout the study. Blood samples was collected during peak malaria transmission seasons between April and October 2017. The study included patients of all age groups with malaria symptoms including vomiting, fever, headache, chills, sweats, nausea and fatigue. Institutional Review Board of the University of Central Punjab, Pakistan approved the study. 5 mL of intravenous blood was drawn into an EDTA tube by venipuncture from patients giving written consent and stored at −20°C. 3% Giemsa stained thin and thick blood smears were prepared and examined by a trained technician under the oil immersion (1000×) for the diagnosis of *Plasmodium*, according to WHO guidelines (Asif, 2008).

About 50 μL of each blood sample from 38 malaria positive patients has been taken and gDNA was extracted according to the protocols described in the TIANamp blood DNA kit (Beijing Biopeony Co. Ltd). For DNA preparations, 1 μL from each gDNA preparation was taken in 4 μL of $\mathrm{ddH_2O}$ to make a 1:5 dilution for use as PCR template. Dilutions of aliquots of only $\mathrm{ddH_2O}$ were made in parallel for use as negative controls. For the species confirmation, deep amplicon sequencing (Illumina MiSeq) of the 18s rDNA was performed (Shaukat et al., 2018). Out of thirty eight samples, thirty six were *P. vivax* and one sample (DHFR102 –Table 1) has a mixed infection of *P. vivax* and *P. falciparum*.

2.2. Deep amplicon sequencing of the dihydrofolate reductase locus

A 468 bp fragments encompassing parts of F57L/I (TTA/ATA), S58R (AGA), T61M (ATG), S117N/T (AAC/ACC) and I173L/F (CTT/TTT) SNPs of 38 *P. vivax* positive isolates were used for deep amplicon sequencing of the dihydrofolate reductase locus. Primers has been modified from the normal primers set (PvdhfrS _For, PvdhfrS_Rev) previously described by Auliff et al. (2006) and Brega et al. (2004). Adapters were added to each primer to allow the successive annealing and N is the number of random nucleotides included between the primers and adopter sequence (Supplementary Table S1).

Four forward (PvdhfrS_For, PvdhfrS_For-1N, PvdhfrS_For-2N, PvdhfrS_For-3N) and four reverse primers (PvdhfrS_Rev, PvdhfrS_Rev-1N, PvdhfrS_Rev-2N, PvdhfrS_Rev-3N) were mixed in equal proportion. The primers were then used for the first round PCR under the following conditions: 5× KAPA HiFi Hot START Fidelity buffer, 10 mM dNTPs, 10 uM forward and reverse adopter primer, 0.5 U KAPA HiFi Hot START Fidelity Polymerase (KAPA Biosystems, USA), 13.25 μL $\mathrm{ddH_2O}$ and 1 μL of lysate. The thermocycling condition of the PCR were 95 °C for 2 min, followed by 35 cycles of 98 °C for 20 s, 60 °C for 15 s, 72 °C for 15 s and a final extension of 72 °C for 5 min. PCR products were purified with AMPure XP Magnetic Beads (1×) using a special magnetic stand and plate according to the protocols described by Beckman coulter, Inc.

After the purification, a second round PCR was performed using eight forward and twelve reverse barcoded primers. Repetitions of the same forward and reverse barcoded primers in different samples were avoided. The second round PCR conditions were: 5× KAPA HiFi Hot START Fidelity buffer, 10 mM dNTPs, 0.5 U KAPA HiFi Hot START

Table 1
Relative allele frequencies of the dihydrofolate reductase pyrimethamine resistance associated mutations in thirty-eight *P. vivax* isolates from the Punjab province of Pakistan. Each *P. vivax* isolate was characterised by the susceptible [S58R(AGC), S117N(AGC), I173L (ATT)] and resistance mutations [S58R(AGA)/S117N(AAC), S58R(AGA), S117N(AAC) and I173L(CTT)]. The relative allele frequency of the resistant versus susceptible SNPs is based on the allele identification using Illumina MiSeq deep amplicon sequencing technology.

Isolates	Total no of Illumina Miseq reads	Total no of susceptible reads	Total no of resistant reads	Susceptible alleles	S58R/S117N resistant alleles	S58R resistant alleles	S117N resistant alleles	I173L resistant alleles	Region
DHFR1	1229	1229		100.0					Lahore
DHFR2	7249	7249		100.0					Lahore
DHFR3	2983	2983		100.0					Lahore
DHFR4	1649	1649		100.0					Lahore
DHFR5	5829	5829		100.0					Lahore
DHFR6	11,744	11,744		100.0					Sheikhupura
DHFR8	11,279	11,279		100.0					Sheikhupura
DHFR9	5812	5812		100.0					Kasur
DHFR11	7985	7985		100.0					Sheikhupura
DHFR 14	9056	76	9040	0.2			99.8		Gujranwala
DHFR15	9018	9018		100.0					Gujrat
DHFR16	15,398	15,398		100.0					Lahore
DHFR 17	17,447		17,447		100.0				Lahore
DHFR 26	6657	3025	3632	45.4	52.0	0.1	2.5		Lahore
DHFR 28	12,831		12,831					100.0	Lahore
DHFR29	5755	5755		100.0					Sheikhupura
DHFR 33	6793	366	6427	5.4	94.1		0.5		Lahore
DHFR34	2511	2511		100.0					Lahore
DHFR35	5600	5600		100.0					Multan
DHFR 39	1456	34	1422	2.3	93.9			3.8	Lahore
DHFR44	13,240	13,240		100.0					Gujranwala
DHFR 45	10,265	9383	882	91.4			8.6		Faisalabad
DHFR49	28,680	28,680		100.0					Multan
DHFR52	12,875	12,875		100.0					Gujrat
DHFR 55	2528	192	2336	7.6			92.4		D.G.Khan
DHFR79	4608	4608		100.0					Multan
DHFR80	10,031	10,031		100.0					Bhakkar
DHFR87	9223	9223		100.0					Gujranwala
DHFR 93	12,892	30	12,889	0.0	99.9				Sheikhupura
DHFR 102	2392	733	1659	30.6	69.3				D.G.Khan
DHFR108	2429	2429		100.0					Narowal
DHFR110	24,342	24,342		100.0					Okara
DHFR 112	7342		7342		100.0				Layyah
DHFR114	47,726	47,726		100.0					Multan
DHFR 118	6701	56	6697	0.1	2.1		97.8		D.G.Khan
DHFR 121	27,705	47	27,658	0.2	98		1.8		R.Y. Khan
DHFR122	23,213	23,213		100.0					R.Y. Khan
DHFR 128	10,029	3808	6221	38.0	61.3		0.7		D.G. Khan

Fidelity Polymerase (KAPA Biosystems,USA), 13.25 μL ddH$_2$O and 2 μL of first round PCR product as DNA template. The barcoded forward (N501 to N508) and reverse (N701 to N712) primers (10 uM each) were obtained from Illumina MiSeq protocols (Supplementary Table S2). The thermocycling conditions of the PCR were 98 °C for 45 s, followed by 7 cycles of 98 °C for 20 s, 63 °C for 20 s, and 72 °C for 2 min. PCR products were purified with AMPure XP Magnetic Beads (1 ×) according to the protocols described by Beckman coulter, Inc. The pool library was checked with a KAPA qPCR library quantification kit (KAPA Biosystems, USA). The prepared library was then run on an Illumina MiSeq Sequencer using a 500-cycle pair end reagent kit (MiSeq Reagent Kits v2, MS-103-2003) at a concentration of 15 nM with addition 25% Phix Control v3 (Illumina, FC-11-2003). The MiSeq separated all sequences by sample during post-run processing using recognised indices and to generate FASTAQ files.

2.3. Deep amplicon sequencing data handling and bioinformatics filter to remove sequencing induced mutations

For the analysis of dihydrofolate reductase resistance to pyrimethamine, Illumina Miseq analysis was performed with a Command Prompt pipeline using Mothur v1.39.5 software and the Illumina Mi-seq SOPs (Schloss et al., 2009). In summary, raw paired-end reads were made into contigs, and those that were too long (> 468 bp) or had ambiguous bases were discarded as a trace amplicon contamination. A consensus sequences was made by *P. vivax* dihydrofolate reductase sequences from the NCBI BLASTN search and aligning by MUSCLE using Geneious Pro 5.4 software (Drummond et al., 2012), then trim up to the region amplified by the primers. The MiSeq reads were aligned to the consensus sequences, as a result, millions of pyrimethamine susceptible and resistance reads of the dihydrofolate reductase locus were generated from 38 *P. vivax* isolates (Table 1).

For the analysis of selective sweep and phylogenetic models, MiSeq reads of 14 *P. vivax* resistant isolates (generated from the above pipeline) were first aligned in Geneious software (Kearse et al., 2012) and remove the SNP only occurring once, considered to be artefacts due to sequencing errors (Chaudhry et al., 2015). The aligned sequences were then imported into the CD-HIT software (Huang et al., 2010) to calculate the number of haplotypes (unique sequence generated from millions of MiSeq reads) present in each isolate (Supplementary Table S3).

2.4. Genetic diversity estimation and selective neutrality test of the dihydrofolate reductase locus

Genetic diversity estimation and selective neutrality tests were calculated using the DnaSP 5.10 software package (Librado and Rozas, 2009). Briefly, sequence polymorphism estimation is calculated through the haplotype frequency (H_f), haplotype diversity (H_d), nucleotide diversity (π), the mean number of pairwise differences (k), the number of segregating sites (S) and the mutation parameter based on an infinite site equilibrium model and the number of segregating sites (θ_S). Tests for selective neutrality were analysed to determine whether the observed frequency distribution of sequence polymorphism departed from neutral expectations. The neutrality tests included Tajima's D (Tajima, 1989) and Fay & Wu's H (Fay and Wu, 2000) methods. Considering the value of Tajima's D and Fay & Wu's H for the calculation of the signature or genetic footprint of selection: if there is purifying selection, mutations will occur and accumulate at a silent site, thus there are likely to be multiple segregating sites, but the Tajima's D and Fay and Wu's H value will be negative. In short, the Tajima's D and Fay & Wu's H value provides insight into the evolutionary history of a particular locus. If the *P* value is statistically significant, departure from neutrality determined with the use of coalescent simulations of 10,000 replicates.

2.5. Split and the network tree analysis of pyrimethamine resistance associated SNPs in the dihydrofolate reductase locus

A split tree of dihydrofolate reductase haplotypes was generated based on the genetic distance model (JukesCantor) using the UPGMA method employed in SplitTrees4 software (Huson and Bryant, 2006). A split tree of the dihydrofolate reductase haplotypes was also constructed using UPGMA method in MEGA5 software (Tamura et al., 2011). The program jModeltest 12.2.0 was used to select the appropriate model of nucleotide substitutions for UPGMA analysis (Posada, 2008). According to Bayesian information criterion, the best scoring was JukesCantor (JK + G + I). Branch supports were obtained using 1000 bootstraps of the data. The most probable ancestral node was determined by rooting the tree to a closely related outgroup (*Plasmodium falciparum*). The network tree of dihydrofolate reductase haplotypes was produced based on the Neighbour Joining algorithm built on a sparse network and the epsilon parameter is set to zero default in Network 4.6.1 software (Fluxus Technology Ltd.).

2.6. Validation of deep amplicon sequencing using known proportions of resistant and susceptible dihydrofolate reductase alleles

Before applying the deep amplicon sequencing method to large scale surveys, it is necessary to show that the resistance allele frequency generated by Illumina MiSeq accurately reflects the genotypic frequency. The dihydrofolate reductase susceptible (DHFR2) and resistance (DHFR14, DHFR17, DHFR28) isolates were selected (Table 1) and run individually through Illumina MiSeq to identify the homozygous susceptible alleles [S: S58R(AGC), S117N(AGC), I173L (ATT)] and homozygous resistant alleles [R: S58R (AGA), S117N (AAC) and I173L (CTT)]. Once identified, pools of gDNA were made based on the known allele frequencies of susceptible and resistance mutations. These were prepared for deep amplicon sequencing and analysis as described above (Section 2.2). There was no statistically significant difference between the expected and observed frequencies of susceptible and resistance alleles in a chi-square test, which suggests that the method was accurate in measuring resistance allele frequencies (Fig. 1). For the pools of 100% susceptible or resistance alleles (Mix1:S-100, Mix2: R (S117N)-100, Mix3: R(I173N)-100], the expected and observed results were perfectly matched (Fig. 1). For pools of 50% or 25% susceptible and 50% or 75% resistance alleles [Mix4:S + R(S117N)-50/50, Mix5:S + R(I173N)-50/50 or Mix6:S + R(S58R/S117N-25/75], the expected and observed results were highly comparable, with some variations between replicates (Fig. 1). Nevertheless, the data provided a sufficient accurate estimation of allele frequencies appropriate for the work undertaken in this study.

3. Results

3.1. Occurrence of pyrimethamine resistance associated SNPs in P. vivax isolates

A 468 bp fragment of the *P. vivax* dihydrofolate reductase locus was amplified from 38 isolates across the Punjab province of Pakistan (Table 1). The relative resistance allele frequencies of eight mutations [F57L/I (TTA/ATA), S58R (AGA), T61M (ATG), S117N/T (AAC/ACC) and I173L/F (CTT/TTT)] were determined by deep amplicon sequencing. The data set that was generated comprised mostly of three resistance associated SNPs [S58R (AGA), S117N (AAC) and I173L (CTT)] (Table 1). The double mutations of S58R (AGA) and S117N (AAC) were identified in ten isolates at frequencies between 2.1 and 100%. The S117N (AAC) SNP was identified in eight isolates at frequencies between 1.8 and 99.8%. The I173L (CTT) SNP was detected in two isolates at frequencies between 3.8 and 100% (Table 1). The pyrimethamine resistance associated SNP S58R (AGA) was present in one isolate at very low frequency of 0.1%, whilst neither F57L/I (TTA/ATA) nor T61M (ATG) SNPs were detected in any isolate examined. Overall results indicated that 14 isolates give an evidence of positive selection pressure on the dihydrofolate reductase locus (Table 1).

3.2. Dihydrofolate reductase locus diversity and neutrality analysis in P. vivax isolates

The genetic diversity and neutrality analysis of the dihydrofolate reductase locus was assessed from the 14 selected isolates encompassing the S58R (AGC/AGA), S117N (AGC/AAC), I173L (ATT/CTT) resistance mutations (Table 1). Based on the analysis of each population separately, *P. vivax* showed a high level of genetic diversity with a haplotype diversity (H_d) ranging from 0.159 to 0.822, a high number of polymorphic sites (S), and the number of alleles ranging from 1 to 33 (Table 2). Overall, there was little difference in the genetic diversity between 14 isolates except DHFR14. There was a significant departure of Fay and Wu ‘s (*H*) and Tajima (*D*) statistic from neutrality in five isolates (DHFR17, DHFR45, DHFR93, DHFR112, DHFR121), providing evidence of the genetic footprint of selection at the *P. vivax* dihydrofolate reductase locus.

3.3. Distribution of P. vivax dihydrofolate reductase haplotypes

A total 42 unique haplotypes of the dihydrofolate reductase locus were identified among all 14 isolates. Twenty-one haplotypes encoded S58R (AGA)/S117N (AAC) resistance mutations, 8 haplotypes encoded S117N (AAC) and one haplotype encoded S58R (AGA) or I173L (CTT) resistance mutations (Fig. 2, Tables 1 and 2). Based on the analysis of each isolate separately, two isolates (DHFR118, DHFR128) contained a maximum of 4 S58R (AGA)/S117N (AAC) and S117N (AAC) resistance haplotypes, 4 isolates (DHFR26, DHFR33, DHFR39, DHFR55) contained a maximum of 3 S58R (AGA)/S117N (AAC), S117N (AAC), S58R (AGA) and I173L (CTT) resistance haplotypes and 2 isolates (DHFR14, DHFR102) contained a maximum of 2 S58R (AGA)/S117N (AAC) and S117N (AAC) resistance haplotypes. A single I173L (CTT) resistance-conferring haplotype was present in the DHFR28 isolate (Table 2, Supplementary Table S3). In all these nine isolates, a single resistance-conferring haplotype present at high frequency range of 95 and 100% (Fig. 2), demonstrating evidence of hard selective sweep patterns.

In contrast, one population (DHFR121) contained a maximum of 14 S58R (AGA)/S117N (AAC) and S117N (AAC) resistance haplotypes, two isolates (DHFR93, DHFR112) contained a maximum of 8 S58R (AGA)/S117N (AAC) and S117N (AAC) resistance haplotypes and two isolates (DHFR17, DHFR45) contained a maximum of 3 S58R (AGA)/S117N (AAC) and S117N (AAC) resistance haplotypes (Table 2, Supplementary Table S3). Those five isolates had equally high frequencies of dihydrofolate reductase resistance-conferring haplotypes (Fig. 2),

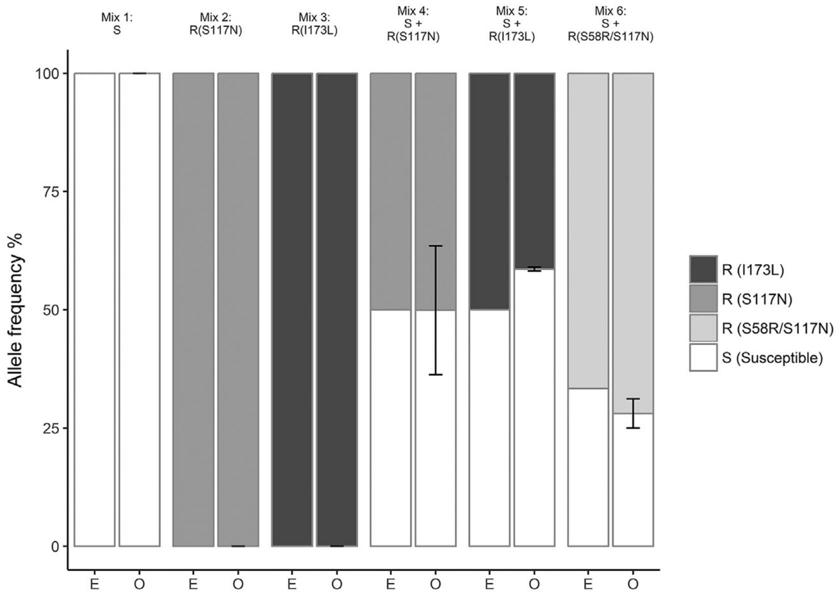

Fig. 1. Average frequency of pyrimethamine resistance in mock pools made from mixing homozygous susceptible alleles [S: S58R(AGC), S117N(AGC), I173L (ATT)] and homozygous resistant alleles [R: S58R (AGA), S117N (AAC), I173L (CTT)]. The symbol mix represents the mixing of homozygous susceptible-S and homozygous resistant-R alleles. In the X-axis, E represents the expected allele frequencies and O represent the observed allele frequencies. White shade indicates susceptible alleles [S: S58R(AGC), S117N(AGC), I173L (ATT)], light grey shade indicates S58R (AGA)/S117N (AAC) resistance alleles, medium grey shade indicates S117N (AAC) resistance allele and dark black shade indicate I173L (CTT) resistance allele. Error bars for observed frequencies represent the standard error of the mean. The mixing of the homozygous susceptible and homozygous resistant were not statistical significant (Chi-square test; Mix1: exact match; Mix2: exact match; Mix3: exact match; Mix4: $\chi^2(1) = 1.0089 \times 10–30$, $p = 1$; Mix5: $\chi^2(1) = 1.1719$, $p = .28$; Mix6: $\chi^2(1) = 0.4259$, $p = .51$).

demonstrating evidence of soft selective sweep patterns.

3.4. *Phylogenetic relationships between pyrimethamine resistance associated SNPs in P. vivax isolates*

The split and network trees were produced to examine the phylogenetic relationship between forty-two dihydrofolate reductase haplotypes. The resistance haplotypes were distributed across the trees consistent with the emergence of resistance from different susceptible ancestral backgrounds (Figs. 3 and 4). When analysing the individual resistance mutations, the split tree of the S58R (AGA)/S117N (AAC) mutation reveals that twenty-one haplotypes (HR39, HR32, HR33, HR26, HR40, HR41, HR42, HR28, HR37, HR5, HR9, HR15, HR34, HR31, HR13, HR24, HR30, HR6, HR38, HR27, HR25) were located in a single distinct clade II (Fig. 3), indicating a single origin for this mutation (Supplementary Fig. S1). Susceptible haplotypes were not share with resistance haplotypes in clade II but arose separately in the closest clade III (Fig. 3). The network tree analysis showed that the 21 resistant haplotypes were present in eleven different isolates collected from five major cities of Lahore, Sheikhupura, D.G. Khan, Layyah and R.Y. Khan (Fig. 4). Only one haplotype (HR5) was present at a high frequency in all 11 different isolates from the five cites, showing its spread between the cities (Fig. 4).

For the S117N (AAC) mutation, the split tree analysis showed 8 resistant haplotypes (HR1, HR7, HR18, HR4, HR35, HR19, HR23, HR14) located in single clade I (Fig. 3), again indicating a single origin of this mutation (Supplementary Fig. S1). Each of these resistance haplotypes was closely related to one or more susceptible haplotypes. The network tree analysis showed that the 8 resistance associated haplotypes were present in 9 different isolates collected from six major cities of Gujranwala, Lahore, Faisalabad, D.G. Khan, Sheikhupura and Layyah (Fig. 4). Three haplotypes (HR1, HR7, HR18) were present at a high frequency in the data set from all 8 different isolates samples from six cites. The HR1 resistance haplotype was present only in a single isolate (DHFR14) collected from Gujranwala city, indicating that this mutation has not spread to any other city. The HR7 and HR18 haplotypes were present in three and six different isolates respectively, sampled from the five cites excluding Gujranwala (Fig. 4), indicating the spread of this mutations between these cities (Fig. 4). The overall phylogenetic relationship supports the view that S58R (AGA)/S117N (AAC) and S117N (AAC) mutations arose at multiple times from a single origin and spread to the multiple different locations in the Punjab

Table 2
Total number of susceptible and resistant haplotype, neutrality analysis and genetic diversity of dihydrofolate reductase locus from fourteen *P. vivax* isolates.

	Total no. of haplotype neutrality tests genetic diversity									
Isolates	Total haplotypes	Susceptible haplotypes	Resistant haplotypes	Fay and Wu 's (H)	Tajima (D)	Haplotype diversity (H_d)	Segregating sites (S)	Nucleotide diversity (Π)	Mutation parameter based on S (Θ_S)	Mean number of pairwise difference (k)
DHFR14	4	2	2	0.62273	−0.68111	0.159	1	0.00037	0.00062	0.159
DHFR17	4	0	4	−2.81536*	−2.15697*	0.497	10	0.00019	0.00576	0.847
DHFR26	4	1	3	0.35747	1.61499	0.671	5	0.00476	0.00308	2.117
DHFR28	1	0	1	N/A						
DHFR33	4	1	3	−0.46069	−0.30808	0.455	5	0.00276	0.00308	1.229
DHFR39	5	2	3	−0.68094	−0.37304	0.800	9	0.00654	0.00718	2.834
DHFR45	7	4	3	−3.09551*	−1.80391	0.762	17	0.00413	0.00932	1.773
DHFR55	5	2	3	−1.69653	−1.22199	0.757	11	0.00446	0.00686	1.991
DHFR93	10	2	8	−3.61296**	−2.37349**	0.655	17	0.00315	0.01042	1.351
DHFR102	6	4	2	−0.82183	0.1152	0.737	6	0.00423	0.00409	1.778
DHFR112	8	0	8	−2.95443*	−2.22226***	0.822	13	0.00289	0.00791	1.289
DHFR118	5	1	4	−0.13002	−0.94342	0.538	6	0.00284	0.00471	1.216
DHFR121	16	2	14	−5.28936**	−2.63234***	0.707	33	0.00344	0.01681	1.476
DHFR128	5	1	4	−2.5697	−0.92415	0.676	10	0.00444	0.00623	1.979

Statistically significant departure from neutrality determined with the use of simulations of the coalescent at $p < .01$***, $p < .02$**, $p < .05$*.

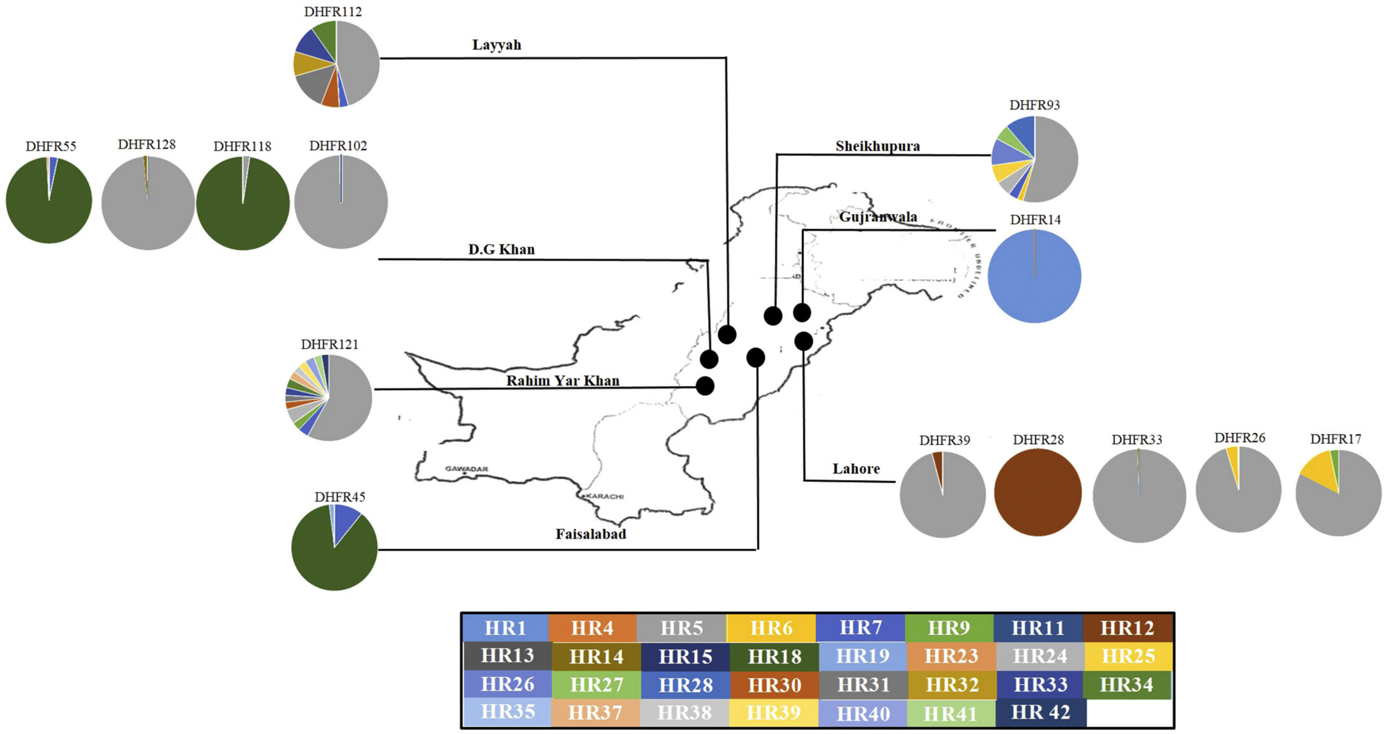

Fig. 2. Relative allele frequencies of thirty-one individual resistant haplotypes in fourteen isolates. Susceptible haplotypes were not indicated in the figure but shown in Table 2. The colors in the pie chart circles indicate the haplotype frequency and their distribution in each individual population in the insert map.

province of Pakistan through the flow of drug resistance alleles.

Interestingly, the I173L (CTT) resistance SNP was present on a single (HR12) haplotype located in clade I of the split tree (Fig. 3, Supplementary Fig. S1). This resistance haplotype was more closely related to one or more susceptible haplotypes and represented in two isolates (DHFR28, DHFR39) from the city of Lahore (Fig. 4). The data showed that HR12 haplotype was present at a high frequency in both isolates (Fig. 4). In conclusion, I173L (CTT) mutation was present on a single haplotype and the phylogenetic relationship suggests that it arose rarely and has not spread to different cities.

4. Discussion

Our study used molecular genetic approaches to provide relevant insight into the emergence and spread of pyrimethamine resistance after intensive positive selection pressure at the dihydrofolate reductase locus of *P. vivax* in the Punjab province of Pakistan. We chose this region because patients are treated in a sporadic manner often with generic pyrimethamine drugs of unknown quality. Antifolate combinations of sulfadoxine and pyrimethamine drugs, have been widely used for the therapy of haemoprotozoa, because they are relatively safe and inexpensive. The drugs, introduced in 1948 and 1967 respectively, exert their antimicrobial actions against *P. falciparum and P. vivax* by targeting critical enzymes, dihydropteroate synthase and dihydrofolate reductase, in their folate metabolism pathway (Wongsrichanalai et al., 2002). Pyrimethamine resistance in *P. falciparum* emerged in the late 1980s, and is now widespread with causative point mutations at the dihydrofolate reductase locus (Petersen et al., 2011). Until recently, it was assumed that pyrimethamine was effective against *P. vivax*, but the widespread resistance in *P. falciparum* shows the need to investigate the emergence and spread of pyrimethamine resistance in *P. vivax* (Hawkins et al., 2007). There is a clear need to understand how the resistance mutations against antifolate drugs emerge and spread. The frequency with which pyrimethamine resistance in *P. vivax* emerges and the extent to which it spreads are important considerations for its prevention and control.

4.1. *Pyrimethamine resistance mutations in P. vivax and their effect on positive selection pressure*

Pyrimethamine resistance has been investigated in a number of different geographical regions and strong evidence exists that F57L/I (TTA/ATA), S58R (AGA), T61M (ATG), S117N/T (AAC/ACC) and I173L/F (CTT/TTT) SNPs are responsible for resistance in *P. vivax* (Hawkins et al., 2007). Subsequent work has consistently shown that F57L/I (TTA/ATA), S58R (AGA), S117 N/T (AAC/ACC) single mutations and S58R (AGA)/S117N/T (AAC/ACC) double mutations are widely distributed in different geographical regions of south Asia (Auliff et al., 2006; Brega et al., 2004; de Pecoulas et al., 1998; Hastings et al., 2005; Imwong et al., 2003; Kaur et al., 2006; Kuesap et al., 2011; Lu et al., 2012; Mint Lekweiry et al., 2012; Ranjitkar et al., 2011; Schunk et al., 2006). Previous studies in Pakistan have shown that the S58R (AGA)/S117N (AAC) double mutation and S117N (AAC) single mutation conferring pyrimethamine resistance were present in different cities of the Punjab, Sindh and KPK provinces (Khattak et al., 2013; Zakeri et al., 2011). In the present study, the S58R (AGA)/S117N (AAC) double mutation was also present in 10/38 isolates. The single mutations of S117N (AAC) and S58R (AGA) were present in 8/38 and 1/38 isolates, respectively. A few studies have shown the I173L (CTT) mutation in south Asia (Auliff et al., 2006; Brega et al., 2004), but we found no evidence for the presence of this mutation in Pakistan. In the present study, we have identified for the first time the I173L (CTT) mutation in 2/38 isolates. This mutation has previously been reported in just two isolates of *P. vivax* collected from patients in Malo Island, Vanuatu (Auliff et al., 2006) and in a traveller on return to France from Afghanistan (Brega et al., 2004), but has not been detected in other surveys from south Asia.

There are couple of expectations in the differences of the frequency of pyrimethamine conferring mutations between geographical regions, as highlighted in our study in *P. vivax*. One explanation may be the variable drug doses, for example if the I173L (CTT) mutations are only selected at low doses of pyrimethamine, while S58R (AGA)/S117N/T (AAC/ACC) double mutations occur at higher doses previously

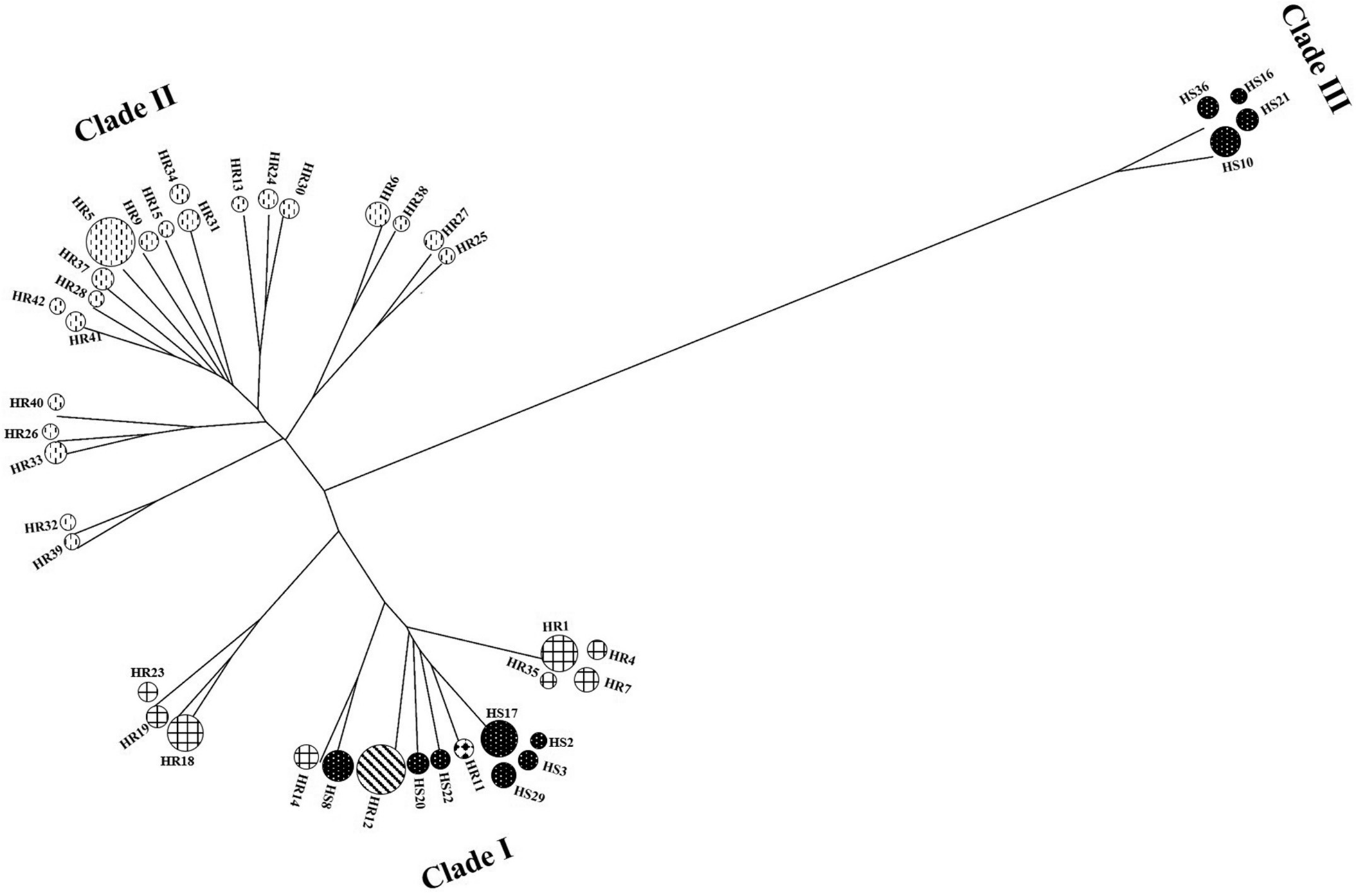

Fig. 3. Split tree of the forty-two dihydrofolate reductase haplotypes was generated with the UPGMA method using the Jukes Cantor (JC + G) model of substitution in SplitsTrees4 software (Huson and Bryant, 2006). The circles represent the different haplotypes and the size of each circle is proportional to the number of sequences generated in that haplotypes from fourteen isolates. The mutation carried each haplotype was shaded as follows: eleven susceptible haplotypes shown by black shading; eight P117N resistant haplotypes were hatched line shading; twenty one P58R/P117N double mutant resistant haplotypes shown by vertical dots shading; one P173L resistant haplotype shown by diagonal line shading and one S58R resistant haplotype containing S58R(AGA)/S117N(AGC)/I173L (ATT) SNPs have solid diamond shading.

described in nematode parasite *Haemonchus contortus* (Barrère et al., 2012). An alternative explanation may be that the mutations confer a fitness cost or disadvantage depending on the parasites genetic background previously described in another nematode parasite *Teladorsagia circumcincta* (Elard et al., 1998). A third explanation is that the differences may be due to bottlenecking effects resulting in to the loss of less common mutations, for example, if climatic changes disrupt the life cycle of the *P. vivax* mosquito vector. A fourth explanation is may be due to the genetic drift of pyrimethamine resistance or susceptible alleles with movement of patients to new places.

4.2. Nature of selective sweeps associated with the emergence of pyrimethamine resistance alleles in P. vivax

Several studies have assessed the selective sweep patterns in pyrimethamine resistance mutations of *P. falciparum* at the dihydrofolate reductase locus from different geographical regions. These emphasise a reduction in polymorphism around the dihydrofolate reductase locus with a single resistance haplotype indicative of a hard selective sweeps (McCollum et al., 2007; Nair et al., 2003; Pearce et al., 2005; Roper et al., 2003). A few studies have examined the presence of multiple haplotypes in Kenyan and Cameroon isolates of *P. falciparum* indicative of soft selective sweeps (McCollum et al., 2008; McCollum et al., 2006). There is a single report for a selective advantage to parasites bearing resistance conferring mutations at the *P. vivax* dihydrofolate reductase, providing evidence for a high level of polymorphism around the locus with multiple resistance haplotypes indicative of a soft selective sweeps (Hawkins et al., 2008). However, there is currently little understanding of the nature of selective sweeps associated with the emergence of pyrimethamine resistance in *P. vivax*. In the present study, although both soft and hard selective sweeps were present, there is a predominance of hard sweeps (Fig. 2). A single resistance haplotype at a high frequency was detected in the DHFR28 population and 2 to 3 resistance haplotypes were present in eight isolates (DHFR55, DHFR128, DHFR118, DHFR102, DHFR14, DHFR39, DHFR33, DHFR26), only one of these predominating at a high frequency (Fig. 2). The selective sweeps on these nine individual isolates were effectively hard with no evidence of the genetic footprint of selection. Our results are consistent with the hypothesis that a single mutation with a high frequency emerged in these nine isolates.

In contrast, 4 to 8 resistance haplotypes at high frequencies were identified in five isolates (DHFR112, DHFR121, DHFR45, DHFR93, DHFR17) (Fig. 2). The selective sweep on these five individual isolates was effectively soft and a genetic footprint of selection was also detected by significant departures from neutrality test (Table 2). In the soft selective sweeps, it can be more difficult to demonstrate the emergence of resistance mutations with multiple haplotypes at high frequencies, either due to recurrent mutations after the onset of positive selection pressure, or due to pre-existing mutations before the onset of positive selection pressure. In these situations, the pre-exiting mutations are seen in multiple haplotypes after historical recombination, and if selected, lead to a high level of haplotype diversity. Hence, the

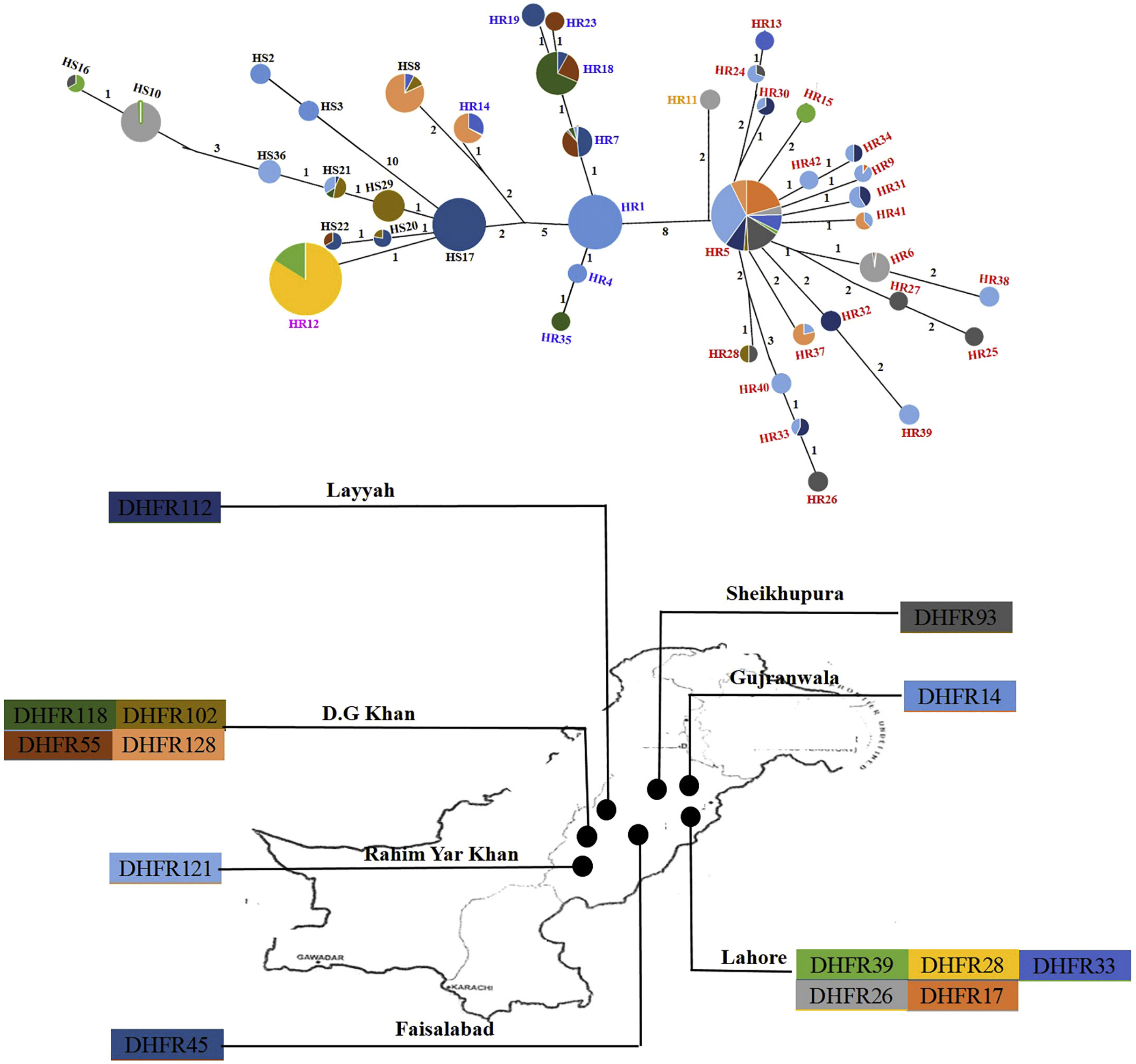

Fig. 4. Network tree of the forty-two dihydrofolate reductase haplotypes was generated with the Neighbour Joining method in the Network 4.6.1 software (Fluxus Technology Ltd.). All unnecessary median vectors and links were removed with the star contractions. The size of each circle represents the haplotype was proportional to the number of sequences generated from different isolates. The colors in the pie chart circles replicate the haplotype frequency and their distribution come from each of the fourteen isolates as indicated on insert map. The number of mutations separating adjacent sequence nodes was indicated along connecting branches and the length of the lines connecting the haplotypes is proportional to the number of nucleotide changes. The mutation carried each haplotype was colored as follows: eleven susceptible haplotypes (HS) containing S58R(AGC)/S117N(AGC)/I173L (ATT) SNPs shown in black; eight P117N resistant haplotypes (HR) shown in blue; twenty one P58R/P117N double mutant resistant haplotypes (HR) shown in red; one P173L resistant haplotype (HR) shown in pink; one S58R resistant haplotype (HR) shown in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presence of soft selective sweeps does not itself prove that recurrent mutations appeared in parasites after the onset of positive selection pressure (Redman et al., 2015). It is most obvious that pre-exiting mutations are similar in each of the isolates examined. Subsequently, if the pre-existing mutations were the only source of the emergence of resistance mutations, similar resistance haplotypes would be present in many isolates (Chaudhry et al., 2016). However, this is not the case in the present study, for example, two isolates (DHFR45, DHFR17) have dramatically different soft selective sweeps. The DHFR45 isolate carried HR7, HR18, HR19 resistance haplotypes, whilst DHFR17 isolate carried different HR5, HR6, HR9 resistance haplotypes (Fig. 2). This striking difference in the soft selective sweeps between the isolates provides evidence for the emergence of resistance by multiple independent recurrent mutations.

4.3. Role of human travel in the spread of pyrimethamine resistance mutations

There is large amount of human movement in the Punjab province of Pakistan, hence migration may play an important role in the spread of resistance mutations. However, the presence of the same resistance haplotypes in two or three isolates cannot itself be taken as conclusive evidence of the spread of resistance mutations (Redman et al., 2015). Nevertheless, the present study suggests that human migration between cities is an important factor in the spread of pyrimethamine resistance in Punjab. The S58R (AGA)/S117N (AAC) and S117N (AAC) mutations were present on multiple diverse resistance haplotypes and their phylogenetic relationship suggests that there is a single independent origin of these two mutations in the isolates examined (Fig. 3). It is notable that the HR5 resistance haplotype of the S58R (AGA)/S117N (AAC) mutation predominates in eleven different isolates sampled from five cities of Punjab and the other two resistance haplotypes (HR18 and HR7) of S117N (AAC) mutation were present in three and six different isolates respectively, sampled from five cities of Punjab (Fig. 4). It is likely that human travelling has contributed to the spread of these resistance haplotypes in the Punjab province from a single origin. Interestingly in the case of the I173L (CTT) mutation, our analysis suggests that it arose rarely from a single origin (Fig. 3). The mutation was present on a single haplotype in two *P. vivax* isolates within the city of Lahore (Fig. 4), indicating that it will spread in future, if control measures are not implemented.

5. Conclusion

P. vivax were examined to investigate effects of positive selection pressure on the emergence and spread of pyrimethamine resistance mutations in the Punjab province of Pakistan. Our results show adaptive mutations [S58R (AGA)/S117N (AAC) and S117N (AAC), S58R (AGA), I173L (CTT)] in response to positive selection pressure in fourteen isolates. Further analysis of those isolates, we have identified S58R (AGA)/S117N (AAC) and S117N (AAC) mutations with multiple diverse resistance haplotypes from a single origin, and the I173L (CTT) mutation with a single resistance haplotype from a single origin. The most common haplotypes (HR5, HR18, HR7) were identified in several isolates in different cities in the Punjab province shows the confirmation of the spread of pyrimethamine resistance mutations. Our findings, therefore, suggest that the emergence of resistance in *P. vivax* is the consequence of both single and multiple independent recurrent mutations. This understanding of the emergence and spread of pyrimethamine resistance mutations is relevant to the implementation of sustainable parasite control strategies. Sustainable control programmes for *P. vivax* are needed, integrating mosquito vector control with the rational use of anti-protozoal drugs and disseminate using appropriate educational methods.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.12.032.

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