HHS Public Access

Author manuscript

Infect Genet Evol. Author manuscript; available in PMC 2019 March 15.

Published in final edited form as:

Infect Genet Evol. 2018 October ; 64: 168–177. doi:10.1016/j.meegid.2018.06.027.

Genetic diversity of the *Plasmodium vivax* multidrug resistance 1 gene in Thai parasite populations

Veerayuth Kittichai[1,2], **Wang Nguitragool**[3], **Huguette Gaelle Ngassa Mbenda**[4], **Jetsumon Sattabongkot**[1,*], and **Liwang Cui**[4,*]

[1]Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand [2]Faculty of Medicine, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand [3]Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand [4]Department of Entomology, Center for Malaria Research, Pennsylvania State University, University Park, Pennsylvania, USA

Abstract

Plasmodium vivax resistance to chloroquine (CQ) was first reported over 60 years ago. Here we analyzed sequence variations in the multidrug resistance 1 gene (*Pvmdr1*), a putative molecular marker for *P. vivax* CQ resistance, in field isolates collected from three sites in Thailand during 2013-2016. Several single nucleotide polymorphisms previously implicated in reduced CQ sensitivity were found. These genetic variations encode amino acids in the two nucleotide-binding domains as well as the transmembrane domains of the protein. The high level of genetic diversity of *Pvmdr1* provides insights into the evolutionary history of this gene. Specifically, there was little evidence of positive selection at amino acid F1076L in global isolates to be promoted as a possible marker for CQ resistance. Population genetic analysis clearly divided the parasites into eastern and western populations, which is consistent with their geographical separation by the central malaria-free area of Thailand. With CQ-primaquine remaining as the frontline treatment for vivax malaria in all regions of Thailand, such a population subdivision could be shaped and affected by the current drugs for *P. falciparum* since mixed *P. falciparum/P. vivax* infections often occur in this region.

Keywords

Plasmodium vivax; Multidrug resistance 1 gene; Genetic diversity; Phylogenetic relationship; Genetic structure

1. Introduction

Chloroquine (CQ) has been the first-line antimalarial drug to treat *Plasmodium vivax* infections for more than 70 years. Whereas CQ resistance in *Plasmodium falciparum* is widespread and appears at high frequencies in most malaria-endemic areas, *P. vivax* CQ resistance is much less common, despite the first report of treatment failure indicative of CQ

[*]**Correspondence:** Jetsumon Sattabongkot, jetsumon.pra@mahidol.ac.th, Liwang Cui, luc2@psu.edu.

resistance in the 1950s (Berliner et al., 1948; Hoekenga, 1952). The slow emergence/spread of CQ resistance in *P. vivax* may be explained by its ability to form gametocytes before symptoms appear, thus enabling mosquito infection before drug treatment. Nonetheless, ineffective treatments of vivax malaria have been reported in many areas (Price et al., 2014), including, but not limited to, Papua New Guinea (Cooper, 1994; Schuurkamp et al., 1992), Ethiopia (Ketema et al., 2011), Indonesia (Baird et al., 1997; Ratcliff et al., 2007; Tjitra et al., 2008), India (Singh, 2000), and Brazil (de Santana Filho et al., 2007). In the Greater Mekong Subregion (GMS) of Southeast Asia, though CQ is generally effective for treatment of vivax malaria, CQ clinical failure indicative of resistance has been reported sporadically in Myanmar (Htun et al., 2017; Marlar et al., 1995; Myat Phone et al., 1993), Thailand (Congpuon et al., 2011; Rijken et al., 2011), Vietnam (Thanh et al., 2015), and at the China-Myanmar border (Liu et al., 2014; Yuan et al., 2015). Because of high recurrence rates of *P. vivax* after CQ treatment in some areas, CQ resistance has been considered an important public concern. In Indonesia, for example, CQ as the front-line treatment for *P. vivax* has been replaced by artemisinin-combination therapy (Baird et al., 1997).

Previous studies have suggested that mutations within the multidrug resistant 1 gene (*Pvmdr1*) may be used as markers for CQ resistance surveillance (Chung et al., 2015; Huang et al., 2014; Mekonnen et al., 2014). *In vitro* drug susceptibility assays also revealed association between higher genomic copy number of *Pvmdr1* and the increase in CQ IC_{50} (Marfurt et al., 2007; Suwanarusk et al., 2008). More recently, M908L and T958M mutations were shown to be associated with reduced *in vitro* CQ sensitivity (Chehuan et al., 2013). In the clinical settings, association was found between the copy number of *Pvmdr1* harboring Y976F/F1076L mutations and treatment failure in severe *P. vivax* malaria cases (Fernandez-Becerra et al., 2009; Melo et al., 2014). However, some studies failed to detect a link between *Pvmdr1* mutations and reduced CQ sensitivity (Hamedi et al., 2016; Schousboe et al., 2015; Shalini et al., 2014). Association between Y976F/F1076L mutations and *P. vivax* CQ resistance was mostly reported based on the cut-off IC_{50} values of an estimated minimal effective concentration of CQ at 100 ng/ml of the whole blood, a value that is applied for *in vitro* drug susceptibility in *P. falciparum* (Baird et al., 1997; Rungsihirunrat et al., 2015). There is not yet a CQ concentration cutoff for defining CQ resistance in *P. vivax*. Thus, with the unclear link between *P. vivax* resistance and *Pvmdr1* mutations (point mutations and copy number variation) (Suwanarusk et al., 2008), the resistance cut-off value needs future validation.

To better understand the diversity and evolution of *Pvmdr1* in *P. vivax* populations in Thailand, we collected blood samples from *P. vivax* malaria patients from the western and eastern national borders where malaria remains endemic. In these areas, CQ has been administered with primaquine (PQ) as the standard radical cure for *P. vivax* malaria since 2007. Through sequencing, we determined the extent of genetic diversity and identified potential mechanisms affecting the evolution of *Pvmdr1*. We also report haplotype clusters representative of major parasite variations and the population structure of the Thai parasites with respect to the global isolates.

2. Materials and methods

2.1. Study sites and samples collection

Malaria transmission in Thailand is perennial and seasonal, which follows the pattern of rainfall with two transmission peaks: one in July-September and one in November (Cui et al., 2003). Samples were collected in three provinces: Tak and Kanchanaburi in the west bordering Myanmar, Ubon Ratchathani in the northeast bordering Cambodia and Laos (Fig. 1). There are different distribution patterns of malaria vectors between western and eastern regions of Thailand (CDC, 2015; Kittichai et al., 2017). While major vectors in western Thailand are *Anopheles dirus*, *An. minimus* and *An. maculatus* complexes, in eastern Thailand such as Ubon Ratchathani *An. barbirostris* is widely distributed. The latter species has been shown to be a vector for *P. vivax* (Sriwichai et al., 2016). Whereas the western border has been the most malaria prevalent, an outbreak occurred in Ubon Ratchathani in 2016, which reported the highest number of malaria cases in Thailand (CDC, 2015). In total, 88 finger-prick blood samples were collected from symptomatic patients in Kanchanaburi (37 during 2013-2014), Tak (22 during 2013-2015), and Ubon Ratchathani (29 during 2015-2016). To reduce the possibility of imported cases, patients were all from local villages who did not have travel histories in the previous month. Genomic DNA was extracted from the bloods using DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA). Malaria diagnosis was performed by light microscopy and confirmed by nested PCR targeting the 18S rRNA gene (Snounou et al., 1993). The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (TMEC11-033).

2.2. PCR amplification and sequencing of the Pvmdr1 gene

PCR of the *Pvmdr1* gene was performed with *Pfu* DNA polymerase (Promega) and primer sets MF1/MR5 and MF4/MR7 which generated two amplicons of 2,770 bp and 2,884 bp, respectively (Supplementary Table S1). PCR products were purified using the AccuPrep PCR purification kit (Bioneer Corporation, South Korea), and sequenced in both directions with primers described in the Table S1. All primers were designed based on the reference *Pvmdr1* sequence from the Salvador I strain (PVX_080100). *S*equences were assembled and edited manually using BioEdit 7.2.5 (Hall, 1999). Sequence alignment was done using the program Muscle 3.8 (Edgar, 2004).

2.3. *Domain prediction in* Pvmdr1

Nucleotide-binding domains (NBDs) and transmembrane domains (TMs) of the Pvmdr1 protein were identified by mapping the sequence to the P-glycoprotein structure model from PDB: 4Q9H, which shares 31% identity. The suitable protein template was searched via the SWISS-MODELL server (https://swissmodel.expasy.org/interactive).

2.4. Global data collection

A total of 113 full-length or near full-length *Pvmdr1* sequences were retrieved from GenBank and PlasmoDB (plasmodb.org) representing parasite isolates from 13 countries: 13 from Thailand, 7 from China, 7 from Papua New Guinea (PNG), 2 from India, 8 from Madagascar, 15 from Mexico, 22 from Columbia, 30 from Peru, 5 from Brazil, and one each

from Vietnam, North Korea, Mauritania and El Salvador (Fig. 1). Each sequence was trimmed to remove low-quality segments, yielding 4,257 bp fragments from the 4,395 bp *Pvmdr1* open reading frame. Sequences obtained in this study are available in GenBank with the accession numbers KX421155-KX421190 and KX868005-KX868056.

2.5. Sequence polymorphism and diversity

Sequence diversity was estimated using either short sequences through the sliding window technique or the nearly full-length (4,257 bp) sequence. The nucleotide diversity of *Pvmdr1* was estimated by DnaSP v5.10 (Librado and Rozas, 2009). Singletons, parsimony, number of segregation sites including both synonymous and nonsynonymous mutations, and haplotypes (H) were determined. For genetic diversity, the average nucleotide diversity ($\theta\pi$) and haplotype diversity (Hd) were measured across different sites within Thailand as well as across different countries.

2.6. Intragenic Recombination

Detection of recombination events was done using several programs incorporated in DnaSP v5.10 and the RDP4 package (Martin et al., 2010). The minimum numbers of recombination events (Rm) were assessed by the gametic test in DnaSP v5.10. Recombination breakpoints were detected using RDP, MaxChi, GENECONV, BootScan, Chimeara, SIScan, PhylPro, LARD and 3Seq algorithms implemented in the RDP4 package. LDhat in the RDP4 package was used to scan the recombination rate (Rho, ρ) and the mutation rate (Theta, θ). The population-scaled recombination to mutation ratio (ρ/θ) was calculated.

2.7. Tests of departure from neutrality and codon-based tests of selection

Tajima's D and Fu & Li's F* and D* tests were used to assess the departure from neutrality using the DnaSP v5.10 software. If the Watterson population nucleotide diversity (θ) and average pairwise nucleotide diversity (π) differ, a significant positive D value is expected, suggesting a balancing selection or a population size shrinking. Likewise, Fu & Li's F* and D* tests are implemented to evaluate the difference between the number of singletons and the average pairwise nucleotide diversity. If a significant positive value is found, a balancing selection is indicated. However, if the Fu & Li's statistics are negative, directional selection is implicated. Sliding window analysis also was used to determine nucleotide diversity and to perform all neutrality tests across the loci. Deletions/insertions were excluded from the analyses.

To study the evolutionary force acting on the *Pvmdr1* gene, a hypothesis of dN>dS was tested using a bootstrap method with 1,000 replicates. Two-tailed Z-test of selection was used to estimate variance and make a substitution model using the Nei-Gojobori method in MEGA7 (Kumar et al., 2016). While a dN/dS ratio exceeding one is considered as a result of positive selection,a ratio less than one is an indicator of a purifying selection. A P-value of < 0.05 was considered as the level of significance.

A panel of maximum likelihood codons-based tests in the HyPhy package implemented in the online web-server Data Monkey (http://www.datamnkey.org) was conducted in order to determine the existence of specific codons targeted by selection (Kumar et al., 2016).

2.8. Phylogenetic relationship

To determine the genetic interrelationships of all the parasite isolates, phylogenetic analysis was done with aligned *Pvmdr1* sequences using PHYLOViZ 2.0 (Francisco et al., 2012). A phylogenetic tree was generated using the neighbor-joining algorithm. The minimum number of substitutions or errors that influence the tree's branching was measured by the Hamming distance. The Sal I reference strain was represented as the wild type. Haplotype clusters were defined manually using nine global single nucleotide polymorphisms (SNPs) with frequencies of ≥ 5%.

2.9. Population structure and genetic differentiation

Haplotype clustering analysis based on the polymorphic sites in *Pvmdr1* was used to analyze the *P. vivax* populations with reference to the dynamics of the parasite transmission bearing the varied SNP patterns. The pattern was shown by the Thai provinces and countries. STRUCTURE 2.3.2 software was used to assess clustering of haplotypes (Pritchard et al., 2000) based on the admixture model. Twenty iterations were run for each cluster (K= 1-12) with a burn-in of 50,000 steps and then 500,000 Markov Chain Monte Carlo (MCMC) steps. The optimal number of clusters (K) was determined according to a published method based on the change in the posterior probability of the data [Ln P(D)] (Evanno et al., 2005). FSTAT v.2.9.3 (Goudet, 1995) software was used to assess pairwise genetic differentiation (Weir & Cockerham *Fst* values) using *Fst*. LIAN 3.7 (Haubold and Hudson, 2000) software was used to calculate the linkage disequilibrium (*LD*) using 50,000 iterations for burn-in followed by 100,000 MCMC iterations.

3. Results

3.1. Genetic diversity of the Pvmdr1 gene in Thai and global parasite isolates

Based on the alignment with the model structure PDB: 4Q9H, the predicted structure of the Pvmdr1 protein comprises a NBD1, the first six TMs (TM1-6), a NBD2 and the second six TMs (TM7-12). Since the genetic diversity of *Pvmdr1* has been evaluated mostly based on partial sequences, we sequenced the near complete *Pvmdr1* gene in 88 *P. vivax* clinical samples from three provinces in Thailand (Table 1). The sequences covering 4,257 bp (1,419 amino acids) contained a total of 34 SNPs, out of which 21 were nonsynonymous and 13 synonymous. The 21 nonsynonymous mutations are located in TM3, NBD1, NBD7-11 and NBD2 (Table 1). 14 out of the 21 nonsynonymous mutations were novel SNPs (K454I, L470H, K672N, N740 D, A763V, L845F, A861E, L936F, E996Q, P1177T, G1232C, G1265W, S1274R, and K1393N). Of the nine most prevalent SNPs, five (L845F, M908L, T958M, Y976F, and F1076L) were found in TM7-12 (Supplementary Fig. S1). In particular, two mutations M908L and T958M previously associated with reduced *in vitro* CQ sensitivity (Chehuan et al., 2013) reached high frequencies (96.59% and 100%, respectively). Although Ubon Ratchathani has the least number of nonsynonymous mutations of the three Thai sites, it has the highest frequency for the L845F mutant (58.62%). In contrast, the S513R and K1393N combination was the most prevalent in the two western provinces (Table 1). Y976F and F1076L were found in 9-27% and 27-41% of isolates across all three sites, respectively.

In total, 15 of the 21 nonsynonymous substitutions were parsimony-informative (9, 12 and 8 were parsimony-informative in Tak, Kanchanaburi and Ubon Ratchathani, respectively) (Table 2). Nineteen singletons were found across the three areas. The nucleotide diversity of the near full-length *Pvmdr1* gene was 0.00085 ± 0.00005 (Table 2). Sliding window plot of nucleotide diversity showed two peaks at the nucleotide positions 1,453-1,529 (NBD1) and 3,147-3,222 (TM11), but the two peaks did not reach statistical significance (Fig. 2). Forty eight haplotypes were detected and the haplotype diversity was high (0.961 ± 0.011). Based on the 9 SNPs with ≥ 5% frequency in global isolates (Table 3), nine haplotypes were found among the Thai isolates. Three haplotypes (Hap_10, Hap_29, and Hap_30) were province-specific, while six haplotypes (Hap_2, Hap_5, Hap_11, Hap_17, Hap_20, and Hap_21) with frequencies of 1-11.44% of total isolates were shared among the three provinces (Table 3, Supplementary Table S2). In terms of regions, seven haplotypes were specific to the western border of Thailand (Hap_7, Hap_10, Hap_11, Hap_17, Hap_18, Hap_20, and Hap_29), whereas only one haplotype Hap_30 was unique in the eastern region.

We used 201 *Pvmdr1* sequences from 13 countries to assess its global genetic diversity. These sequences contained 46 polymorphic sites, comprising 14 synonymous and 30 nonsynonymous substitutions. All the nonsynonymous mutations were located in TM1, TM3-5, NBD1, TM7-11 and NBD2. Sixteen of those polymorphic sites were parsimony-informative. The nucleotide diversity was 0.00069 ± 0.00004 in global isolates (Table 2). When the analysis was conducted according to the countries, the number of parsimony-informative sites in the Thai population (0.00085) was higher than the rest of countries, and this finding was also consistent with the haplotype number and the degree of haplotype diversity. Sliding window analysis showed that the highest peak was at the nucleotide positions 3,150-3,233 (TM11), coinciding with the second highest peak of the Thai isolates (Fig. 2). Out of the 9 global SNPs with ≥ 5% frequency, 7 were found in the 88 Thai isolates from the current study (Table 1). Based on these 9 SNPs, there were 30 haplotypes distributed across all the continents (Table 3, Supplementary Table S2). Most of them were country-shared haplotypes with a frequency of 1-18% of total isolates/haplotypes. Ten out of the 30 haplotypes (with frequency ≤ 1% of total isolates) were country-specific: Hap_13 for Madagascar; Hap_22-30 for China, Peru, India, North Korea and Thailand, respectively. Four haplotypes were from South America: Hap_3, Hap_16, Hap_23, and Hap_26. Eleven haplotypes were shared by countries or continents; Hap_1 and Hap_4 between Southeast Asia (SEA: Thailand) and the South America, Hap_6 between South America and Africa, Hap_7 and Hap_18 between Thailand and Africa, Hap_12 and Hap_14-15 between Thailand and China. Hap_8 was shared by four subcontinents: SEA (Thailand), South Asia (India), East Asia (China), and the Southwestern Pacific (Papua New Guinea, PNG). Similarly, Hap_9 was shared by three continents: SEA, Africa and the PNG. Seven haplotypes were shared between the western Thailand and the others, but none was shared with the eastern Thai parasite population.

3.2. Departure from neutrality and selection

In order to see whether the Thai *Pvmdr1* gene follows the neutral equilibrium model of molecular evolution, Tajima D, as well as Fu & Li's F* and D* were computed for the full-length *Pvmdr1* gene, which all yielded negative values in the Thai populations (Table 4).

Only Fu & Li's F* and D* were statistically deviating from zero (Table 4). Similarly, in the worldwide populations, all the neutrality tests yielded negative values and they were all statistically deviating from zero (Table 4). These findings suggest an excess of low-frequency polymorphisms within the Thai populations as well as the global populations, possibly as a result of directional selection or population expansion. The dN-dS statistic generated by the Z-test was also negative in the total populations, signifying purifying selection on *Pvmdr1*, albeit the level of significance was not reached (Table 4). To zoom in on particular regions of the gene, sliding window analysis was performed for Tajima's D (Fig. 2), which showed insignificant positive values at TM11 among the global sequences. When the Thai isolates were analyzed alone, two peaks higher than 1 were observed for both Tajima's D and Fu & Li's F*: The first peak was within NBD1, whereas the second peak of significant positive Tajima's D value was observed at the nucleotide positions 3098-3272 (TM11 encompassing the F1076L mutation). TM11 also matched the highest value of nucleotide and haplotype diversity when partitioning the gene into TM domains (Supplementary Table S3). Interestingly, two codon-based tests of selection revealed that the mutations S513R and F1076L were under positive selection and a few others sites under negative selection (Supplementary Table S4).

3.3. Recombination

As recombination can break the linkage, several methods were used to detect the number of recombination events and the locations of breakpoints in the genome. Using seven recombination detection algorithms within the RDP4 software, we only detected one recombination breakpoint by the MaxChi algorithm in the global isolates (Supplementary Fig. S2). For country-wise sequences, DnaSP detected six recombination events in Thailand, two in Colombia and one each in Peru and Madagascar (Table 4), while three methods in the RDP package (BootScan, MaxChi and 3Seq1) only detected one recombination event. The country-wise recombination signal, measured by using the estimated recombination (ρ)/ estimated mutation rates (θ) ratio, showed that the ratio in most countries were >1 (Table 4).

3.4. Genetic substructure

The global population genetic structure using the 9 major SNPs (with minor allele frequencies of ≥ 5%) showed optimal clustering at K = 3 (Fig. 3), which are colored as green, blue and red. Whereas all parasite populations had the M908L and T958M mutations at high frequencies, the three clusters also featured discordant, additional mutations (Supplementary Fig. S3). The green cluster represented parasites from South America with prevalent mutations at V221L (Fig. 1). The red cluster containing parasites from China and western Thailand included mutations of S513R, G698S, and K1393N. The blue cluster contained S513R, G698S, L845F, Y976F and F1076L, and it was of multiple origins mostly found in eastern Thailand, Madagascar, and PNG. The expected heterozygosity (H_E) corresponding to these clusters was 0.027 (green), 0.048 (red), and 0.063 (blue), respectively (Supplementary Fig. S3). Phylogenetic analysis based on the Neighbor-Joining method among the global parasite isolates also showed a clear lineage separation consistent with the three population clusters (Fig. 4). For the majority of the Thai's isolates, the red and the blue clusters were also clearly separated.

Moderate to high *Fst* values (0.15-0.76) were observed among the populations from different countries, indicating genetic differentiation of *P. vivax* populations. *Fst* showed a high degree of genetic difference among clusters (*Fst* > 0.25), which was 0.37 between the red and the green clusters, 0.43 between the blue and the green clusters, and 0.41 between the red and the blue clusters. Within Thailand, no significant genetic differentiation was detected between the two western sites, Tak and Kanchanaburi (*Fst* =0.0283). In contrast, the parasites from eastern Thailand was moderately different from Tak (*Fst* = 0.2009) and Kanchanaburi (*Fst* = 0.1505). The result was consistent with the genetic substructure described above.

LD was found in the isolates from Ubon Ratchathani, but not form Tak and Kanchanaburi (Table 6). Likewise, the global isolates displayed limited LD except for those from Brazil and PNG. The low LD might be due to the presence of recombination and could suggest a population expansion/isolation.

4. Discussion

Although malaria incidence has significantly declined over the past decade, the problem of drug resistance for both *P. falciparum* and *P. vivax* poses a major challenge in malaria control and elimination. The P-glycoprotein encoded by *mdr1*, an ABC transporter protein, plays an important role in multidrug detoxification in various organisms including the malarial parasites. In *P. vivax*, though genetic evidence is lacking, SNPs as well as gene duplication of *Pvmdr1* are associated with resistance to CQ (Lu et al., 2011; Suwanarusk et al., 2008). This study detected 12 of 34 SNPs in Pvmdr1 occurring at high frequency (≥ 5%) in *P. vivax* populations in Thailand, among which seven were nonsynonymous. Similar to what has been found in world *P. vivax* populations, the number of *Pvmdr1* SNPs in different Thai parasite populations also varied. Seven SNPs were consistent with those reported from Tak Province (Rungsihirunrat et al., 2015), but different from those reported from Laos (including N133K, S139S and E1261K) (Imwong et al., 2008). Among the SNPs found in this study, one novel SNP (L845F) was found in the eastern province Ubon Ratchathani, whereas another novel SNP (K1393N) was found in Kanchanaburi (Barnadas et al., 2008; Orjuela-Sanchez et al., 2009; Rungsihirunrat et al., 2015). Widespread SNPs such as T958M, Y976F and F1076L, associated with CQR in *P. vivax* were used in epidemiological surveillance worldwide (Huang et al., 2014; Lu et al., 2011). In previous studies, *Pvmdr1* SNPs and gene duplication events (~9%) were reported from countries of the GMS, including Thailand, Myanmar and Laos, albeit the sample sizes are small (Imwong et al., 2008; Lu et al., 2011). High frequency (>62%) F1076L plus gene duplication of the *Pvmdr1* was reported in western Thailand, whereas Cambodian parasite isolates near eastern Thailand harbored high frequency Y976F but no gene amplification (Lin et al., 2013). In this study, we did not investigate *pvmdr1* gene amplification, and thus some sequences used for analysis could be from different copies of the gene in case of duplicated *Pvmdr1*. Given that *pvmdr1* duplication was mostly associated with mefloquine use and the history of this event was relatively short (Khim et al., 2014), the *pvmdr1* gene copies should not have diverged much, which should not have a major effect on our analysis. Findings from *in vitro* drug susceptibility studies in *P. vivax* vary (de Santana Filho et al., 2007; Lu et al., 2011; Orjuela-Sanchez et al., 2009; Rungsihirunrat et al., 2015; Shalini et al., 2014), and there is no clear

association between any SNP or increased gene copy number and reduced antimalarial drug susceptibility. How *P. vivax* responds to the antimalarial drug pressure remains controversial (Barnadas et al., 2008; Lu et al., 2011; Orjuela-Sánchez P et al., 2009). This could be studied by performing co-evolution analysis of *Pfmdr1* gene in *P. vivax*/*P. falciparum* co-endemic regions, given mixed-species infections are frequent and collateral selection can happen.

Our study aimed to analyze the extent of sequence diversity of and potential selection on the *Pvmdr1* gene from Thai *P. vivax* populations. Similar to what was found for the global *P. vivax* populations, low nucleotide diversity (0.00069 ± 0.00004) was found in Thailand, suggesting the presence of functional/structural constraint on this gene. however, sliding window analysis of Tajima's D, Fu & Li's D* and Fu & Li's F* observed positive values in two domains NBD1 (S513R) and TM11 (F1076L) of *Pvmdr1* among the Thai isolates, but only in one domain (TM11) in the global isolates (Fig. 2). This latter result might signify that these two domains of the Pvmdr1 protein might have experienced a balancing selection in Thai populations or a reduction of population size. The multidrug transporter protein is predicted to function by coupling ATP hydrolysis in NBDs to the conformation change in the TM domains leading to extrusion of foreign compounds (Dawson and Locher, 2006). Mutations in both NBDs and TM may therefore negatively impact the function of the transporter protein more than the F1076L alone (Lu et al., 2011; Shalini et al., 2014). Significant signature of selection on *Pvmdr1* has not been reported to date, which might be correlated with the unique biology of the parasite such as early gametocyte production before antimalarial drug treatment (Koepfli et al., 2011). However, in the present study, the mutations S513R and F1076L were shown to be under positive selection with two codon-based tests of selection. This observation might be justified since selection often targets specific amino acids instead of the whole protein, which sometimes is masked by purifying selection also acting on the gene (Koepfli et al., 2011).

Two population genomics studies of the global *P. vivax* populations divided the New and Old World populations as well as detected parasite population substructures according to countries (Hupalo et al., 2016; Pearson et al., 2016). Interestingly, two population clusters in western and eastern Thailand with little gene flow were identified which are separated by the central, malaria-free region. *Fst* and genetic substructure results both support such a notion. An earlier study identified contrasting genetic structure between Asian and South American populations, and also revealed substantial population differentiation between populations from Thailand and Laos (Imwong et al., 2007). The significant population differentiation between the eastern and western Thai *P. vivax* populations is similar to this finding. Strong LD within the eastern populations suggested parasite expansion or bottleneck events, which was not found in the two western populations. Furthermore, the eastern parasite population did not share any haplotypes with other parts of the world, further indicating that parasite population from this area might have undergone a unique evolutionary process. The predicted population expansion and bottleneck events are consistent with the malaria outbreaks detected in the eastern province in recent years. Phylogenetic analysis showed three major clusters of haplotypes, where both population-specific and shared haplotypes were observed. It would be interesting to test whether these clusters are correlated with different sensitivities to CQ. It is noteworthy that CQ resistance has only been sporadically reported in the GMS (Myat Phone et al., 1993), where CQ and PQ remain as the standard

treatment for uncomplicated *P. vivax* infections. Thus, the reasons for the differences in the *Pvmdr1* gene mutations and their prevalence in the GMS could be related to the epidemiology of sympatric *P. falciparum* parasites, which are often present in mixed parasite species infections and are treated differently among endemic countries. In addition, the different distribution of major malaria vectors also may contribute to the differentiation of parasites in the eastern and western provinces. Furthermore, different immunity of human hosts may also account for the differences in mutation patterns and their prevalence.

In summary, this study revealed action of directional selection in the evolutionary course of *Pvmdr1* gene in Thai *P. vivax* populations. Particular domains of the gene showing high genetic diversity were found to be under balancing selection or subject to a population size decline. The eastern-western division pattern of the *P. vivax* parasite populations based on the *Pvmdr1* SNPs is consistent with the spread of both *Pfmdr1* (Veiga et al., 2016) and K13 propeller mutations in *P. falciparum* (Menard et al., 2016; Talundzic et al., 2015). Therefore, in addition to the selection from CQ-PQ treatment, we speculate that *P. vivax* might have been under drug selection pressure from treatment of mixed *P. falciparum* infections. Obviously, in an area with complex interplays among the multiple vector species, human host and co-endemic *P. vivax* and *P. falciparum*, the evolution of drug resistance is also influenced by the multitude of factors. Regardless of the population subdivision between eastern and western Thailand, much higher numbers of recombination events were detected in the Thai *P. vivax* population, which suggests the presence of a relatively large parasite population despite intensified control efforts in recent years. This is in contrast to the rapidly shrinking *P. falciparum* population in this region, and highlights that effective targeting the *P. vivax* reservoir is essential to achieve the goal of malaria elimination in Thailand by 2024.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement:

We would like to thank all the volunteers who participated in the research project as well as all the surveillance workers. This study was supported by grants from the Fogarty International Center (D43 TW006571) and the National Institute of Allergy and Infectious Diseases (U19 AI089672), National Institutes of Health, USA.

References

Baird JK, Leksana B, Masbar S, Fryauff DJ, Sutanihardja MA, Suradi, Wignall FS, Hoffman SL, 1997 Diagnosis of resistance to chloroquine by Plasmodium vivax: timing of recurrence and whole blood chloroquine levels. The American journal of tropical medicine and hygiene 56, 621–626. [PubMed: 9230792]

Barnadas C, Ratsimbasoa A, Tichit M, Bouchier C, Jahevitra M, Picot S, Menard D, 2008 Plasmodium vivax resistance to chloroquine in Madagascar: clinical efficacy and polymorphisms in pvmdr1 and pvcrt-o genes. Antimicrobial agents and chemotherapy 52, 4233–4240. [PubMed: 18809933]

Berliner RW, Earle DP, Taggart JV, Zubrod CG, Welch WJ, Conan NJ, Bauman E, Scudder ST, Shannon JA, 1948 Studies on the Chemotherapy of the Human Malarias. Vi. The Physiological Disposition, Antimalarial Activity, and Toxicity of Several Derivatives of 4-Aminoquinoline. The Journal of clinical investigation 27, 98–107.

CDC, 2015 Annual Report, Malaria situation in Thailand Bureau of Vector-Borne Diseases, Department of disease control Nonthaburi, Thailand: ministry of public health., Bangkok, Thailand

Chehuan YF, Costa MR, Costa JS, Alecrim MG, Nogueira F, Silveira H, Brasil LW, Melo GC, Monteiro WM, Lacerda MV, 2013 In vitro chloroquine resistance for Plasmodium vivax isolates from the Western Brazilian Amazon. Malaria journal 12, 226. [PubMed: 23819884]

Chung DI, Jeong S, Dinzouna-Boutamba SD, Yang HW, Yeo SG, Hong Y, Goo YK, 2015 Evaluation of single nucleotide polymorphisms of pvmdr1 and microsatellite genotype in Plasmodium vivax isolates from Republic of Korea military personnel. Malaria journal 14, 336. [PubMed: 26337571]

Congpuon K, Satimai W, Sujariyakul A, Intanakom S, Harnpitakpong W, Pranuth Y, Cholpol S, Bualombai P, 2011 In vivo sensitivity monitoring of chloroquine for the treatment of uncomplicated vivax malaria in four bordered provinces of Thailand during 2009-2010. Journal of vector borne diseases 48, 190–196. [PubMed: 22297279]

Cooper RD, 1994 Studies of a chloroquine-resistant strain of Plasmodium vivax from Papua New Guinea in Aotus and Anopheles farauti s.l. The Journal of parasitology 80, 789–795. [PubMed: 7931914]

Cui L, Mascorro CN, Fan Q, Rzomp KA, Khuntirat B, Zhou G, Chen H, Yan G, Sattabongkot J, 2003 Genetic diversity and multiple infections of Plasmodium vivax malaria in Western Thailand. The American journal of tropical medicine and hygiene 68, 613–619. [PubMed: 12812356]

Dawson RJ, Locher KP, 2006 Structure of a bacterial multidrug ABC transporter. Nature 443, 180–185. [PubMed: 16943773]

de Santana Filho FS, Arcanjo AR, Chehuan YM, Costa MR, Martinez-Espinosa FE, Vieira JL, Barbosa M, Alecrim WD, Alecrim M, 2007 Chloroquine-resistant Plasmodium vivax, Brazilian Amazon. Emerging infectious diseases 13, 1125–1126. [PubMed: 18214203]

Edgar RC, 2004 MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC bioinformatics 5, 113. [PubMed: 15318951]

Evanno G, Regnaut S, Goudet J, 2005 Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14, 2611–2620. [PubMed: 15969739]

Fernandez-Becerra C, Pinazo MJ, Gonzalez A, Alonso PL, del Portillo HA, Gascon J, 2009 Increased expression levels of the pvcrt-o and pvmdr1 genes in a patient with severe Plasmodium vivax malaria. Malaria journal 8, 55. [PubMed: 19341456]

Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carrico JA, 2012 PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC bioinformatics 13, 87. [PubMed: 22568821]

Goudet J, 1995 FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. Journal of Heredity 86, 485–486.

Hall TA, 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98.

Hamedi Y, Sharifi-Sarasiabi K, Dehghan F, Safari R, To S, Handayuni I, Trimarsanto H, Price RN, Auburn S, 2016 Molecular Epidemiology of P. vivax in Iran: High Diversity and Complex Sub-Structure Using Neutral Markers, but No Evidence of Y976F Mutation at pvmdr1. PloS one 11, e0166124. [PubMed: 27829067]

Haubold B, Hudson RR, 2000 LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage Analysis. Bioinformatics 16, 847–848. [PubMed: 11108709]

Hoekenga MT, 1952 Treatment of malaria with a single dose of amodiaquine or chloroquine. Journal of the American Medical Association 149, 1369–1371. [PubMed: 14938184]

Htun MW, Mon NCN, Aye KM, Hlaing CM, Kyaw MP, Handayuni I, Trimarsanto H, Bustos D, Ringwald P, Price RN, Auburn S, Thriemer K, 2017 Chloroquine efficacy for Plasmodium vivax in Myanmar in populations with high genetic diversity and moderate parasite gene flow. Malaria journal 16, 281. [PubMed: 28693552]

Huang B, Huang S, Su XZ, Tong X, Yan J, Li H, Lu F, 2014 Molecular surveillance of pvdhfr, pvdhps, and pvmdr-1 mutations in Plasmodium vivax isolates from Yunnan and Anhui provinces of China. Malaria journal 13, 346. [PubMed: 25179752]

Hupalo DN, Luo Z, Melnikov A, Sutton PL, Rogov P, Escalante A, Vallejo AF, Herrera S, Arevalo-Herrera M, Fan Q, Wang Y, Cui L, Lucas CM, Durand S, Sanchez JF, Baldeviano GC, Lescano AG, Laman M, Barnadas C, Barry A, Mueller I, Kazura JW, Eapen A, Kanagaraj D, Valecha N,

Ferreira MU, Roobsoong W, Nguitragool W, Sattabonkot J, Gamboa D, Kosek M, Vinetz JM, Gonzalez-Ceron L, Birren BW, Neafsey DE, Carlton JM, 2016 Population genomics studies identify signatures of global dispersal and drug resistance in Plasmodium vivax. Nature genetics 48, 953–958. [PubMed: 27348298]

Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay M, Newton PN, Kim JR, Nandy A, Osorio L, Carlton JM, White NJ, Day NP, Anderson TJ, 2007 Contrasting genetic structure in Plasmodium vivax populations from Asia and South America. International journal for parasitology 37, 1013–1022. [PubMed: 17442318]

Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, Nosten F, Anderson TJ, Dondorp A, Day NP, White NJ, 2008 Gene amplification of the multidrug resistance 1 gene of Plasmodium vivax isolates from Thailand, Laos, and Myanmar. Antimicrobial agents and chemotherapy 52, 2657–2659. [PubMed: 18443118]

Ketema T, Getahun K, Bacha K, 2011 Therapeutic efficacy of chloroquine for treatment of Plasmodium vivax malaria cases in Halaba district, South Ethiopia. Parasites & vectors 4, 46. [PubMed: 21453465]

Khim N, Andrianaranjaka V, Popovici J, Kim S, Ratsimbasoa A, Benedet C, Barnadas C, Durand R, Thellier M, Legrand E, Musset L, Menegon M, Severini C, Nour BY, Tichit M, Bouchier C, Mercereau-Puijalon O, Menard D, 2014 Effects of mefloquine use on Plasmodium vivax multidrug resistance. Emerging infectious diseases 20, 1637–1644. [PubMed: 25272023]

Kittichai V, Koepfli C, Nguitragool W, Sattabongkot J, Cui L, 2017 Substantial population structure of Plasmodium vivax in Thailand facilitates identification of the sources of residual transmission. PLoS neglected tropical diseases 11, e0005930. [PubMed: 29036178]

Koepfli C, Ross A, Kiniboro B, Smith TA, Zimmerman PA, Siba P, Mueller I, Felger I, 2011 Multiplicity and diversity of Plasmodium vivax infections in a highly endemic region in Papua New Guinea. PLoS neglected tropical diseases 5, e1424. [PubMed: 22206027]

Kumar S, Stecher G, Tamura K, 2016 MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular biology and evolution 33, 1870–1874. [PubMed: 27004904]

Librado P, Rozas J, 2009 DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. [PubMed: 19346325]

Lin JT, Patel JC, Kharabora O, Sattabongkot J, Muth S, Ubalee R, Schuster AL, Rogers WO, Wongsrichanalai C, Juliano JJ, 2013 Plasmodium vivax isolates from Cambodia and Thailand show high genetic complexity and distinct patterns of P. vivax multidrug resistance gene 1 (pvmdr1) polymorphisms. The American journal of tropical medicine and hygiene 88, 1116–1123. [PubMed: 23509126]

Liu H, Yang HL, Tang LH, Li XL, Huang F, Wang JZ, Li CF, Wang HY, Nie RH, Guo XR, Lin YX, Li M, Xu JW, 2014 Monitoring Plasmodium vivax chloroquine sensitivity along China-Myanmar border of Yunnan Province, China during 2008-2013. Malaria journal 13, 364. [PubMed: 25224069]

Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, Lee HW, Chen JH, Wang Y, Sattabongkot J, Han ET, 2011 Genetic polymorphism in pvmdr1 and pvcrt-o genes in relation to in vitro drug susceptibility of Plasmodium vivax isolates from malaria-endemic countries. Acta tropica 117, 69–75. [PubMed: 20933490]

Marfurt J, Mueller I, Sie A, Maku P, Goroti M, Reeder JC, Beck HP, Genton B, 2007 Low efficacy of amodiaquine or chloroquine plus sulfadoxine-pyrimethamine against Plasmodium falciparum and P. vivax malaria in Papua New Guinea. The American journal of tropical medicine and hygiene 77, 947–954. [PubMed: 17984359]

Marlar T, Myat Phone K, Aye Yu S, Khaing Khaing G, Ma S, Myint O, 1995 Development of resistance to chloroquine by Plasmodium vivax in Myanmar. Transactions of the Royal Society of Tropical Medicine and Hygiene 89, 307–308. [PubMed: 7660445]

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P, 2010 RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26, 2462–2463. [PubMed: 20798170]

Mekonnen SK, Aseffa A, Berhe N, Teklehaymanot T, Clouse RM, Gebru T, Medhin G, Velavan TP, 2014 Return of chloroquine-sensitive Plasmodium falciparum parasites and emergence of

chloroquine-resistant Plasmodium vivax in Ethiopia. Malaria journal 13, 244. [PubMed: 24964730]

Melo GC, Monteiro WM, Siqueira AM, Silva SR, Magalhaes BM, Alencar AC, Kuehn A, del Portillo HA, Fernandez-Becerra C, Lacerda MV, 2014 Expression levels of pvcrt-o and pvmdr-1 are associated with chloroquine resistance and severe Plasmodium vivax malaria in patients of the Brazilian Amazon. PloS one 9, e105922. [PubMed: 25157811]

Menard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, Rahim-Awab G, Barnadas C, Berry A, Boum Y, Bustos MD, Cao J, Chen JH, Collet L, Cui L, Thakur GD, Dieye A, Djalle D, Dorkenoo MA, Eboumbou-Moukoko CE, Espino FE, Fandeur T, Ferreira-da-Cruz MF, Fola AA, Fuehrer HP, Hassan AM, Herrera S, Hongvanthong B, Houze S, Ibrahim ML, Jahirul-Karim M, Jiang L, Kano S, Ali-Khan W, Khanthavong M, Kremsner PG, Lacerda M, Leang R, Leelawong M, Li M, Lin K, Mazarati JB, Menard S, Morlais I, Muhindo-Mavoko H, Musset L, Na-Bangchang K, Nambozi M, Niare K, Noedl H, Ouedraogo JB, Pillai DR, Pradines B, Quang-Phuc B, Ramharter M, Randrianarivelojosia M, Sattabongkot J, Sheikh-Omar A, Silue KD, Sirima SB, Sutherland C, Syafruddin D, Tahar R, Tang LH, Toure OA, Tshibangu-wa-Tshibangu P, Vigan-Womas I, Warsame M, Wini L, Zakeri S, Kim S, Eam R, Berne L, Khean C, Chy S, Ken M, Loch K, Canier L, Duru V, Legrand E, Barale JC, Stokes B, Straimer J, Witkowski B, Fidock DA, Rogier C, Ringwald P, Ariey F, Mercereau-Puijalon O, Consortium K, 2016 A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms. The New England journal of medicine 374, 2453–2464. [PubMed: 27332904]

Myat Phone K, Myint O, Myint L, Thaw Z, Kyin Hla A, Nwe Nwe Y, 1993 Emergence of chloroquine-resistant Plasmodium vivax in Myanmar (Burma). Transactions of the Royal Society of Tropical Medicine and Hygiene 87, 687. [PubMed: 8296378]

Orjuela-Sánchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alecrim Md HA, d.P., 2009 Analysis of single-nucleotide polymorphisms in the crt-o and mdr1 genes of Plasmodium vivax among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrobial agents and chemotherapy 53, 3561–3564. [PubMed: 19451296]

Orjuela-Sanchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alecrim M, del Portillo HA, 2009 Analysis of single-nucleotide polymorphisms in the crt-o and mdr1 genes of Plasmodium vivax among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrobial agents and chemotherapy 53, 3561–3564. [PubMed: 19451296]

Pearson RD, Amato R, Auburn S, Miotto O, Almagro-Garcia J, Amaratunga C, Suon S, Mao S, Noviyanti R, Trimarsanto H, Marfurt J, Anstey NM, William T, Boni MF, Dolecek C, Tran HT, White NJ, Michon P, Siba P, Tavul L, Harrison G, Barry A, Mueller I, Ferreira MU, Karunaweera N, Randrianarivelojosia M, Gao Q, Hubbart C, Hart L, Jeffery B, Drury E, Mead D, Kekre M, Campino S, Manske M, Cornelius VJ, MacInnis B, Rockett KA, Miles A, Rayner JC, Fairhurst RM, Nosten F, Price RN, Kwiatkowski DP, 2016 Genomic analysis of local variation and recent evolution in Plasmodium vivax. Nature genetics 48, 959–964. [PubMed: 27348299]

Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ, 2014 Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. The Lancet. Infectious diseases 14, 982–991. [PubMed: 25213732]

Pritchard JK, Stephens M, Donnelly P, 2000 Inference of population structure using multilocus genotype data. Genetics 155, 945–959. [PubMed: 10835412]

Ratcliff A, Siswantoro H, Kenangalem E, Wuwung M, Brockman A, Edstein MD, Laihad F, Ebsworth EP, Anstey NM, Tjitra E, Price RN, 2007 Therapeutic response of multidrug-resistant Plasmodium falciparum and P. vivax to chloroquine and sulfadoxine-pyrimethamine in southern Papua, Indonesia. Transactions of the Royal Society of Tropical Medicine and Hygiene 101, 351–359. [PubMed: 17028048]

Rijken MJ, Boel ME, Russell B, Imwong M, Leimanis ML, Phyo AP, Muehlenbachs A, Lindegardh N, McGready R, Renia L, Snounou G, Singhasivanon P, Nosten F, 2011 Chloroquine resistant vivax malaria in a pregnant woman on the western border of Thailand. Malaria journal 10, 113. [PubMed: 21545737]

Rungsihirunrat K, Muhamad P, Chaijaroenkul W, Kuesap J, Na-Bangchang K, 2015 Plasmodium vivax drug resistance genes; Pvmdr1 and Pvcrt-o polymorphisms in relation to chloroquine sensitivity

from a malaria endemic area of Thailand. The Korean journal of parasitology 53, 43–49. [PubMed: 25748708]

Schousboe ML, Ranjitkar S, Rajakaruna RS, Amerasinghe PH, Morales F, Pearce R, Ord R, Leslie T, Rowland M, Gadalla NB, Konradsen F, Bygbjerg IC, Roper C, Alifrangis M, 2015 Multiple Origins of Mutations in the mdr1 Gene--A Putative Marker of Chloroquine Resistance in P. vivax. PLoS neglected tropical diseases 9, e0004196. [PubMed: 26539821]

Schuurkamp GJ, Spicer PE, Kereu RK, Bulungol PK, Rieckmann KH, 1992 Chloroquine-resistant Plasmodium vivax in Papua New Guinea. Transactions of the Royal Society of Tropical Medicine and Hygiene 86, 121–122. [PubMed: 1440763]

Shalini S, Chaudhuri S, Sutton PL, Mishra N, Srivastava N, David JK, Ravindran KJ, Carlton JM, Eapen A, 2014 Chloroquine efficacy studies confirm drug susceptibility of Plasmodium vivax in Chennai, India. Malaria journal 13, 129. [PubMed: 24685286]

Singh RK, 2000 Emergence of chloroquine-resistant vivax malaria in south Bihar (India). Transactions of the Royal Society of Tropical Medicine and Hygiene 94, 327. [PubMed: 10975013]

Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN, 1993 High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and biochemical parasitology 61, 315–320. [PubMed: 8264734]

Sriwichai P, Samung Y, Sumruayphol S, Kiattibutr K, Kumpitak C, Payakkapol A, Kaewkungwal J, Yan G, Cui L, Sattabongkot J, 2016 Natural human Plasmodium infections in major Anopheles mosquitoes in western Thailand. Parasites & vectors 9, 17. [PubMed: 26762512]

Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, Prasetyorini B, Kenangalem E, Piera KA, Lek-Uthai U, Anstey NM, Tjitra E, Nosten F, Cheng Q, Price RN, 2008 Amplification of pvmdr1 associated with multidrug-resistant Plasmodium vivax. The Journal of infectious diseases 198, 1558–1564. [PubMed: 18808339]

Talundzic E, Okoth SA, Congpuong K, Plucinski MM, Morton L, Goldman IF, Kachur PS, Wongsrichanalai C, Satimai W, Barnwell JW, Udhayakumar V, 2015 Selection and spread of artemisinin-resistant alleles in Thailand prior to the global artemisinin resistance containment campaign. PLoS pathogens 11, e1004789. [PubMed: 25836766]

Thanh PV, Hong NV, Van NV, Louisa M, Baird K, Xa NX, Peeters Grietens K, Hung le X, Duong TT, Rosanas-Urgell A, Speybroeck N, D'Alessandro U, Erhart A, 2015 Confirmed Plasmodium vivax Resistance to Chloroquine in Central Vietnam. Antimicrobial agents and chemotherapy 59, 7411–7419. [PubMed: 26392501]

Tjitra E, Anstey NM, Sugiarto P, Warikar N, Kenangalem E, Karyana M, Lampah DA, Price RN, 2008 Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia. PLoS medicine 5, e128. [PubMed: 18563962]

Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnadig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA, 2016 Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nature communications 7, 11553.

Yuan L, Wang Y, Parker DM, Gupta B, Yang Z, Liu H, Fan Q, Cao Y, Xiao Y, Lee MC, Zhou G, Yan G, Baird JK, Cui L, 2015 Therapeutic responses of Plasmodium vivax malaria to chloroquine and primaquine treatment in northeastern Myanmar. Antimicrobial agents and chemotherapy 59, 1230–1235. [PubMed: 25512415]

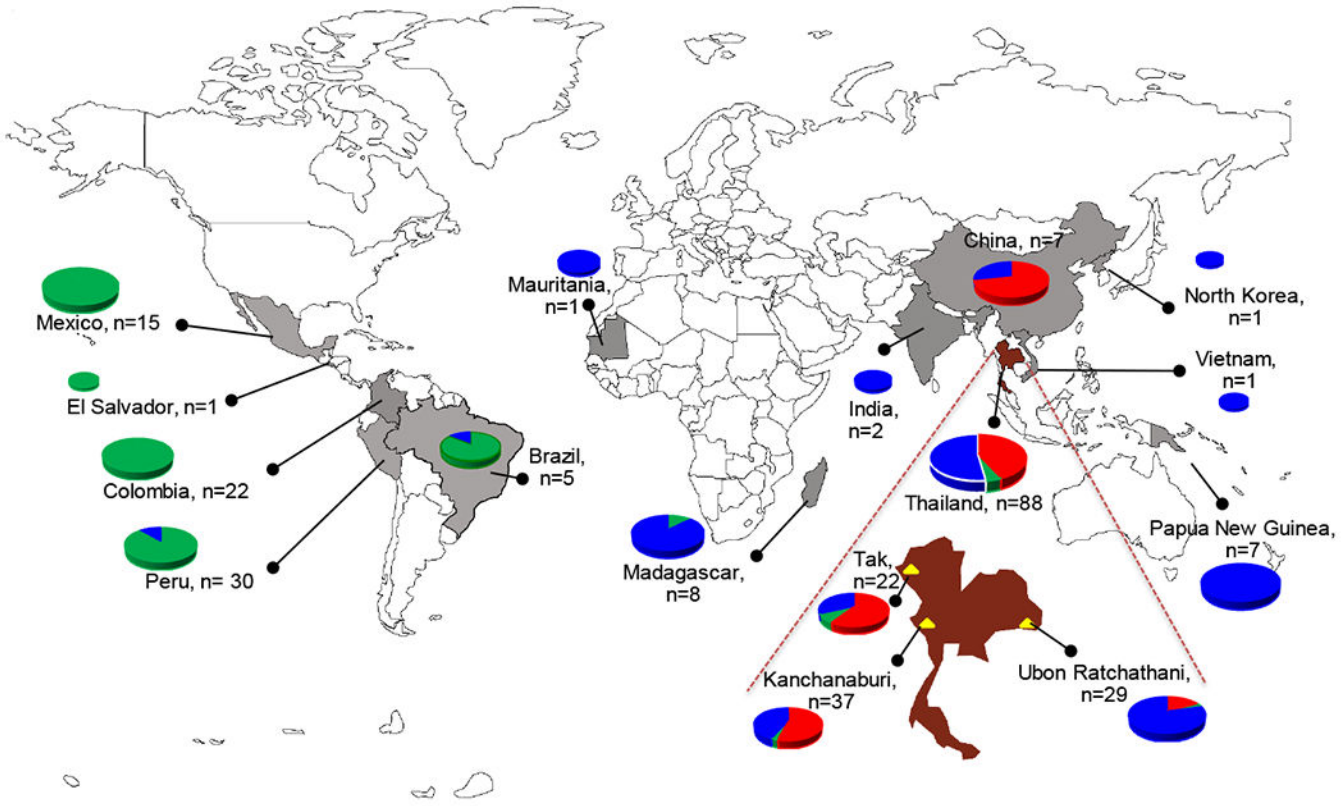

Fig. 1. Map indicating locations of the sampling sites for parasite isolates from three provinces in Thailand and other regions worldwide.

In addition to the origin of the samples, the distribution of the three clusters of *Pvmdr1* haplotypes in each country or region based on the STRUCTURE plot (Blue, Green and Red) in Fig. 3 is shown as pie charts. The world map was modified from the one available on: www.freeworldmap.net.

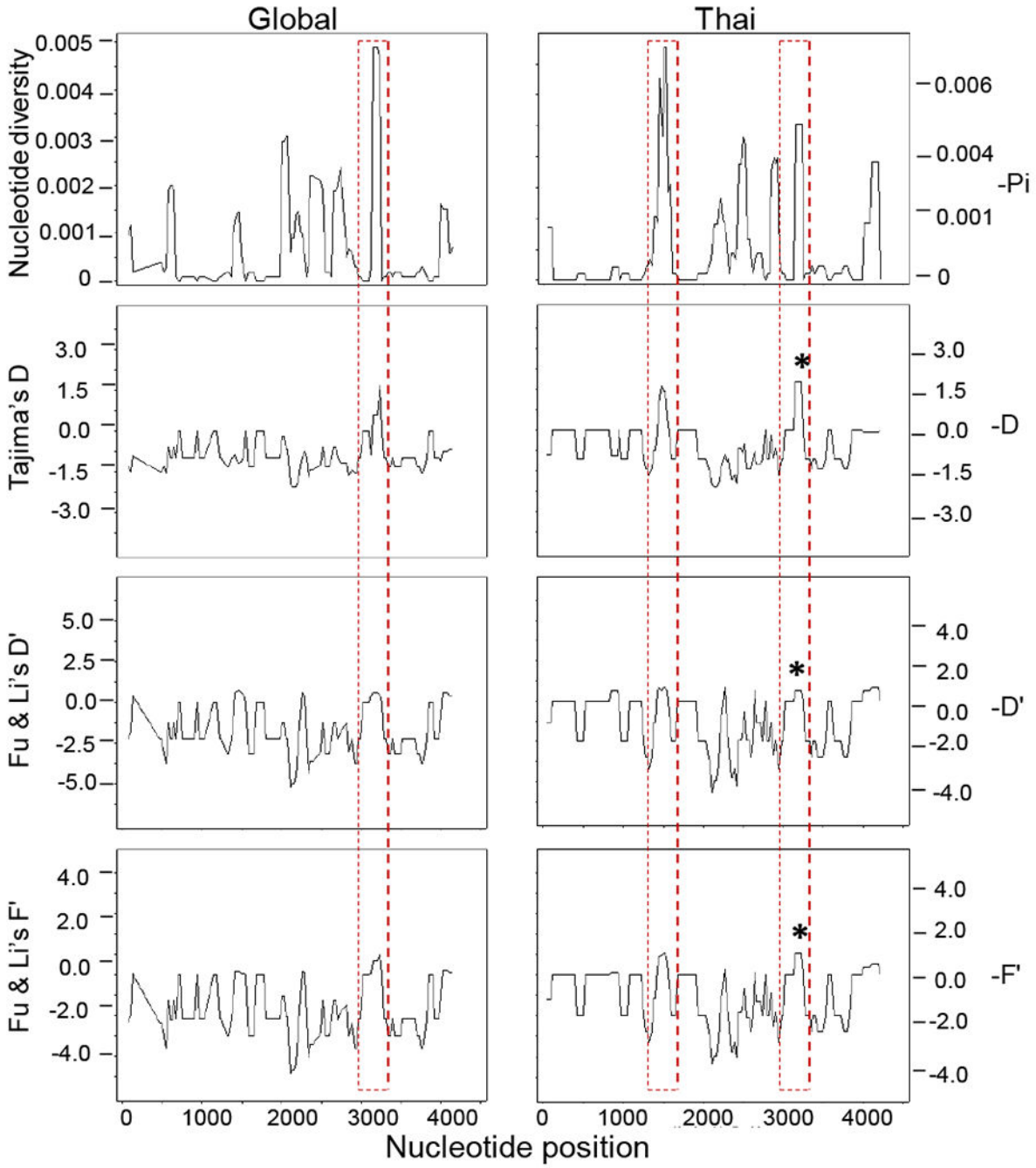

Fig. 2. Sliding window plots of nucleotide diversity and results of the neutrality tests from the global (left) and Thai (right) *Pvmdr1* sequences.

Peaks of diversity consistently shown in all analyses suggestive of balancing selection or population decline, thus, an excess of alleles at intermediate frequencies are marked by red dotted rectangles and correspond to positive values. The star plotted above the peaks depicts the statistical significance of the test at the level 0.05. Sliding window analysis used a window size of 100 bp and a step size of 10 bp. Nucleotide positions correspond to those of Sal I sequence.

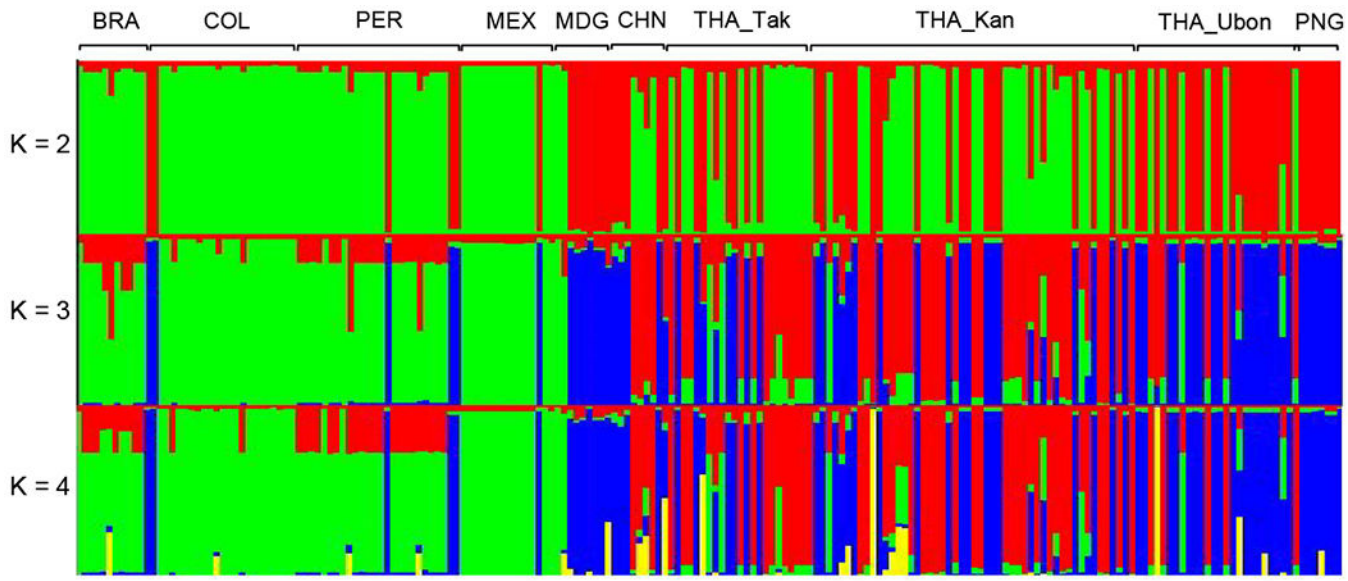

Fig. 3. Structure analysis of the global *Pvmdr1* SNPs by the admixture model.

Plots representative of the genetic structure of *Pvmdr1* sequences in different countries and regions at K = 2, 3, and 4 respectively. Country codes include of THA_Tak: Tak, THA_Kan: Kanchanaburi, THA_Ubon: Ubon Ratchathani, MEX: Mexico, COL: Colombia, PER: Peru, BRA: Brazil, MDG: Madagascar, CHN: China, and PNG: Papua New Guinea.

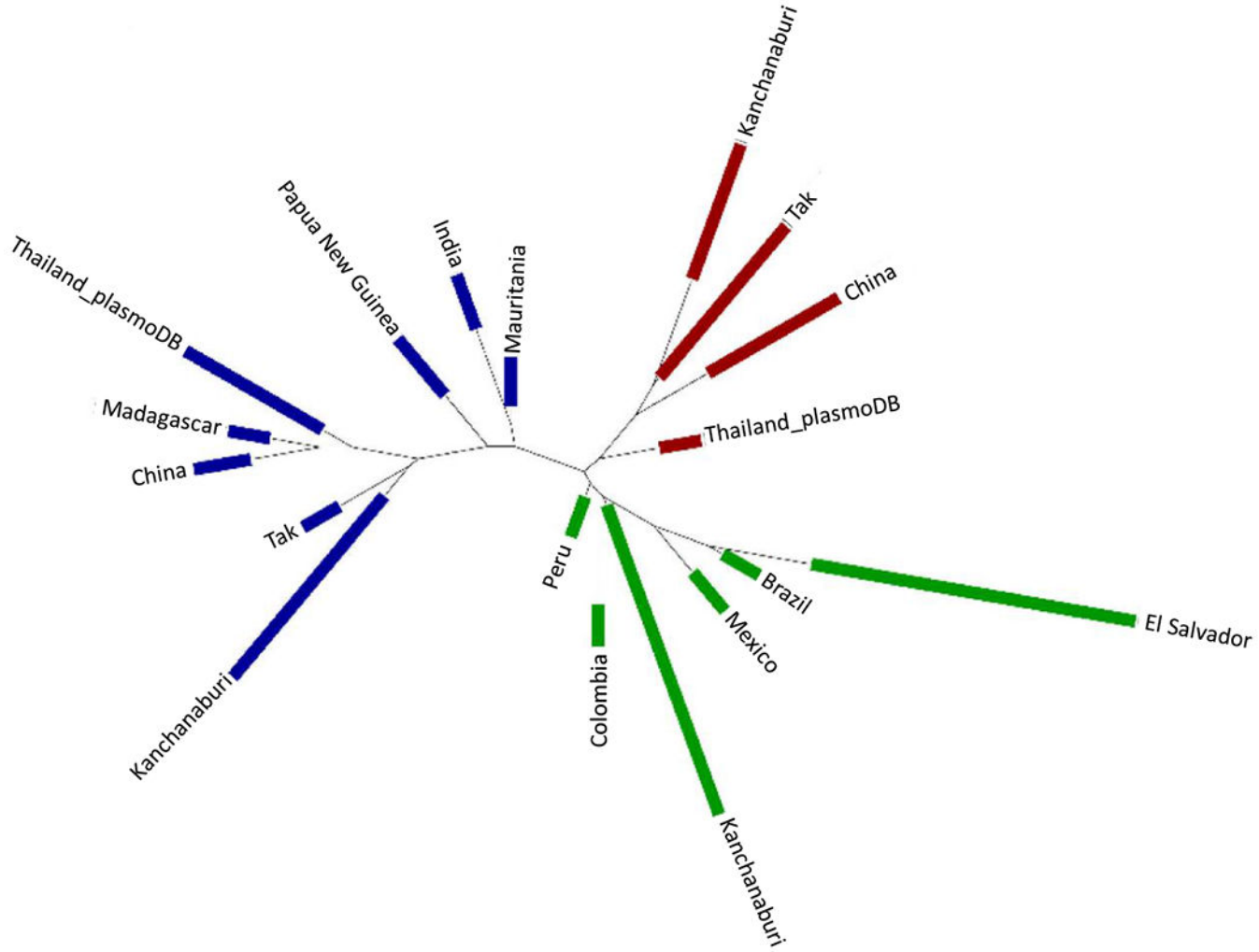

Fig. 4. Phylogenetic analysis of *Pvmdr1* gene in worldwide isolates.

The distribution of the three clusters of *Pvmdr1* haplotypes based on the Neighbor-Joining phylogenetic tree is shown. Clusters 1, 2 and 3 are colored in red, green and blue, respectively.

Table 1

SNPs identified in the three *P. vivax* populations in Thailand and their prevalence (%).

SNPs	Amino acids*	Tak (N=22)	Kanchanaburi (N=37)	Ubon Rachathani (N=29)	Total (N=88)
ACC-126-ACT	(T42T)			3.45	1.14
AAG-132-AAA	(K44K)	4.55	5.41	13.79	7.95
GGC-516-GGT	(G172G)		5.41		2.27
AAC-936-AAT	(N312N)			6.90	2.27
ACG-1065-ACA	(T355T)	4.55			1.14
AAA-1367-ACA	(K456**I**)		2.70		1.14
CTT-1409-CAT	(L470**H**)	4.55			1.14
CTA-1477-TTA	(L493L)	13.64	2.70	17.24	10.23
AGT-1539-AGA	S513**R**	63.64	37.84	17.24	37.50
ACA-1587-ACG	T529T	86.36	97.30	68.97	85.23
AAG-2016-AAC	(K672**N**)		2.70		1.14
AAT-2218-GAT	(N740**D**)		2.70		1.14
CGG-2266-AGG	(R756R)	4.55	8.11		4.55
GCG-2288-GTG	(A763**V**)	4.55	8.11		4.55
CCA-2424-CCG	(P808P)		2.70	3.45	2.27
CTC-2533-TTC	(L845**F**)		2.70	58.62	20.45
GCG-2582-GAG	(A861**E**)		10.81		4.55
ATG-2722-CTG	M908**L**	95.45	97.30	96.55	96.59
AGC-2739-AGT	S913S		2.70		1.14
TTA-2808-TTT	(L936**F**)	4.55			1.14
ACG-2873-ATG	T958**M**	100.00	100.00	100.00	100.00
TAC-2927-TTC	Y976**F**	9.09	27.03	20.69	20.45
TTT-2936-TCT	F979**S**		2.70		1.14
ATG-2938-GTG	M980**V**		2.70		1.14
GAG-2986-CAG	(E996**Q**)		2.70		1.14
TTT-3226-CTT	F1076**L**	31.82	40.54	27.59	34.09
ATC-3414-ATT	(I1138I)			3.45	1.14
CCA-3529-ACA	(P1177**T**)	4.55			1.14
GGT-3694-TGT	(G1232**C**)			3.45	1.14
GGG-3793-TGG	(G1265**W**)			3.45	1.14
AGT-3822-AGG	(S1274**R**)		2.70		1.14
TCC-4074-TCT	S1358S	27.27	10.81		11.36
AAG-4179-AAC	(K1393**N**)	9.09	29.73		14.77
GAG-4188-GAA	(E1396E)	9.09	10.81		6.82

*New mutations identified in this study are in parenthesis, and non-synonymous mutations are in bold.

Table 2

Sequence polymorphisms and summary statistics of the near full-length *Pvmdr1* gene in different populations*.

Population	No. isolates	Polymorphic sites	Singletons	Amino acid changes*	Syn	Non-syn	π ± SD	Haplotypes	
								H	Hd ± SD
Thailand (this study)	88	34	19	15	13	21	0.00085 ± 0.00005	48	0.961 ± 0.011
Tak	22	15	7	9	6	9	0.00073 ± 0.00008	17	0.961 ± 0.029
Kanchanaburi	37	26	14	12	10	16	0.00090 ± 0.00007	27	0.961 ± 0.022
Ubon Ratchathani	30	14	6	8	7	7	0.00072 ± 0.00083	16	0.927 ± 0.025
Thailand (plasmodb)	13	5	4	1	1	4	0.00025 ± 0.00007	5	0.692 ± 0.110
China	7	10	8	2	3	7	0.00075 ± 0.00018	6	0.952 ± 0.096
PNG	7	1	0	1	0	1	0.00014 ± 0.0003	2	0.571 ± 0.110
India	2	4	4	0	3	1	0.00091 ± 0.00046	2	1.000 ± 0.500
Madagascar	8	14	10	4	4	10	0.00110 ± 0.00023	8	1.000 ± 0.063
Mexico	15	7	6	1	3	4	0.00024 ± 0.00013	4	0.371 ± 0.153
Peru	30	8	4	4	2	6	0.00032 ± 0.00007	7	0.692 ± 0.057
Brazil	5	4	4	0	2	2	0.00038 ± 0.00011	4	0.900 ± 0.161
Colombia	22	63	59	4	24	39	0.00161 ± 0.00108	7	0.814 ± 0.049
Other	4	9	8	1	2	7	0.00111 ± 0.00026	4	1.000 ± 0.177
Global	**201**	**46**	**30**	**16**	**14**	**30**	**0.00069 ± 0.00004**	**53**	**0.907± 0.013**

*For amino acid changes, only the parsimony-informative changes are included here. Syn, synonymous changes; non-syn, nonsynonymous changes; H, number of haplotypes; Hd, haplotype diversity; SD, standard deviation.

Table 3

Haplotypes of the near full-length *Pvmdr1* gene from global *P. vivax* isolates.

2° Structure		TM4	NBD1		TM7>	TM8	TM9	TM10	TM11	NBD2		
Haplotype	**Cluster**	**V221L**	**S513R**	**G698S**	**L845F**	**M908L**	**T958M**	**Y976F**	**F1076L**	**K1393N**	**N**	**Frequency (%)**
Hap_1	Green	V	S	G	L	L	M	Y	F	K	37	18.41
Hap_2	Blue	V	R	G	L	L	M	Y	F	K	23	11.44
Hap_3	Green	L	S	G	L	L	M	Y	F	K	20	9.95
Hap_4	Blue	V	S	G	L	L	M	F	L	K	17	8.46
Hap_5	Blue	V	S	G	F	L	M	Y	L	K	19	9.45
Hap_6	Green	V	S	G	L	M	M	Y	F	K	15	7.46
Hap_7	Blue	V	S	G	L	L	M	Y	L	K	7	3.48
Hap_8	Blue	V	S	S	L	L	M	Y	L	K	9	4.48
Hap_9	Blue	V	S	S	L	L	M	F	L	K	6	2.99
Hap_10	Red	V	S	G	L	L	M	Y	F	N	5	2.49
Hap_11	Red	V	R	G	L	L	M	Y	F	N	5	2.49
Hap_12	Red	V	R	S	L	L	M	Y	F	K	4	1.99
Hap_13	Blue	V	R	S	L	L	M	F	L	K	4	1.99
Hap_14	Red	V	S	S	L	L	M	Y	F	N	3	1.49
Hap_15	Red	V	S	S	L	L	M	Y	F	K	3	1.49
Hap_16	Green	V	S	G	L	M	T	Y	F	K	3	1.49
Hap_17	Red	V	R	G	L	M	M	Y	F	K	3	1.49
Hap_18	Blue	V	R	G	L	L	M	F	L	K	3	1.49
Hap_19	Red	V	R	S	L	L	M	Y	F	N	2	1.00
Hap_20	Blue	V	S	G	L	L	M	Y	L	N	2	1.00
Hap_21	Blue	V	S	G	F	L	M	Y	F	K	2	1.00
Hap_22	Blue	V	-	S	L	L	M	F	L	K	1	0.50
Hap_23	Green	V	S	G	L	L	T	Y	F	K	1	0.50
Hap_24	Blue	V	-	G	L	L	M	Y	L	K	1	0.50
Hap_25	Blue	V	S	S	F	L	M	Y	L	K	1	0.50
Hap_26	Green	V	S	G	L	L	M	-	F	K	1	0.50
Hap_27	Red	V	R	S	L	L	M	Y	F	-	1	0.50
Hap_28	Red	V	-	G	L	L	M	Y	F	K	1	0.50
Hap_29	Red	V	R	G	L	L	M	F	F	N	1	0.50
Hap_30	Blue	V	S	G	L	L	M	F	F	K	1	0.50
Allele frequency (≥ 5%)		**9.95**	**22.89**	**17.41**	**9.45**	**89.55**	**98.01**	**16.42**	**34.83**	**8.96**	**201**	

Hap_16 is Sal I. The assignment of each haplotype to one of the three clusters (colored as blue, green and red) is indicated.

Table 4

Neutrality and recombination tests of the near full-length *Pvmdr1* gene in different populations.

Population	No. isolates	Neutrality tests			Rm rates		Rm events
		Fu & Li's F	Fu & Li's D	Tajima's D	Z test	ρ/θ ratios	
Thailand (this study)	88	−3.290 **	−3.548 **	−1.486	−1.464	3.301	6
Tak	22	−1-114	−0.950	−0.973	−2.624	29.927	3
Kanchanaburi	36	−2.153	−2.057	−1.442	−0.755	3.914	3
Ubon Ratchathani	30	−0.962	−1.000	−0.426	0.155 *	1362.087	5
Thailand (plasmodb)	13	−1.704	−1.610	−1.182	−1610	52.094	0
China	7	−1.168	−1.053	−1.109	−0.653	0.080	0
PNG	7	1.101	−0.953	1.342	1.034	na	0
India	2	na	na	na	−1.562	na	0
Madagascar	8	−1.056	−0.961	−0.916	−0.077	2.132	1
Mexico	15	−2.345	−2.120	−1.849 *	−1.187	0.059	0
Peru	30	−1.381	−1.211	−1.118	−1.565	43.900	1
Brazil	5	−1.113	−1.094	−1.094	−0.542	3386.638	0
Colombia	22	−4.107 **	−3.997 **	−2.412 #	−1.931	3.230	2
Others	4	−0.488	−0.492	−0.492	0.022 *	na	0
Global	**201**	**−5.474** **	**−6.403** **	**−2.146** **	**−1.298**	**1.756**	6

# $p < 0.01$

** $p < 0.02$

* $p < 0.05$

ρ estimated recombination rate; Rm; recombination

θ estimated mutation rate; na, not analyzed; Other: El Salvador, Mauritania, North Korea, and Vietnam

Infect Genet Evol. Author manuscript; available in PMC 2019 March 15.

Table 5

Genetic differentiation of parasite populations based on *Pvmdr1* gene in the global isolates.

	Thailand[1]	**Peru**	**India**	**Brazil**	**China**	**Colombia**	**Mexico**	**PNG**	**Thailand[2]**	**Madagascar**
Thailand[1]	-	0.00002	NA	0.00236	0.00018	0.00002	0.00002	0.00002	0.00002	NA
Peru	0.1535***	-	NA	0.01042	0.00002	0.00002	0.00002	0.00002	0.00002	NA
India	0.0982	0.4185	-	NA	NA	NA	NA	NA	NA	NA
Brazil	0.1968^{NS}	0.2490^{NS}	0.4683	-	0.00220	0.00009	0.00027	0.00116	0.00033	NA
China	0.2018**	0.4259***	0.0906	0.3940^{NS}	-	0.00002	0.00004	0.00798	0.64489	NA
Colombia	0.2857***	0.2544***	0.5544	0.4291**	0.5217***	-	0.00002	0.00002	0.00002	NA
Mexico	0.2568***	0.3875***	0.6481	0.5508*	0.5514**	0.5127***	-	0.00002	0.00002	NA
PNG	0.2761***	0.5934***	0.4426	0.7385^{NS}	0.2699^{NS}	0.6831***	0.7564***	-	0.01720	NA
Thailand[2]	0.1983***	0.3982***	0.2923	0.4255*	−0.0301^{NS}	0.5103***	0.5376***	0.2736^{NS}	-	NA
Madagascar	0.2051	0.4976	0.0644	0.4335	0.1568	0.5658	0.5469	0.1662	0.2360	-

[#]Country-wise comparison included Fst (below diagonal) and p-value (above diagonal). Thailand[1] and Thailand[2] are from this study and PlasmoDB, respectively. NS, not significant; NA, not analyzed.

Table 6

Linkage disequilibrium (LD) of the near full-length *Pvmdr1* gene in the global isolates.

Origin	I_A^S	*p* value	*LD*
Thailand (this study)	0.0043	9.16×10^{-03}	Significant
Ubon Ratchathani	0.0072	9.32×10^{-03}	Significant
Tak	0.0006	3.94×10^{-01}	NS
Kanchanaburi	0.0044	5.61×10^{-02}	NS
Thailand (plasmodb)	−0.0015	6.20×10^{-01}	NS
China	0.0205	2.83×10^{-02}	Significant
India	NA	1.00×10^{-05}	Significant
Madagascar	0.0302	3.03×10^{-03}	Significant
PNG	0.0008	1.00e + 00	NS
Mexico	0.0443	3.00×10^{-05}	Significant
Columbia	0.0128	4.20×10^{-04}	Significant
Peru	0.0349	$<1.00 \times 10^{-03}$	Significant
Brazil	−0.0005	8.08×10^{-01}	NS

*Significant LD at $p < 0.05$ based on 100,000 Monte Carlo Markov chain re-samplings; NA, not analyzed because of the small sample size; NS, not significant.