Limited Polymorphism of the \textit{Plasmodium vivax} Merozoite Surface Protein 1 Gene in Isolates from Turkey

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Abstract. The 200-kD merozoite surface protein of \textit{Plasmodium vivax} (PvMSP-1) is one of the leading vaccine candidates against \textit{P. vivax} malaria. However, the gene encoding PvMSP-1 (pvmsp1) is highly polymorphic and is a major obstacle to effective vaccine development. To further understand polymorphism in pvmsp1, we obtained 30 full-length pvmsp1 sequences from southeastern Turkey. Comparative analysis of sequences from Turkey and other areas showed substantially limited polymorphism. Substitutions were found at 280 and 162 amino acid sites in samples from other regions and those from Turkey, respectively. Eight substitutions were unique to Turkey. In one of them, D/E at position 1706 in the C-terminal 19-kD region, the K/E change at 1709 was the only polymorphism previously known. Limited diversity was also observed in microsatellites. Data suggest a recent population bottleneck in Turkey that may have obscured a signature for balancing selection in the C-terminal 42-kD region, which was otherwise detectable in other areas.

INTRODUCTION

Malaria imposes a huge public health burden in tropical and subtropical countries with one million deaths every year.\(^1\) Of the five human malaria parasites, \textit{Plasmodium vivax} is the most prevalent in Asia, Melanesia, the Middle East, South and Central America, accounting for 70–80 million cases annually.\(^2\) Although \textit{P. vivax} is often regarded as a benign and self-limiting infection, it can lead to debilitating illness and remains a major cause of morbidity in malaria-endemic countries. Emergence of drug-resistant \textit{P. vivax} makes the control of \textit{P. vivax} malaria more difficult.\(^3\) Thus, there is an increasing demand for developing effective vaccines against \textit{P. vivax} malaria with potential targets directed against the asexual blood stages, which are responsible for clinical manifestations of the disease.\(^4\) The 200-kD merozoite surface protein 1 (MSP-1), which is abundantly expressed on the surface of merozoites, is one of the leading asexual blood stage vaccine candidates.\(^5\) MSP-1 is conserved in all \textit{Plasmodium} species\(^6\) and is essential for parasite survival.\(^7\)

\textit{Plasmodium falciparum} MSP-1 undergoes proteolytic processing, producing four major polypeptides of 83-kD, 30-kD, 38-kD, and 42-kD.\(^8\) Coincident with erythrocyte invasion, the C-terminal 42-kD protein is further cleaved to produce the N-terminal 33-kD and C-terminal 19-kD fragments, with all, except one, processed fragments shed.\(^8\) The C-terminal 19-kD fragment, which contains cysteine-rich epidermal growth factor–like domains, remains anchored to the merozoite membrane and is carried into the invaded erythrocytes. Both the 42-kD and 19-kD polypeptides are considered to be promising vaccine candidates for \textit{P. falciparum} and \textit{P. vivax}.\(^9\) However, undoubtedly, the gene encoding MSP-1 (mspl) is highly polymorphic,\(^10-11\) and, thus, presents a major obstacle to effective vaccine development. \textit{Plasmodium vivax} mspl (pvmsp1) shows extensive allelic variation and is subject to balancing selection,\(^4,12\) suggesting an involvement of parasite evasion from host immune attack.

According to inter-allelic sequence variation, pvmsp1 contains six highly polymorphic regions interspersed with conserved blocks.\(^13\) Of note is the limited polymorphism in the C-terminal 19-kD in pvmsp1 with only one amino acid substitution, K/E at 1709,\(^14\) in contrast to five major amino acid substitutions in \textit{P. falciparum} mspl.\(^11\) With its polymorphic nature, pvmsp1, particularly the highly polymorphic poly Q region in block 6,\(^13\) previously referred to as conserved block (CB) 5,\(^13\) has frequently been used as a molecular marker to monitor genetic diversity of \textit{P. vivax} in different populations.\(^16-20\) However, investigations on polymorphism of the whole pvmsp1 have until now been limited.\(^15\)

In this study, we report polymorphism of pvmsp1 from isolates in Sanliurfa, southeastern Turkey, where malaria has long been one of the most common infectious diseases and \textit{P. vivax} has continuously been identified as the only \textit{Plasmodium} species. Our previous study has identified a high prevalence of persons with naturally acquired antibodies to PvMSP1 in southeastern Turkey.\(^24\) We obtained 30 full-length pvmsp1 sequences from the same study area. Comparative analysis of polymorphism in \textit{P. vivax} populations from Sanliurfa and other areas showed substantially limited pvmsp1 polymorphism in parasite populations in Turkey with some polymorphism unique to this country.

MATERIALS AND METHODS

Parasite isolates and DNA extraction. \textit{Plasmodium vivax} isolates were obtained in two towns, Siverek and Harran, in Sanliurfa Province, southeastern Turkey (Figure 1), where malaria persists throughout the year with high rates during July–November.\(^24\) According to the World Health Organization, in 2006 \textit{P. vivax} transmission was reported in seven provinces of Turkey and 84% of the cases were from Diyarbakir and Sanliurfa.\(^1\) In the past decade, malaria incidence rapidly decreased in this area because of government malaria control efforts that used chloroquine and primaquine.\(^1\) Turkey shows a strong political commitment to the Tashkent Declaration, endorsed in 2005, and malaria surveillance activities have

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been intensified throughout the country, and priority has been
given to provinces in southeastern Anatolia.

A total of 31 blood samples were obtained from patients
diagnosed microscopically with \textit{P. vivax} infection at several
National Malaria Control Centers within Siverek and Harran:
20 from Siverek during July–December in 2007 and 11 from
Harran during September–November in 2008. Although these
samples were limited in number (\(n = 31\)), they include 32%
of total \textit{P. vivax} cases (\(n = 96\)) in the study area: 47 in 2007
and 49 in 2008 (Figure 2). Mean age of patients was 21 years
(range = 2–55 years) and 61% were male. Giemsa-stained
thick blood smears were used to calculate parasitemia (para-
sites/microliter of blood) as described.\textsuperscript{25} Mean \(\pm\) SD parasite
density was 5,057 \(\pm\) 757 parasites/\(\mu\)L (range = 480–14,720 par-
asites/\(\mu\)L). The patients were selected randomly from differ-
ent ethnic and racial groups. All samples were collected after
informed consent was obtained from patients or their par-
ents. Sampling authorization was obtained from the Turkish
Ministry of Health Sanliurfa Bureau, and ethical approval was
obtained from the Research Institute for Microbial Diseases,
Osaka University.

An aliquot of venous blood (100–200 \(\mu\)L) was taken by
finger prick, spotted onto Whatman\textsuperscript{31} ETCHR filter paper
(Whatman, Piscataway, NJ), and air-dried. DNA was extracted
from filter blots using the EZ1 BioRobot\textsuperscript{TM} (Qiagen, Hilden,
Germany) according to the manufacturer’s instructions.

**Sequencing.** Full-length \textit{pvmsp1} (5.2–5.3 kb) was amplified
by polymerase chain reaction (PCR) using Takara LA Taq
(Takara Bio, Otsu, Japan) in a 20-\(\mu\)L reaction mixture as
described\textsuperscript{26} with primers PVF0 and PVR0.\textsuperscript{13} Forty cycles of
amplification (20 seconds at 93°C and 5 minutes at 62°C)
were preceded by denaturation at 93°C for 1 minute and
followed by final elongation at 72°C for 10 minutes. The
PCR product was diluted 10-fold, and a 2-\(\mu\)L aliquot was
used as template for a second PCR amplification of 20
cycles in a 50-\(\mu\)L reaction mixture using primers PVF0-2
(5′-CGTACATCTTTAAACCCCACACT-3′) and PVR0.
The PCR products were purified by using QIAquick (PCR
Product Purification kit; Qiagen). DNA sequencing was
performed directly from two independent PCR products, using
the BigDye\textsuperscript{®} Terminator v3.1 Cycle Sequencing Kit (Applied
Biosystems, Foster City, CA) and an ABI 3130 Genetic Analyzer
(Applied Biosystems). Sequencing primers were designed to
cover specific regions in both directions as described.\textsuperscript{13} Four
microsatellite loci(MS8, MS9, MS15, and 3.502)\textsuperscript{27,28} were also
sequenced after PCR amplification by using specific primers
shown in Supplementary Table 1. The PCR conditions were
the same as for \textit{pvmsp1} and a second amplification was not
performed. Contiguous sequences were constructed by using
ATGC version 4.01 (Genetyx Corp., Tokyo, Japan). Mixed
genotype infections judged from overlapping peak at given
positions in an electrophelogram were excluded from further
analysis. Sequences obtained in this study have been deposited
to DNA Database of Japan/European Molecular Biology/
GenBank under accession numbers AB564559–AB564588.

**Statistical analysis.** The \textit{pvmsp1} sequences obtained in this
study were analyzed with previously published full-length
sequences (n = 43) from Thailand (n = 20), Brazil (n = 9), South Korea (n = 4), India (n = 1), El Salvador (n = 1), Bangladesh (n = 5), Vanuatu (n = 2), and Côte d’Ivoire (n = 1) (GenBank accession numbers AF435593–AF435599, AF435601–AF435620, AF435622–AF435625, AF435627, AF435629–AF435632, AF435634–AF435639, and DQ220742) (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Between-population comparison of full-length *pvmsp1* was made by using samples from Turkey, Thailand and Brazil. Isolates from Thailand were obtained from Tak Province in 1997–1998, and isolates from Brazil were obtained in Rondonia in 1995 and 1997.15 Partial sequences of the C-terminal 42-kD region from isolates from India (n = 28)19 were obtained from GenBank. Partial *pvmsp1* sequences were also obtained from Azerbaijan (n = 36),20 Iran (n = 191),21,22,30 Afghanistan (n = 57),21,22,30 Pakistan (n = 33),30 Myanmar (n = 135),31 Thailand (n = 33),31 China (n = 33),31 and Brazil (n = 78)46 to analyze polymorphism in the tandem Gln (Q) repeat region in block 620 or CB5 previously defined.15 Frequency distribution of poly Q haplotypes was made for six countries, from which > 20 sequences were available. Sequences were aligned by using CLUSTAL W45 implemented in MEGA software version 439 with manual corrections.

Sequence polymorphism was estimated by using the S, the number of polymorphic nucleotide sites; the number of singleton polymorphic sites; the number of polymorphic amino acid sites; the number of haplotypes and haplotype diversity $\theta_h$, the observed average number of pairwise nucleotide difference per site; and $\theta_S$, the standardized number of polymorphic nucleotide sites (S) per site in the sample expected under neutrality.36 Tajima’s D statistic was estimated for testing departure from neutrality with focus on allele frequency spectrum.37 We used Tajima’s D, which compares $\theta_S$ and $\theta_S$. Under neutrality, the value is expected to be 0; significantly positive values suggest recent population bottlenecks or balancing selection, and negative values suggest population growth or directional selection. We also used Fu and Li’s D* and F* tests to test for excess or lack of singleton nucleotides by comparing estimates of $\theta_S$ based on the number of singletons versus that derived from S (the D* index) or $\theta_S$ (the F* index).38 An excess of intermediate-frequency polymorphisms or a lack of rare variants (including singleton nucleotides) results in positive values for D* and F*. All estimates were calculated by using DnaSP software version 4.1049 and MEGA software.

The mean numbers of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) were estimated by using the Nei and Gojobori method40 with the Jukes and Cantor correction as implemented in MEGA. Standard error was determined by 1,000 bootstrap replications, and dN and dS were compared by using a Z-test of selection implemented in MEGA. If dN was significantly greater than dS, balancing selection appears to be acting. If dS was greater than dN, purifying selection is predicted. The McDonald-Kreitman test41 was also used to assess a signature for selection, in which the ratio of nonsynonymous and synonymous substitutions was compared between polymorphic (within species) and fixed difference (between closely related species) by using DnaSP. Under neutrality these ratios will be similar, whereas an excess of intraspecific nonsynonymous polymorphisms is suggestive of balancing selection. Ten *mspl* sequences from *P. cynomolgi,*32 a monkey malaria parasite closely related to *P. vivax,* were used for between-species comparison. Fisher’s exact test was used to test for statistical significance. Microsatellite haplotypes (alleles) were determined by counting the number of microsatellite repeats.

RESULTS

**Polymorphism of *pvmsp1* in isolates from Turkey.** The PCR amplification of the whole *pvmsp1* was successful for 31 *P. vivax* isolates, and 30 full-length sequences were obtained: n = 20 from Siverek and n = 10 from Harran. One isolate from Harran had mixed genotype infection and was not used for further analysis. An alignment of the deduced amino acid sequence of *pvmsp1* from Turkey and other areas (n = 73) showed a number of sequence regions with insertions/deletions and/or tandem repeats of degenerative repeat units, which were scattered throughout the gene (Supplementary Figure 1). Because these sequence regions were not reliably aligned, they were excluded from further analysis, except for a region containing poly Q tandem repeats in block 6, which was used for repeat number polymorphism analysis (see below).

In the sequence region analyzed (4,728 basepairs), there were 543 and 299 polymorphic nucleotide sites in worldwide (n = 73) and Turkey samples, respectively (Table 1). The number of polymorphic sites in Turkey was less than two-thirds of that in Thailand and Brazil. In the 1,576 amino acid sites analyzed, amino acid substitutions were found in 280 and 162 sites in worldwide and Turkey samples, respectively (Supplementary Figure 1). Of the 162 amino acid changes, 8 were newly identified and not observed in other areas. These are N/S at amino acid position 60 (after the Sal-1 sequence), D/N at 931, K/N at 956, S/K at 958, G/T at 959, T/S at 968, P/A at 971, and D/E at 1706. Within Turkey, the number of polymorphic nucleotide and amino acid sites was somewhat lower in Harran than in Siverek. Of note, one of the eight changes that occurred in the C-terminal 19-kD region (D/E substitution at 1706) is a new report because, up to now, only the K/E change at 1709 was the sole polymorphism reported.31,14 The K/E change was not detected in samples from Turkey.

The number of *pvmsp1* haplotypes was only three in Turkey with haplotype diversity ($h$) of 0.536, which is much lower than that in Thailand and Brazil ($h = 0.974$ and 0.944, respectively). Moreover, the three haplotypes found in Turkey were unique to this country. Both $\theta_h$ and $\theta_S$ were also lower in Turkey (56% and 67%, respectively) than worldwide values (Table 1).

**Polymorphism in *pvmsp1* poly Q region.** The *pvmsp1* contains a highly polymorphic sequence region, which is characterized by the presence (Belem type) or absence (Sal-1 type) of tandem repeats of Gln (Q) residues in amino acid positions 726–748 in the Belem sequence (GenBank accession no. AF435594). Polymorphism of this region in isolates from Turkey was compared with that reported from 8 countries (Table 2). All samples from Turkey had Belem type and no崇尚 difference (between closely related species) by using DnaSP. Under neutrality these ratios will be similar, whereas an excess of intraspecific nonsynonymous polymorphisms is suggestive of balancing selection. Ten *mspl* sequences from *P. cynomolgi,*32 a monkey malaria parasite closely related to *P. vivax,* were used for between-
Table 1

<table>
<thead>
<tr>
<th>Country</th>
<th>No.</th>
<th>H</th>
<th>h</th>
<th>No. Sal-1 type (%)</th>
<th>No. Belem type (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>30</td>
<td>3.536</td>
<td>0.077</td>
<td>0 (0)</td>
<td>30 (100)</td>
<td>This study</td>
</tr>
<tr>
<td>Azerbaijan</td>
<td>36</td>
<td>0.614</td>
<td>0.088</td>
<td>9 (25)</td>
<td>27 (75)</td>
<td>15</td>
</tr>
<tr>
<td>Iran</td>
<td>191</td>
<td>0.848</td>
<td>0.016</td>
<td>80 (42)</td>
<td>111 (58)</td>
<td>41–43</td>
</tr>
<tr>
<td>Afghanistan</td>
<td>57</td>
<td>0.821</td>
<td>0.039</td>
<td>31 (54)</td>
<td>26 (46)</td>
<td>44</td>
</tr>
<tr>
<td>Pakistan</td>
<td>33</td>
<td>0.873</td>
<td>0.027</td>
<td>13 (39)</td>
<td>20 (61)</td>
<td>43</td>
</tr>
<tr>
<td>Myanmar</td>
<td>135</td>
<td>0.794</td>
<td>0.016</td>
<td>111 (82)</td>
<td>24 (18)</td>
<td>20</td>
</tr>
<tr>
<td>Thailand</td>
<td>33</td>
<td>0.739</td>
<td>0.057</td>
<td>28 (85)</td>
<td>5 (15)</td>
<td>23,26</td>
</tr>
<tr>
<td>China</td>
<td>33</td>
<td>0.712</td>
<td>0.059</td>
<td>17 (52)</td>
<td>16 (48)</td>
<td>47</td>
</tr>
<tr>
<td>Brazil</td>
<td>78</td>
<td>0.859</td>
<td>0.016</td>
<td>25 (32)</td>
<td>35 (68)</td>
<td>3,26</td>
</tr>
</tbody>
</table>

*H* = no. haplotypes; *h* = haplotype diversity ± SD.

**Departure from neutrality.** Three tests were applied to detect departure from neutrality in *P. vivax*. The Z-test for a difference between dN and dS showed significantly higher dS than dN (Figure 3) in Turkey, Thailand, and Brazil. When the whole sequence (4,728 basepairs) was divided into three regions (the N-terminal, central, and C-terminal regions), dS was significantly higher than dN in the N-terminal and central regions for all three countries, whereas in the C-terminal 42-kD region, dN was significantly higher than dS in Saudi Arabia, Brazil, and India but not in Turkey. In the 19-kD C-terminal region, dN > dS was not significant because there were only two substitutions in this region (Supplementary Figure 1).

Using Tajima’s D statistics and Fu and Li’s statistics (D* test and F* test), we found that Tajima’s D value was weakly (but not significantly) positive only in Turkey. Fu and Li’s D* and F* values were significantly positive for Turkey but not for Thailand and Brazil. Within Turkey, samples from Harran yielded significantly positive values for the three statistics. If we consider recent rapid reduction in the incidence of malaria in the study areas (Figure 2), these results suggest a recent bottleneck in *P. vivax* populations in Turkey.

The McDonald and Kreitman test showed significantly higher intraspecific nonsynonymous substitutions than synonymous substitutions in parasite populations from Turkey, Thailand, and Brazil (Table 3), suggesting balancing selection acting on *pvmsp1* in all areas. No signature of balancing selection was evident for *P. cynomolgi*, as reported. Sequence regions showing balancing selection in *pvmsp1* were the central and C-terminal 42-kD regions. A population from India also showed significantly higher intraspecific nonsynonymous substitutions than synonymous substitutions in the 42-kD region. Although high intraspecific nonsynonymous substitutions over synonymous substitutions in a locus is also observed when constraints are relaxed, no singleton alleles were found in samples from Turkey (Table 1), making the occurrence of relaxed constraints in *pvmsp1* unlikely.

**Polymorphism in microsatellites.** The number of microsatellite repeats (alleles) was only three in four loci examined, with *h* = 0.55 (range = 0.476–0.626) (Table 4). These values are considerably lower than *h* values from other areas: 0.72–0.79 in India, Laos, Thailand, and Colombia; 0.86 in Vietnam; 0.79 in Sri Lanka; and 0.80 in Brazil.

**DISCUSSION**

The present analysis of *pvmsp1* polymorphism showed a remarkably lower diversity in *P. vivax* populations in Turkey.
than in populations in Thailand and Brazil. Nucleotide diversity was 56–67% of worldwide samples. Nonetheless, there were eight new single nucleotide polymorphisms identified that were unique to Turkey isolates. This finding indicates that additional pvmsp1 sequences from various areas are still likely to be required to fully document polymorphism of the gene. Among the eight single nucleotide polymorphisms, D/E substitutions at 1706 are notable because they reside in the C-terminal 19-kD polypeptide, a candidate vaccine molecule. Given that the amino acid change is not radical, i.e., D and E are acidic residues, it may not cause a dramatic effect on the function of the polypeptide during erythrocyte invasion by the merozoite. However, the substitution may potentially lead to a change of antibody binding because the position is surface exposed.45

The observed low diversity of pvmsp1 in Turkey has implications regarding acquisition of immunity against P. vivax malaria. It is believed that repeated infections are required for persons in malaria-endemic areas to effectively mount anti-malarial protective immunity. This belief largely stems from

![Figure 3](image-url)

**Figure 3.** Rate of synonymous (dS) and nonsynonymous (dN) substitutions per synonymous and nonsynonymous sites in the *Plasmodium vivax* merozoite surface protein gene (pvmsp1) from Turkey, Thailand, and Brazil. The pvmsp1 sequence was separated into three regions: the N-terminal, central, and C-terminal regions. The C-terminal 42-kD polypeptide region was further divided into the 33-kD and 19-kD regions. *P < 0.05; **P < 0.01; and ***P < 0.001.

<table>
<thead>
<tr>
<th>pvmsp1 region</th>
<th>Substitution type</th>
<th>BSFD</th>
<th>Turkey</th>
<th>P. cynomolgi</th>
<th>Thailand</th>
<th>P. cynomolgi</th>
<th>Brazil</th>
<th>P. cynomolgi</th>
<th>India</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole gene</td>
<td>Synonymous</td>
<td>173</td>
<td>69</td>
<td>196</td>
<td>155</td>
<td>105</td>
<td>195</td>
<td>156</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.005</td>
<td>P = 0.77</td>
<td>P = 0.007</td>
<td>P = 0.72</td>
<td>P = 0.017</td>
<td>P = 0.53</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S’ region</td>
<td>Synonymous</td>
<td>62</td>
<td>47</td>
<td>80</td>
<td>51</td>
<td>65</td>
<td>79</td>
<td>52</td>
<td>79</td>
</tr>
<tr>
<td>1,851 bp</td>
<td>P = 0.369</td>
<td>P = 0.784</td>
<td>P = 0.244</td>
<td>P = 0.579</td>
<td>P = 0.820</td>
<td>P = 0.413</td>
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<tr>
<td>Central</td>
<td>Synonymous</td>
<td>58</td>
<td>20</td>
<td>68</td>
<td>51</td>
<td>37</td>
<td>68</td>
<td>52</td>
<td>68</td>
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<tr>
<td>1,227 bp</td>
<td>P = 0.039</td>
<td>P = 0.862</td>
<td>P = 0.014</td>
<td>P = 0.766</td>
<td>P = 0.037</td>
<td>P = 0.719</td>
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<tr>
<td>3’ region</td>
<td>Synonymous</td>
<td>53</td>
<td>2</td>
<td>48</td>
<td>53</td>
<td>3</td>
<td>48</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>(42 kD)</td>
<td>P = 0.017</td>
<td>P = 0.682</td>
<td>P = 0.002</td>
<td>P = 0.778</td>
<td>P = 0.005</td>
<td>P = 0.679</td>
<td>P = 0.013</td>
<td>P = 0.784</td>
<td></td>
</tr>
<tr>
<td>33 kD</td>
<td>Synonymous</td>
<td>43</td>
<td>2</td>
<td>39</td>
<td>43</td>
<td>3</td>
<td>39</td>
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<tr>
<td>789 bp</td>
<td>P = 0.012</td>
<td>P = 0.701</td>
<td>P = 0.004</td>
<td>P = 0.754</td>
<td>P = 0.006</td>
<td>P = 0.699</td>
<td>P = 0.009</td>
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<tr>
<td>19 kD</td>
<td>Synonymous</td>
<td>10</td>
<td>0</td>
<td>9</td>
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<tr>
<td>309 bp</td>
<td>P = 0.454</td>
<td>P = 0.863</td>
<td>P = 1.0</td>
<td>P = 1.0</td>
<td>NA</td>
<td>P = 0.863</td>
<td>P = 1.0</td>
<td>P = 0.879</td>
<td></td>
</tr>
</tbody>
</table>

*pvmsp1 = P. vivax merozoite surface protein 1; BSFD = between-species fixed difference; WSPD = within-species polymorphic difference; NA = not available; bp = basepairs. P values < 0.05 are shaded in grey.
epidemiologic observations from areas highly endemic for *P. falciparum* malaria in sub-Saharan Africa, where a number of distinctive genotypes are circulating and mixed genotype infections are commonly seen. Strain-specific protective immunity (SSPI) or allele-linked immunity is strongly suggested to be involved in the slow acquisition of protective immunity against *P. falciparum* malaria. In a rodent malaria model, SSPI has been shown to exist, and parasite MSP-1 has strongly been suggested to be the principal candidate molecule for the control of SSPI against *P. chabaudi* malaria. If strain-specific immunity is true for *P. vivax*, it is predicted that acquisition of immunity may be faster in Turkey than in other malaria-endemic areas, such as Thailand and Brazil, where *pvmspl* diversity is relatively high. In Brazil, antibodies against polymorphic regions of PvMSP-1 are slow to develop, compared with the conserved C-terminal 19-KD polypeptide, which suggests that repeated infections are required to elicit antibody responses to variable sequence regions. In Sri Lanka, antibody prevalence was higher to the 33-kD polymorphic region than to the 19-KD region. Cohort studies to correlate protective immunity with antibody responses against PvMSP-1 (polymorphic regions and conserved regions, including the C-terminal 19-KD polypeptide) would be required to infer a role of *pvmspl* polymorphism in immune evasion.

In general, the level of genetic diversity of the parasite may be determined by several variables such as population evolutionary/demographic history, effective population size, gene flow between neighboring populations, and natural selection. In a population that has an old origin and large effective population size, a higher genetic diversity would be expected than a population with a recent origin and small effective population size. Microsatellite diversity was lower in Turkey than in other areas, which suggested a relatively small effective population size in the parasite population studied. In Turkey, human movements from and to neighboring countries is strictly limited, and parasite gene flow accompanied by human movements would be too low to cause introduction of parasite variants not prevalent in Turkey or increase genetic diversity. The frequency distribution pattern of poly Q repeat haplotypes (Supplementary Figure 2) was distinctive between Turkey and neighboring countries (Azerbaijan and Iran), and the distribution pattern was apparently similar between Iran and Afghanistan. Contribution of human movements to increasing genetic diversity has been observed between Iran, Afghanistan and Pakistan. In this study, Fu and Li’s D* and F* tests showed significantly positive values for populations from Turkey, which suggested a recent parasite population bottleneck. Consistent with this finding is the dramatic decrease in annual incidence of malaria in Sanliurfa, where there were two waves of rapid reductions in the early 1980s and after 1999 until the present time (Figure 2).

Additionally, the present population genetic study indicates that a signature of balancing selection on *pvmspl* appears differently among *P. vivax* populations. In Thailand, Brazil, and India, an excess of dN over dS was observed in the C-terminal 42-kD polypeptide; this finding was not detected in Turkey. The McDonald-Kretman test detected balancing selection for the central and C-terminal polypeptides in Turkey, Thailand, and Brazil (and in India for the 42-kD fragment). A potential population bottleneck in the *P. vivax* populations in the study area after a rapid reduction of *P. vivax* cases caused by recent extensive malaria interventions may be associated with the failure of detecting an excess of dN over dS in Turkey.

In conclusion, the present study demonstrates low diversity of *pvmspl* in *P. vivax* isolates from Turkey. The low antigen diversity should be informative for gaining insights of acquired immunity against *P. vivax* malaria. A population bottleneck of *P. vivax* was inferred, which was probably caused by recent malaria intervention efforts in the study area. A signature of balancing selection on *pvmspl* was observed in a parasite population that had experienced a bottleneck, which suggested geographic differences in balancing selection in an antigen gene of *P. vivax*.

Table 4

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>No.</th>
<th>Repeat unit</th>
<th>No repeat types</th>
<th>Haplotype diversity (H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS8</td>
<td>30</td>
<td>CA(C/G)</td>
<td>3</td>
<td>0.476 ± 0.091</td>
</tr>
<tr>
<td>MS9</td>
<td>29</td>
<td>AGG</td>
<td>3</td>
<td>0.626 ± 0.048</td>
</tr>
<tr>
<td>MS15</td>
<td>29</td>
<td>CTT</td>
<td>3</td>
<td>0.488 ± 0.091</td>
</tr>
<tr>
<td>3.502</td>
<td>26</td>
<td>ATGAACGG</td>
<td>3</td>
<td>0.594 ± 0.073</td>
</tr>
</tbody>
</table>

Mean ± SD = 0.546 ± 0.075

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