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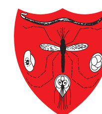

RESEARCH ARTICLE

Genetic diversity of secreted protein with an altered thrombospondin repeat (SPATR) of *Plasmodium knowlesi* clinical isolates from Malaysia

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ABSTRACT

The *Plasmodium knowlesi* secreted protein with an altered thrombospondin repeat (PkSPATR) is an important protein that helps in the parasite's invasion into the host cell. This protein has been regarded as one of the potential vaccine candidates against *P. knowlesi* infection. This study investigates the genetic diversity and natural selection of *PkSPATR* gene of *P. knowlesi* clinical isolates from Malaysia. PCR amplification of the full length *PkSPATR* gene was performed on 60 blood samples of infected *P. knowlesi* patients from Peninsular Malaysia and Malaysian Borneo. The amplified PCR products were cloned and sequenced. Sequence analysis of *PkSPATR* from Malaysia showed higher nucleotide diversity (CDS π: 0.01462) than previously reported *Plasmodium vivax PvSPATR* (π = 0.0003). *PkSPATR* from Peninsular Malaysia was observed to have slightly higher diversity (CDS π: 0.01307) than those from Malaysian Borneo (CDS π: 0.01212). Natural selection analysis on *PkSPATR* indicated significant purifying selection. Multiple amino acid sequence alignment revealed 69 polymorphic sites. The phylogenetic tree and haplotype network did not show any distinct clustering of PkSPATR. The low genetic diversity level, natural selection and absence of clustering implied functional constrains of the PkSPATR protein.

Keywords: *Plasmodium knowlesi*; SPATR; genetic diversity; haplotype analysis.

INTRODUCTION

Malaria is a parasitic infection caused by the protozoan parasite genus *Plasmodium*. The disease is transmitted to humans via the bite of female *Anopheles* mosquitoes. This disease is a major public health problem especially in tropical countries. The World Health Organization reported an estimated 241 million clinical cases globally in 2020 (WHO, 2021). Patients infected with malaria typically have symptoms such as high fever, shaking chills and flu-like illness. Without proper treatment, malaria infection may develop into more severe complications such as anaemia and kidney failure. The simian parasite of long-tailed and pig-tailed macaques, *Plasmodium knowlesi*, is now one of the major zoonotic malaria parasites that infect humans. Since the early 2000s, human knowlesi infection cases have been increasing across Southeast Asia countries, with Malaysia recording the highest number of cases (Ooi *et al*., 2017).

P. knowlesi infection may cause severe complications in humans. The parasite has a rapid replication stage as it takes only 24-hours for the parasite to complete the erythrocytic cycle compared to other *Plasmodium* species. Thus, this may result in massive erythrocyte destruction in infected patients. Frequently observed clinical complications include respiratory distress, jaundice, renal failure and hyperparasitaemia (Antinori *et al*., 2013). In Malaysia, around 10% of the patients suffered severe or complicated malaria with a case fatality rate of 1–2% (Ahmed *et al*., 2016).

The invasion of the *Plasmodium* into host cells at every stage of the parasite life cycle involves the interaction of different proteins with host cell receptors. The invasion process significantly involves the parasite's secretory organelles which are located at the apical end such as micronemes, rhoptries and dense granules. These organelles produce proteins that assist parasite motility and host cell entry (Palaeya *et al*., 2013). Microneme-secreted *Plasmodium* surface proteins containing the thrombospondin repeat (TSR) domain have been associated with various biological functions including parasite motility, attachment to host cell and host cell invasion (Chattopadhyay *et al*., 2003). The TSR domain is known to be present in more than 300 different proteins (Apweiller *et al*., 2001). The *P. falciparum* TSR domain has been evaluated as a vaccine candidate, suggesting that other *Plasmodium* proteins containing this domain can serve as potential vaccines as well (Mahajan *et al*., 2005).

Studies have found a variant version of TSR, known as secreted protein with an altered thrombospondin repeat (SPATR). In addition to the TSR domain, SPATR also possesses a cysteine-rich region which is also known as the Type II EGF-like domain. SPATR is expressed in multiple stages of the *Plasmodium* life cycle and has been

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characterized for its expression, localization and function at different stages of the parasite's life cycle (Chattopadhyay *et al*., 2003). The *P. knowlesi* SPATR (PkSPATR) has been shown to be immunogenic as anti- PkSPATR antibody was observed to bind to recombinant SPATR protein in an enzyme-linked immunosorbent assay (Mahajan *et al*., 2005). In addition, high-activity binding peptides from *P. falciparum* SPATR (PfSPATR) are capable of binding to the erythrocyte and inhibiting *in vitro* invasion of merozoites (Curtidor *et al*., 2008). *Plasmodium berghei* SPATR (PbSPATR)-deficient hepatic merozoites have been shown to be dispensable in hepatocytes but they are unsuccessful in developing blood stage infection (Gupta *et al*., 2020). Due to its multiple biological functions especially in immunological aspects, the PkSPATR can be regarded as a potential vaccine candidate against the *P. knowlesi* infection (Mahajan *et al*., 2005).

In Malaysia, *P. knowlesi* infection is reported in both Malaysian Borneo and Peninsular Malaysia with more than 70% of cases seen in Malaysian Borneo (Hussin *et al.*, 2020), and the majority of severe *P. knowlesi* infections were encountered in Malaysian Borneo (Cox-Singh *et al*., 2008). While the exact causes behind this uneven distribution number of cases remain undefined, geographic variation, distinct parasite populations and human social factors may be the reasons (Yusof *et al*., 2016). Studies have recorded that there are distinct genetic differences between the parasites in these two geographical regions (Fong *et al*., 2015a; Yusof *et al*., 2016) and this might be the important drivers for the differences in infection prevalence. Therefore, the present study's aims to compare the genetic diversity and natural selection of the *PkSPATR* gene in these two Malaysian regions.

MATERIALS AND METHODS

Blood samples

The 60 human blood samples used in this study were collected from *P. knowlesi* infected patients from hospitals in Peninsular Malaysia (n = 29) and Malaysian Borneo (n = 31). The samples were confirmed as *P. knowlesi* infection by microscopic examination of Giemsa-stained thin and thick blood smear and nested polymerase chain reaction (Snounou *et al.,* 1993; Imwong *et al*., 2009). Ethical approval for the use of the blood samples was granted by the Medical Research Subcommittee of Malaysia Ministry of Health (NMRR-15-67223975).

Extraction of DNA

Plasmodium knowlesi genomic DNA was extracted from 100 µl of blood using the QIAGEN Blood DNA Extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The extracted DNA was eluted with 50 µl of elution buffer. The eluted DNA was stored a -20°C until used.

PCR amplification of *PkSPATR* gene

The *PkSPATR* gene was amplified by semi-nested PCR using specifically designed oligonucleotide primers. Primer pairs PkSPATR EXT_F1: 5'- CCGTTCCCTATTCACACA -3' and PkSPATR MINR: 5'-ATATGGAGGGGCACACTT -3' were used in Nest 1 reaction. PkSPATR MINF: 5'- CAAGTACATACCAGGAAG -3' and PkSPATR MINR were used in Nest 2 reaction. These primers were designed based on the *P. knowlesi* SPATR protein sequence from GenBank (Accession No. AM910986.1). The cycling conditions for Nest 1, started with initial denaturation at 95°C for 3 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 75 s, with the final extension at 72°C for 10 min. The PCR product was subjected to Nest 2 with initial denaturation at 95°C for 3 min, followed by 40 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 75 s. The final extension was at 72°C for 10 min. The result of PCR, with an expected band size of 1143 bp, was observed on a 1.7% agarose gel.

Purification of PCR products and DNA cloning

PCR products were purified by using the QIAquick gel purification Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The purified PCR products were then ligated into cloning vector pGEM-T® (Promega Corp., USA) before being transformed into competent *Escherichia coli* TOP10F' cells. Plasmids of recombinant clones harbouring the *PkSPATR* gene fragment were sent to a commercial laboratory (Apical Scientific Sdn. Bhd., Malaysia)for DNA sequencing. The sequencing was performed in both directions of the insert in the plasmid.

Analysis of *PkSPATR* gene sequences

Each sequence was trimmed, joined and aligned using the BioEdit sequence alignment editor ver. 7.2.0 to obtain the full length sequence of *PkSPATR*. Then, all 60 sequences of *PkSPATR* were aligned together with the reference sequence from strain H (GenBank Accession No. AM910986.1:326887-328029). Multiple nucleotide and amino acid sequence alignment of the *PkSPATR* gene from Peninsular Malaysia and Malaysia Borneo isolates were analysed using the MEGA10 program.

***PkSPATR* sequence polymorphism analysis**

DnaSP ver. 5.10.00 software was used to generate the result for nucleotide diversity (π) and haplotype diversity (Hd). To estimate the stepwise diversity across the gene, the value π was established on a sliding window of 100 bases with a step size of 25. The Z-test ($P < 0.05$) in MEGA10 software was used to determine the rates of synonymous (dS) and non-synonymous (dN) substitutions by employing the Nei and Gojobori's method with Jukes and Cantor correction. For genes that undergo positive selection, non-synonymous mutations can be advantageous and the dN will be greater than dS (dN/dS > 1) while genes under purifying (negative) selection dN will be less than dS (dN/dS < 1).

Phylogenetic analysis

A phylogenetic tree was constructed using the Neighbour Joining method described in MEGA10. In constructing the phylogenetic tree, bootstrap replicates of 1000 were used to test the robustness of the tree. *P. vivax SPATR* sequence was used as an outgroup.

Haplotype network

Genealogical relationships between the *PkSPATR* haplotypes were constructed based on amino acid haplotypes using the median-joining method in NETWORK software (version 4.6.1.2, Fluxus Technology Ltd, Suffolk, UK).

RESULTS

***PkSPATR* sequences and gene structure**

All 60 samples were successfully amplified for the *PkSPATR* gene. The sequences were trimmed to obtain the full length sequences of 944 nucleotides. The sequences were deposited into GenBank (Accession Numbers: ON890244-ON890303). The *PkSPATR* gene contains two exons and one intron region. Exon I (231 bp) and exon II (558 bp) are separated by a short intron of 155 bp. The coding sequence (CDS: exon I + exon II) produces an mRNA of 789 bases that translates into a protein of 263 amino acids.

***PkSPATR* sequence diversity**

The sequence polymorphism analysis of *PkSPATR* is summarized in Table 1. Taking the Malaysian samples as a single population (n = 60), exon I had the highest nucleotide diversity (π) with 0.01745, followed by CDS (π: 0.01462) and exon II (π: 0.01342). The haplotype diversity (Hd) of exon I, exon II and total CDS showed values of 0.970, 0.997 and 0.999, respectively. The sliding window plot (window length

Table 1. Estimates of genetic diversity and natural selection of the total coding region (CDS), exon I and exon II of *PkSPATR* gene from Peninsular Malaysia and Malaysian Borneo isolates

Gene/region	N	Sites	Nucleotide diversity (π ± SD)	Haplotype diversity (Hd ± SD)	Z- test *P* value	
					dN > dS	dN < dS
Malaysia						
Exon I	60	231	0.01756 ± 0.00157	0.970 ± 0.015	1.00	<0.01*
Exon II	60	558	0.01342 ± 0.00060	0.997 ± 0.004	1.00	<0.01*
CDS	60	789	0.01462 ± 0.00057	0.999 ± 0.003	1.00	<0.01*
Peninsular Malaysia						
Exon I	29	231	0.02014 ± 0.00217	0.988 ± 0.013	1.00	0.01*
Exon II	29	558	0.01013 ± 0.00074	0.993 ± 0.011	0.26	1.00
CDS	29	789	0.01307 ± 0.00095	0.998 ± 0.001	1.00	<0.01*
Malaysian Borneo						
Exon I	31	231	0.01051 ± 0.00162	0.897 ± 0.049	1.00	<0.01*
Exon II	31	558	0.01279 ± 0.00090	0.996 ± 0.009	0.28	1.00
CDS	31	789	0.01212 ± 0.00069	1.000 ± 0.008	1.00	<0.01*

N: number of isolates, Sites: total number of sites analysed excluding gaps, Hd: haplotype diversity, π: nucleotide diversity.
* significant at $P < 0.05$.

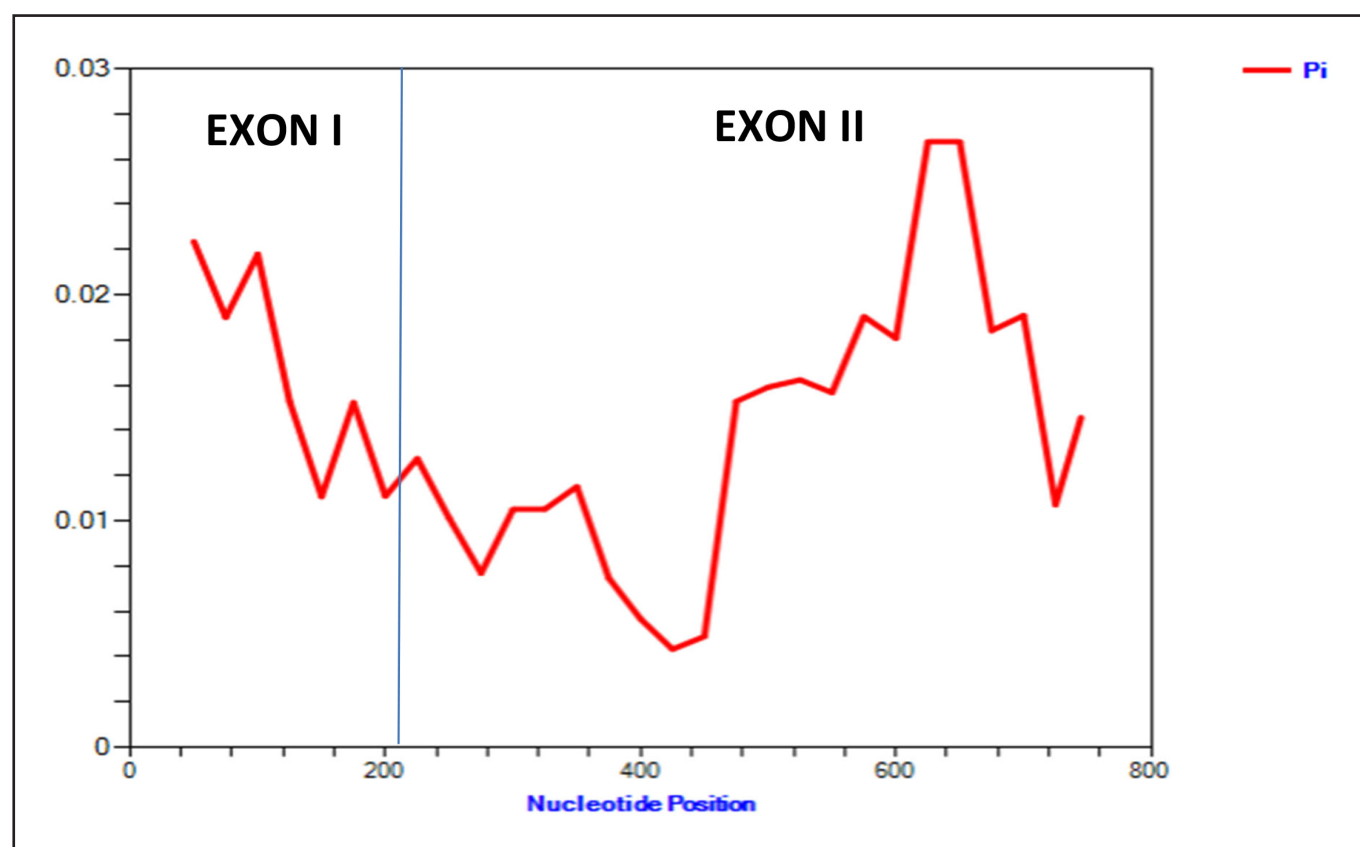

Figure 1. Nucleotide polymorphism in the *PkSPATR* of Malaysia isolates (n = 60). Sliding window plot of the nucleotide diversity (ɔ)/pi along the *PkSPATR* was generated with a window length of 100 bp and step size of 25 bp.

100 bp, step size 25 bp) provides detailed analysis of the π that ranged from 0.004-0.027, with the highest and lowest polymorphic regions located in exon II (Figure 1). The most diverse region was observed to be within nucleotide positions 650-700, while the lowest polymorphic region was within nucleotide positions 400-450.

A separate analysis on Peninsular Malaysia (n = 29) and Malaysian Borneo (n = 31) *PkSPATR* revealed that exon I from Peninsular Malaysia (π: 0.02014) had higher nucleotide diversity than Malaysian Borneo PkSPATR (π: 0.01051). Exon I from Peninsular Malaysia and Malaysian Borneo had Hd of 0.988 and 0.897, respectively, which was slightly lower than exon II (Hd: 0.993 and 0.996 respectively) and CDS (Hd: 0.998 and 1.000 respectively). The π of exon II (π: 0.01013) for Peninsular Malaysia *PkSPATR* was lower than the CDS with (π: 0.01307) in contrast to exon II of Malaysian Borneo (π: 0.01279) that revealed higher π than the CDS (π: 0.01212).

PkSPATR natural selection

The Z-test results of the overall Malaysian *PkSPATR* sequences indicated purifying selection of exon I, exon II and CDS (dN < dS, *P* < 0.01). The Z-test value for Peninsular Malaysia *PkSPATR* showed purifying selection of exon I and CDS (dN < dS, *P* = 0.01 and *P* < 0.01 respectively), Similarly, the Z-test analysis on Malaysian Borneo *PkSPATR* showed purifying selection of exon I and CDS (dN < dS, *P* < 0.01). However, exon II of both the Peninsular Malaysia and Malaysian Borneo *PkSPATR* showed neutral selection (dN > dS, *P* = 0.26, dN > dS, *P* = 0.28). Hence, the purifying selection of Malaysia *PkSPATR* is attributed mainly to exon I and CDS.

PkSPATR polymorphism and haplotypes

By employing the H strain (H1) as a reference sequence, the multiple alignments of PkSPATR amino acid sequences from Peninsular Malaysia and Malaysian Borneo isolates showed 69 polymorphic sites (Figure 2). Among these polymorphic sites, 65 were monomorphic substitutions (changed into 1-amino acid type), 3 showed dimorphic substitutions (changed into 2-amino acid type) and 1 displayed trimorphic substitution (changed into 3-amino acid type).

The multiple alignment in Figure 2 also presented the number of PkSPATR haplotypes in the population. A total of 47 haplotypes were identified, of which 22 were from Peninsular Malaysia and 25 from Malaysian Borneo. No major dominant haplotype(s) were observed, with haplotype frequency (i.e., number of sequences per haplotype) ranging from 1 to 5. The phylogenetic tree based on these 47 haplotypes (Figure 3) did not show a distinct separation of Peninsular Malaysia and Malaysian Borneo PkSPATR as haplotypes from both regions were seen to mix together in the clusters of the tree. A haplotype network was constructed to provide a clearer distribution of the PkSPATR haplotypes (Figure 4). No distinct clusters were seen in the network. The short links connecting the haplotypes indicated close relations between Peninsular Malaysia and Malaysian Borneo PkSPATR. Uniquely, it was seen that Peninsular Malaysia haplotypes H32 and H34 branched out from the node of Malaysian Borneo. It is worthwhile also to mention that H40 was from a patient in Peninsular Malaysia but the network analysis showed its close relation to Malaysian Borneo haplotypes. Upon closer examination of the records, the patient had travelled to the Malaysian Borneo state of Sarawak. He most likely acquired the *P. knowlesi* infection there but was diagnosed upon returning to Peninsular Malaysia.

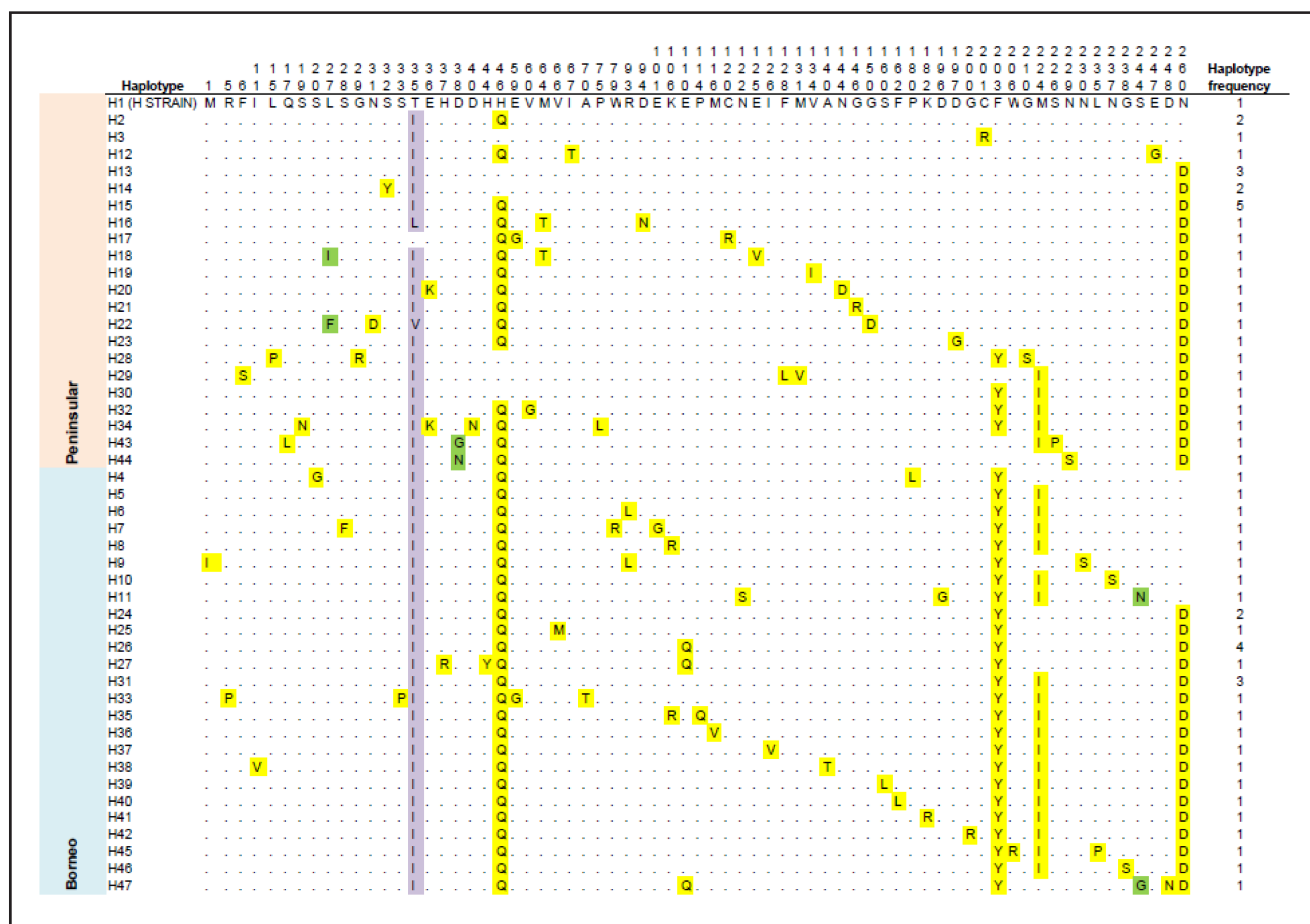

Figure 2. Amino acid sequence polymorphism in *PkSPATR* from Peninsular Malaysia and Malaysian Borneo isolates. Polymorphic amino acid residues are listed for each haplotype. Amino acid residues identical to those of the reference sequence [strain H (haplotype H1)] are marked by dots. Monomorphic, dimorphic and trimorphic amino acid positions are marked in yellow, green and purple shadings respectively. Haplotype frequency for each haplotype is listed in the right panel.

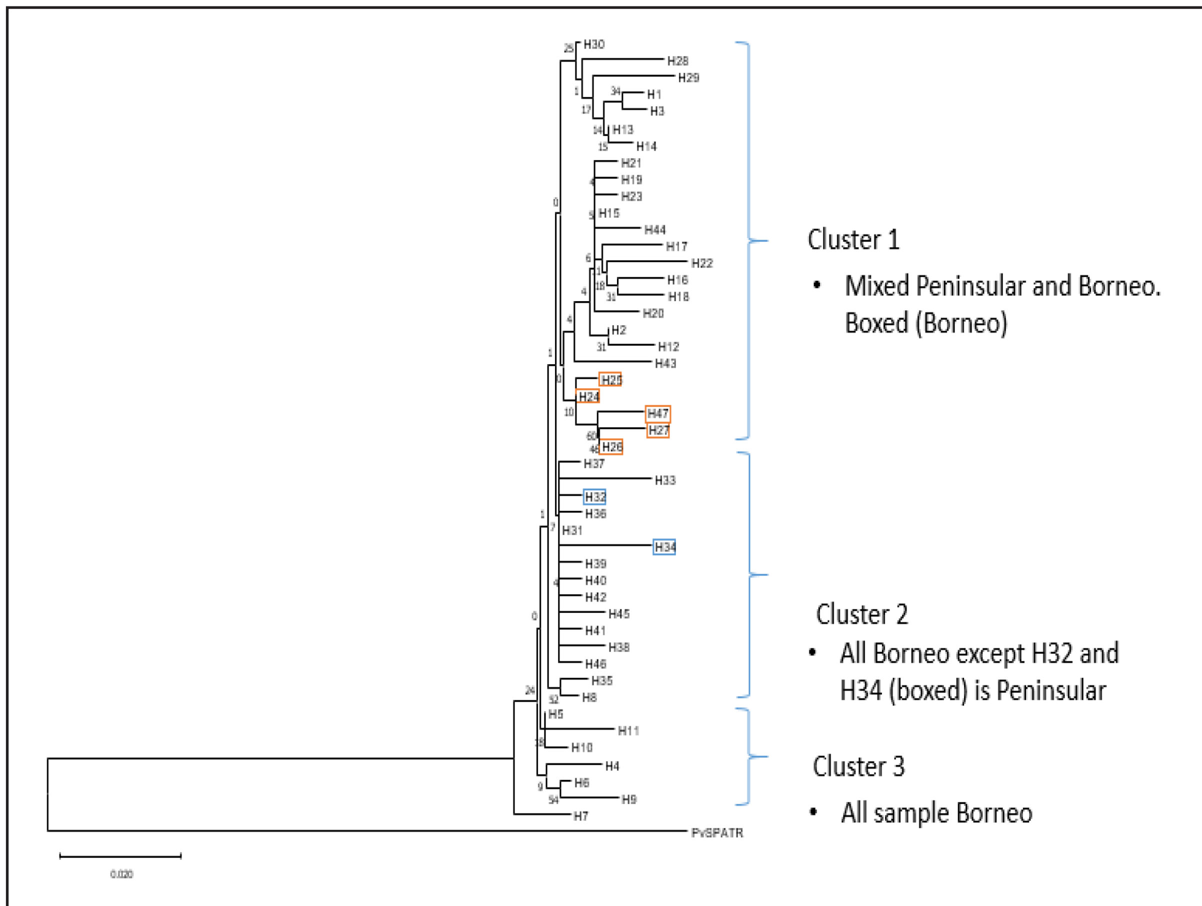

Figure 3. Phylogenetic tree of PkSPATR haplotypes based on Neighbour-Joining Method with numbers at the node indicating percentage supporting 1000 bootstrap replicates. *P. vivax* SPATR is used as an outgroup.

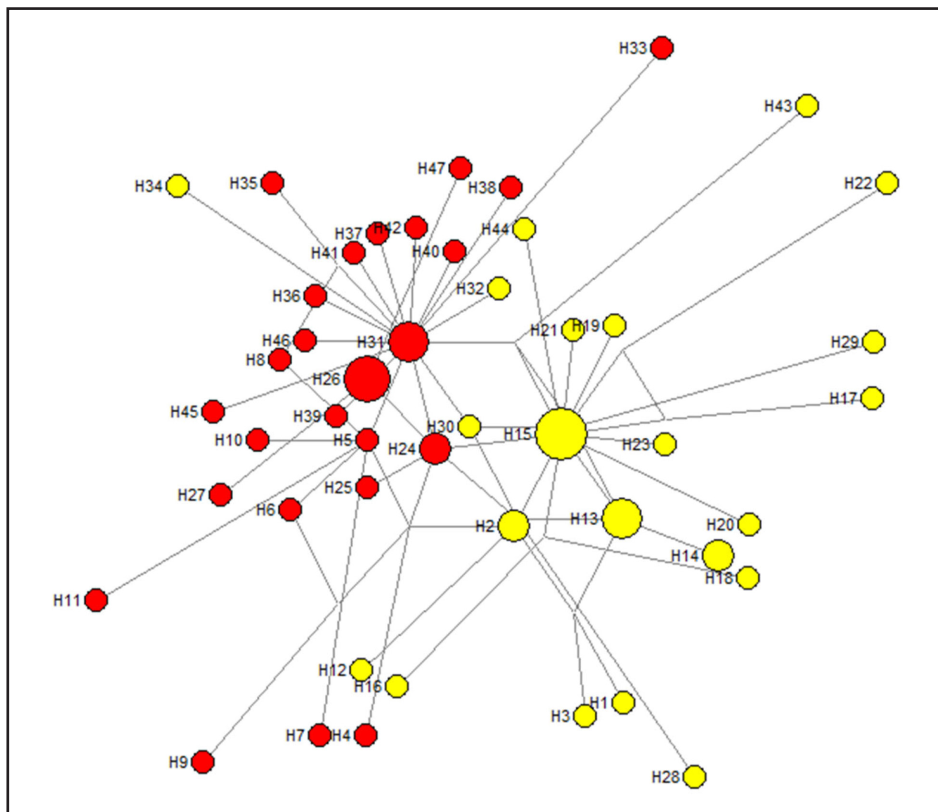

Figure 4. Network analysis of PkSPATR haplotypes. Peninsular Malaysia and Malaysian Borneo haplotypes are indicated by yellow and red nodes respectively. The size of each node reflects the number of isolates in each haplotype.

DISCUSSION

SPATR is a multi-stage protein widely studied for its function in *Plasmodium* parasite invasion into the host cell and its immunological properties. This protein has shown potential as an anti-malarial vaccine candidate. Genetic polymorphism is an important aspect in malaria vaccine design as well as understanding the population history of the parasite and its adaptive potential. Numerous studies on *P. knowlesi* genetic polymorphism have been conducted. However, limited information was reported for *PkSPATR*. The current study is the first to report gene polymorphism and natural selection on *PkSPATR*.

The nucleotide diversity of *PkSPATR* (π = 0.01462) is higher than *P. vivax* SPATR (π = 0.0003) (Garzón-ospina *et al*., 2018). However, the π for *PkSPATR* shows a similar range of low polymorphism with other *P. knowlesi* functional genes such as *PkDBP*α*II* (π = 0.012) (Fong *et al*., 2015a), *PkRAP-1* (π = 0.01298) (Rawa *et al*., 2016) and *PkNBPXa* (π = 0.02186) (Ahmed *et al*., 2016). This low polymorphism signifies the functional importance of PkSPATR. In contrast to that, a relatively higher Hd was observed in PkSPATR. The concurrent low π but high Hd may be the result of protein adaptation to population growth after a phase of low effective population size (Grant & Bowen, 1988; Garg & Mishra, 2018). Besides, the high number of singletons (58 of 69 of the polymorphic sites, in Figure 2), which present new and rare variants, is a possible reason that leads to the high Hd (Ahmed *et al*., 2018b).

The polymorphism analysis of the *PkSPATR* gene under a single population (Malaysia, n = 60) shows significant purifying (negative) selection that indicates an excess of synonymous substitutions against non-synonymous substitutions. Negative selection has been observed in many important *P. knowlesi* proteins such as *PkNBPXa* (Ahmed *et al*., 2016), *PkRAP1* (Rawa *et al*., 2016), *PkMSP1* (Ahmed *et al*., 2018a), *PkAMA1* (Fong *et al*., 2015b; Ng *et al*., 2021) and *PkDBP*π*II* (Fong *et al*., 2015a). Purifying selection is a mechanism that limits polymorphism which may affect the protein's function and be deleterious to the parasite. Besides, the long-term population expansion of *P. knowlesi* may be the possible reason for purifying selection as previous studies on *P. knowlesi* mitochondrial DNA showed evidence of population expansion in Southeast Asia (Lee *et al*., 2011).

Analysis of *PkSPATR* sequence based on subpopulations showed a minimal difference between Peninsular Malaysia and Malaysian Borneo. In general, Peninsular Malaysia *PkSPATR* had a slightly higher level of diversity as compared to Malaysian Borneo's. Thus, the difference in diversity level is not the cause of the higher prevalence of infection in Malaysian Borneo compared to Peninsular Malaysia.

Interestingly, the phylogenetic analysis did not show any clustering of PkSPATR, unlike those of other *P. knowlesi* proteins such as PkNBPXa (Ahmed *et al*., 2016), PkRAP1 (Rawa *et al*., 2016) and PkDBPαII (Fong *et al*., 2015a). Haplotype network analysis also showed the absence of clustering. Although no haplotype sharing was observed, the short link between Peninsular Malaysia and Malaysian Borneo haplotypes indicates close genetic relation of PkSPATR from these two regions and conservation of the protein.

CONCLUSION

The PkSPATR is a conserved protein with minimal polymorphism. Proteins with such characteristics are usually chosen for vaccine design. Therefore, future work on PkSPATR should focus on its immunological properties and protective potential.

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Conflict of Interest

The authors declare no conflict of interest.

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