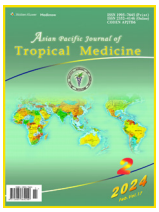

Original Article

Asian Pacific Journal of Tropical Medicine

apjtm.org

doi: 10.4103/apjtm.apjtm_540_23

Genetic diversity of the S-type small subunit ribosomal RNA gene of *Plasmodium knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia

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ABSTRACT

Objective: To determine the genetic diversity of *Plasmodium (P.) knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia, targeting the S-type *SSU rRNA* gene and including aspects of natural selection and haplotype.

Methods: Thirty-nine blood samples infected with *P. knowlesi* were collected in Sabah, Malaysian Borneo and Peninsular Malaysia. The S-type *SSU rRNA* gene was amplified using polymerase chain reaction, cloned into a vector, and sequenced. The natural selection and haplotype of the S-type *SSU rRNA* gene sequences were determined using DnaSP v6 and illustrated using NETWORK v10. This study's 39 S-type *SSU rRNA* sequences and eight sequences from the Genbank database were subjected to phylogenetic analysis using MEGA 11.

Results: Overall, the phylogenetic analysis showed no evidence of a geographical cluster of *P. knowlesi* isolates from different areas in Malaysia based on the S-type *SSU rRNA* gene sequences. The S-type *SSU rRNA* gene sequences were relatively conserved and with a purifying effect. Haplotype sharing of the S-type *SSU rRNA* gene was observed between the *P. knowlesi* isolates in Sabah, Malaysian Borneo, but not between Sabah, Malaysian Borneo and Peninsular Malaysia.

Conclusions: This study suggests that the S-type *SSU rRNA* gene of *P. knowlesi* isolates in Sabah, Malaysian Borneo, and Peninsular Malaysia has fewer polymorphic sites, representing the conservation of the gene. These features make the S-type *SSU rRNA* gene suitable for comparative studies, such as determining the evolutionary relationships and common ancestry among *P. knowlesi* species.

KEYWORDS: *Plasmodium knowlesi*; S-type small subunit ribosomal RNA; Genetic diversity; Natural selection; Haplotype

1. Introduction

The COVID-19 pandemic has lessened access to healthcare services, especially in the African regions. This has directly contributed to a 69 000 increase in malaria deaths, raising the malaria mortality rate to 15 deaths per 100 000 population in 2020[1,2]. Although human malaria cases caused by the five *Plasmodium*

Significance

Information on the genetic diversity of the *Plasmodium (P.) knowlesi* S-type *SSU rRNA* gene remains scarce in Sabah, Malaysian Borneo and Peninsular Malaysia. This study suggests that the S-type *SSU rRNA* gene of *P. knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia is conserved and with fewer polymorphic sites. These characteristics make the S-type *SSU rRNA* gene suitable for comparative studies. The data of this study is beneficial for conservation and environmental management and public health officials, especially in understanding the transmission of *P. knowlesi* in Malaysia.

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How to cite this article: Chong ETJ, Neoh JWF, Lau TY, Chua KH, Lim YAL, Lee PC. Genetic diversity of the S-type small subunit ribosomal RNA gene of *Plasmodium knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia. Asian Pac J Trop Med 2024; 17(2): 84-90.

Article history: Received 2 July 2023 Revision 15 January 2024
Accepted 5 February 2024 Available online 28 February 2024

species, namely *Plasmodium (P.) malariae*, *P. falciparum*, *P. knowlesi*, *P. ovale*, and *P. vivax*, gradually decreased from 2000 to 2019, the situation worsened in 2021, with an estimated 247 million malaria cases and 619 thousand malaria deaths being reported across the 84 endemic countries[3]. Malaysia has reported zero indigenous malaria cases since 2018, but it is estimated that about 1.34 million of the Malaysian population are at risk of contracting malaria[3].

The Southeast Asian regions, including Malaysia, are severely impacted by the deadly *P. knowlesi* parasites, a simian *Plasmodium*[4,5]. From 2013 to 2017, *P. knowlesi* cases accounted for 77.1% of malaria cases in Sabah and Sarawak, Malaysian Borneo, and 40.3% in Peninsular Malaysia[6]. There are several strategies to estimate the spread of these parasites in the country, one of which is to understand the genetic diversity of *P. knowlesi* genes.

Many studies have been conducted to understand the genetic diversity of selected genes of *P. knowlesi* isolates from Malaysian Borneo and Peninsular Malaysia, such as circumsporozoite protein (csp), cytochrome b, gamma protein region][, and merozoite surface protein 1[7–12]. However, the genetic diversity of the small subunit ribosomal RNA (*SSU rRNA*) gene in Malaysia's *P. knowlesi* isolates is rarely reported. The *SSU rRNA* gene encodes the SSU rRNA molecules of ribosomes, which are responsible for translating mRNA to functional proteins in *Plasmodium* species. In parasitology, the *SSU rRNA* gene can be used as a biomarker to detect the presence of blood-stage parasites and is always a preferred target for identifying and determining evolutionary relationships of *Plasmodium* species in infected samples through a phylogenetic approach[13,14]. The *SSU rRNA* gene could also be utilized to differentiate *Plasmodium* parasites undergoing asexual multiplication in the erythrocytes from those in the sexual stages, which helps in understanding the clinical manifestations of the disease.

The *SSU rRNA* genes appear to be multicopy per haploid genome and are located on different chromosomes. In *P. knowlesi*, the A-type *SSU rRNA* gene isoforms are annotated on chromosomes 3 and 10, and they are expressed during the asexual blood stages in the vertebrate host. On the other hand, the S-type *SSU rRNA* gene is mapped to chromosome 13 of the parasite's genome[15,16]. During the sexual stage of *P. knowlesi* parasites, the merozoites differentiate into microgametocytes (male) and macro gametocytes (female), and the S-type *SSU rRNA* gene is expressed in this stage to ensure their survival. The S-type *SSU rRNA* is often considered more interesting since it can provide insights into the parasite's reproductive biology and transmission dynamics.

Despite the extensive use of the *SSU rRNA* gene in various diagnostic and evolutionary studies, information on the genetic diversity of the S-type *SSU rRNA* gene in *P. knowlesi* isolates remains scarce, particularly in Malaysia, where this parasite has a severe impact. Therefore, this study aims to understand the genetic diversity of the S-type *SSU rRNA* gene in *P. knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia, including aspects of natural selection and haplotypes.

2. Material and methods

2.1. *P. knowlesi* samples collection and DNA extraction

Peripheral blood samples were collected from 39 malaria patients infected with *P. knowlesi* parasites in different areas of Sabah, Malaysian Borneo, including Telupid (n=9), Keningau (n=6), Nabawan (n=3), Tambunan (n=5), and Tenom (n=6), as well as in Peninsular Malaysia (n=10) from 2008 to 2013 for this study (Table 1). The presence of the *P. knowlesi* parasites in the collected blood samples was previously verified using the Giemsa stain and cross-validated using the PlasmoNex™ diagnostic system[17]. DNA was extracted from all the blood samples using a procedure previously described[18].

Table 1. Blood samples infected with *Plasmodium knowlesi* were collected from different areas in Sabah, Malaysian Borneo, and Peninsular Malaysia.

Areas		Samples
Sabah, Malaysian Borneo (n=29)	Telupid	SB28, SB39, SB56, SB130, SB131, SB143, SB148, SB151, SB156
	Keningau	KN010, KN013, KN030, KN037, KN045, KN048
	Nabawan	NB028, NB032, NB046
	Tambunan	TB013, TB016, TB049, TB066, TB073
	Tenom	TN003, TN023, TN024, TN026, TN027, TN029
Peninsular Malaysia (n=10)		5687, 5832, 6835, 6854, 6873, 7341, 7412, 7894, 8183, 8939

2.2. S-type SSU rRNA gene amplification using polymerase chain reaction

Polymerase chain reaction (PCR) amplification of the S-type *SSU RNA* gene was performed using *Plasmodium*-specific primers as previously described[19,20]. In brief, a PCR was carried out in a 20 μL reaction mixture containing 1× of GoTaq Buffer (Promega, Madison, USA), 2 mM of $MgCl_2$, 0.2 mM of dNTP mixtures, 0.25 μM of each primer (rPLU5 and Pmk8), 1 unit of Taq DNA polymerase (Promega, Madison, USA), and 100 ng of extracted DNA as a template. The PCR condition was set at 94 ℃ for 4 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 1 min, and 72 ℃ for 1 min, and a final extension step at 72 ℃ for 5 min. The PCR products (≈1 080 bp) were electrophoresed and analysed in 1% agarose gel stained with ethidium bromide.

2.3. Cloning of the PCR amplicons and sequencing

The QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to isolate the 39 successfully amplified PCR amplicons from agarose gel, and the purified PCR products were cloned into a pCR™4-TOPO® TA vector using the TOPO™ TA Cloning™ Kit (Invitrogen, Waltham, USA). The ligated vectors were transformed into *Escherichia (E.) coli* TOP10 strain using a heat-shock approach. The desired plasmid containing the S-type *SSU rRNA* fragment was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Sanger sequencing utilizing the M13 forward and reverse sequencing primers was performed using the Applied Biosystems 3500 Series Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

2.4. SSU rRNA sequences alignment and phylogenetic tree construction

A total of 46 *P. knowlesi SSU rRNA* sequences, including the 39 sequences from this study (S1) and seven S-type and A-type sequences retrieved from the Genbank database (S-type strain from human hosts: DQ350255, DQ350263, and LR701173; S-type strain from *Macaca fascicular* host: DQ350270; A-type from human hosts: AY327549 and AY327557; A-type from *Macaca fascicularis* host: DQ641518) were aligned using the CLUSTAL-W tool in Molecular Evolutionary Genetics Analysis 11 (MEGA 11) software[21]. The *P. coatneyi SSU rRNA* sequence (KC662442) was used as an outgroup. A phylogenetic tree was constructed using a Neighbour-joining method with bootstrap replicates of 1 000 to test the robustness and reliability of the tree.

2.5. Natural selection and haplotype analyses of the S-type SSU rRNA sequences

The aligned S-type *SSU rRNA* sequences of the *P. knowlesi* isolates were trimmed, and a portion of the sequences (total length=939 bp) was subjected to natural selection and haplotype analyses by comparing to the reference *P. knowlesi* strain H (LR701173). Polymorphisms in the S-type *SSU rRNA* sequences were determined using the DnaSP v6 software[22]. Data such as the average number of pairwise nucleotide differences (K), number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) were obtained. A comprehensive analysis of the π was performed on a sliding window of 100 bases with a step size of 25 bp to estimate the stepwise diversity of the S-type *SSU rRNA* sequences. The neutral theory of evolution was tested with Tajima's D as well as Fu and Li's D and F using the tools implemented in the DnaSP v6 software. The median-joining method in NETWORK v10 software (available at: https://www.fluxus-engineering.com/sharenet.htm) was utilized to generate a networking linkage of the S-type *SSU rRNA* haplotypes for *P. knowlesi* isolates.

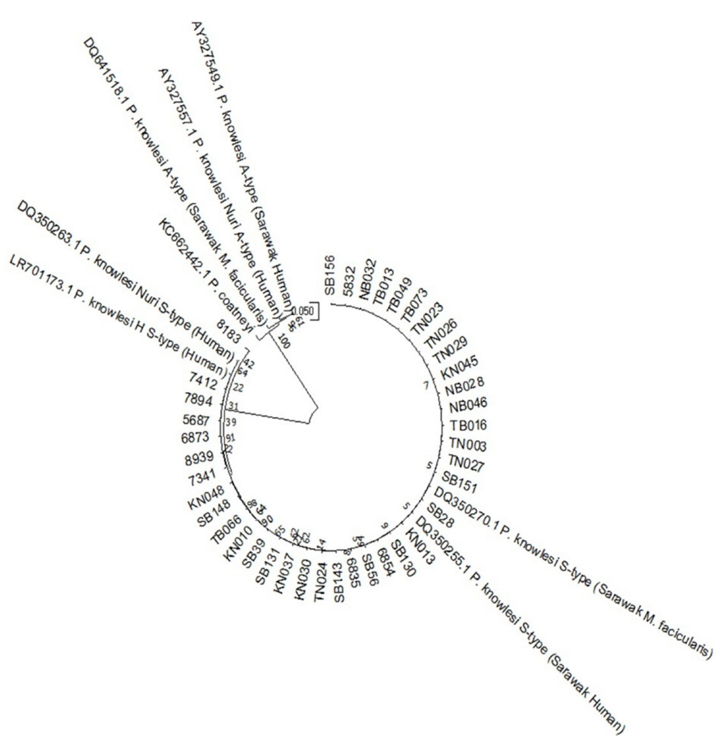

Figure 1. Phylogenetic tree of *SSU rRNA* sequences constructed using a Neighbour-joining method in MEGA 11. A total of 46 S-type and A-type *SSU rRNA* sequences of *Plasmodium knowlesi* and one *Plasmodium coatneyi SSU rRNA* sequence as an outgroup were included in this phylogenetic tree. The number at the nodes indicates the percentage support of 1000 bootstrap replicates.

2.6. Ethical approval

Ethical approval of this study was obtained from the Medical Research and Ethics Committee of the University of Malaya Medical Centre with a reference number: 709.1.

3. Results

3.1. Phylogenetic tree analysis of the SSU rRNA sequences

The phylogenetic tree revealed a distinct separation between the A-type and S-type *SSU rRNA* sequences (Figure 1). When considering the S-type *SSU rRNA* sequences, there is no evidence of a geographical cluster among *P. knowlesi* isolates in Sabah, Malaysian Borneo, and Peninsular Malaysia. Additionally, there is no distinction between *P. knowlesi* isolates from humans and *M. fascicularis* hosts.

3.2. Genetic analysis of the S–type SSU rRNA sequences

The genetic analysis of the 39 S-type *SSU rRNA* sequences in this study revealed that the average number of pairwise nucleotide differences (K) was 5.768 when compared to the S-type *SSU rRNA* sequence of *P. knowlesi* strain H (LR701173), which served as the reference. The overall nucleotide diversity (π) and haplotype diversity (Hd) were 0.006±0.001 and 0.897±0.002, respectively. A comprehensive analysis of the π, using a sliding window length of 100 bp and a step size of 25 bp, revealed that the diversity ranged from 0.001 to 0.010. The nucleotide position at 482-581 bp was the most conserved region among the aligned S-type *SSU rRNA* sequences, whereas the highest peak of nucleotide diversity was observed within 382-481 bp (Figure 2).

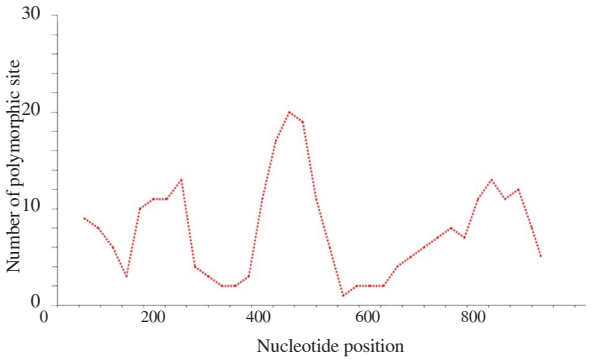

Figure 2. Nucleotide segregating in the portion of the S-type *SSU rRNA* gene of *Plasmodium knowlesi* in this study. The sliding window plot with a window length of 100 bp and a step size of 25 bp for the number of segregating sites within the S-type *SSU rRNA* sequences was generated using DnaSP v6.

3.3. Natural selection and haplotype analyses

In natural selection analysis, Tajima's D was calculated to be -2.406 (P<0.01), while Fu and Li's D and F were -5.103 (P<0.02) and -4.938 (P<0.02), respectively. All the negative values in Tajima's D and Fu and Li's D and F suggest an expansion in population size with purifying selection.

This study identified several novel single nucleotide polymorphisms within the aligned S-type *SSU rRNA* nucleotide sequences (Figure 3). These sequences were further classified into 26 different haplotypes. Notably, half of the haplotypes were shared among the *P. knowlesi* isolates from various areas of Sabah, Malaysian Borneo (haplotype 9). However, the median-joining network analysis revealed no haplotype sharing between the *P. knowlesi* isolates from Sabah, Malaysian Borneo, and Peninsular Malaysia (Figure 4).

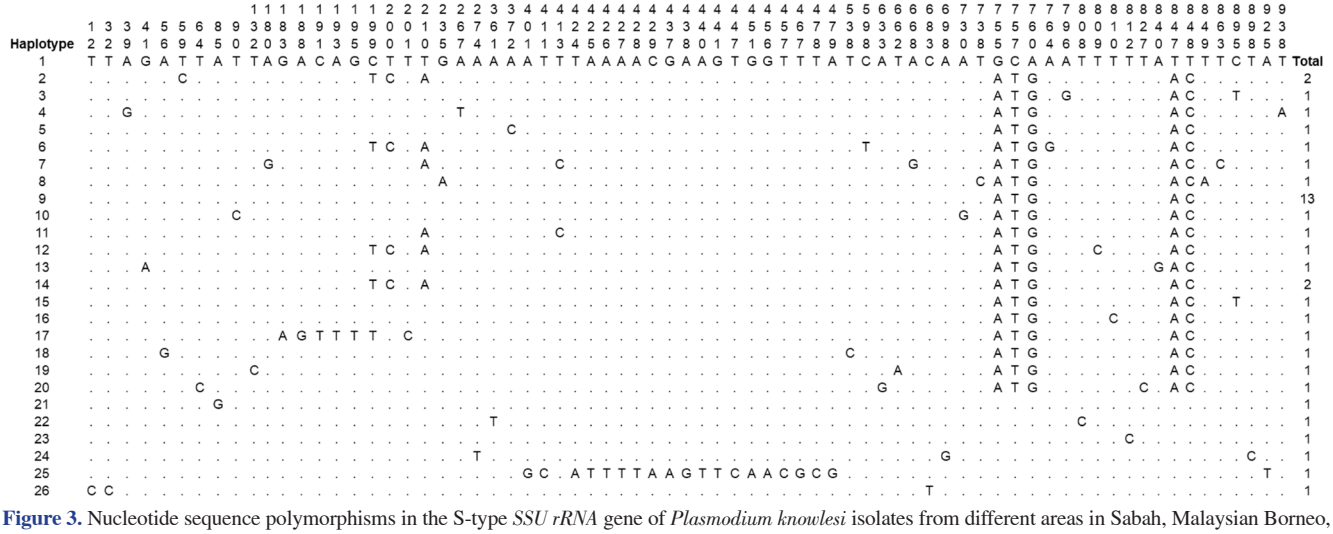

Figure 3. Nucleotide sequence polymorphisms in the S-type *SSU rRNA* gene of *Plasmodium knowlesi* isolates from different areas in Sabah, Malaysian Borneo, and Peninsular Malaysia. Polymorphic nucleotide sites are listed for each haplotype, and the total number of sequences for each haplotype is listed in the right-end column. Nucleotides matched to those of the reference *Plasmodium knowlesi* strain H sequence (accession ID: LR701173) are marked by dots.

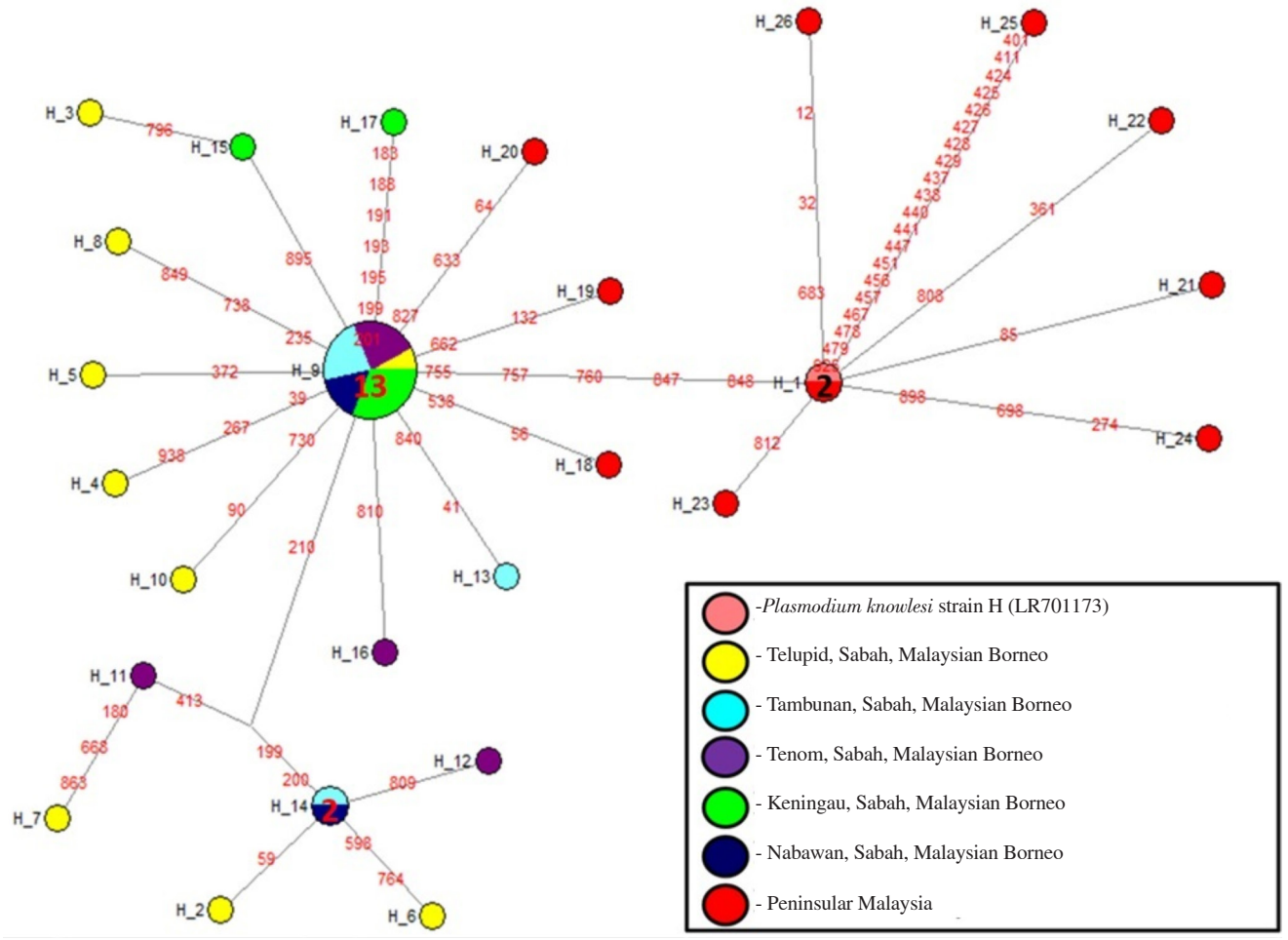

Figure 4. Median-joining network of haplotypes (h= 26) for the S-type *SSU rRNA* gene of *Plasmodium knowlesi* isolates between different areas in Sabah, Malaysian Borneo, and Peninsular Malaysia. The radius of the circle corresponds to the total number of samples for each haplotype, and different colors represent each sampling site. Numbers along the lines are the polymorphism sites between two haplotypes.

4. Discussion

The *SSU rRNA* gene is widely used in molecular approaches to identify bacterial strains and determine parasite species. In malaria research, the *SSU rRNA* gene is particularly valuable for differentiating *Plasmodium* species, especially between the *P. knowlesi* and *P. malariae* parasites that are exceptionally challenging to distinguish under microscopy examinations. Despite being a highly acceptable biomarker for species identification, reports on the genetic diversity of the *SSU rRNA* gene in *P. knowlesi* studies are rare. Therefore, this study assessed the genetic diversity targeting the S-type *SSU rRNA* gene of *P. knowlesi* isolates from Sabah, Malaysian Borneo and Peninsular Malaysia, including the natural selection and haplotype aspects to address this issue.

The phylogenetic tree in this study divided the *SSU rRNA* sequences of the *P. knowlesi* isolates into two distinct parts according to their sexual types. While previous reports have shown transcription switching between stage-specific rRNA genes in *P. berghei*[23], it is worth investigating whether *P. knowlesi* parasites have the ability to modify their genetic contents and selectively regulate the transcription activities when they exist in different sexual types within different hosts or environments to ensure their survival. Considering recent findings on the differential response of male and female *Plasmodium* mature gametocytes to antimalarial drugs[24], understanding this aspect will have significant implications for drug development by clarifying whether certain antimalarial drugs are only effective in treating *P. knowlesi* at specific sexual stages.

The average number of pairwise nucleotide differences (K) in the S-type *SSU rRNA* gene is higher than that of the cytochrome *b* gene but smaller than that of the *csp* gene, as previously reported in *P. knowlesi* isolates from Malaysia[8,9]. However, the S-type *SSU rRNA* gene exhibits lower overall nucleotide diversity (π), indicating that it is considerably more conserved compared to the *csp* and cytochrome *b* genes. In addition, the negative values in Tajima's D and Fu and Li's D and F suggest that the S-type *SSU rRNA* gene is under a purifying effect, which is supported by previous studies[25,26]. These characteristics make the S-type *SSU rRNA* gene a promising target for comparative studies to investigate evolutionary relationships and

determine the common ancestry among different *P. knowlesi* isolates.

Haplotype sharing of the S-type *SSU rRNA* gene was observed only between the *P. knowlesi* isolates collected from different areas in Sabah, Malaysian Borneo (haplotype 9 and haplotype 14). However, none of these haplotypes was identified among the *P. knowlesi* isolates from Sabah, Malaysian Borneo, and Peninsular Malaysia in this study. This could be partly explained by the existence of the South China Sea acting as a geographical barrier that inhibits genetic exchange between *P. knowlesi* isolates from Sabah, Malaysian Borneo, and Peninsular Malaysia. This is supported by a previous study that identified spatially distinct clusters of *P. knowlesi* isolates between Malaysian Borneo and Peninsular Malaysia using multilocus microsatellite genotyping[27]. Nevertheless, a recent whole-genome analysis of *P. knowlesi* isolates from Malaysian Borneo and Peninsular Malaysia revealed substantial genome-wide divergence[26].

One limitation of this study is that the primers used could only amplify a portion of the *SSU rRNA* gene. As a result, determining the genetic diversity and natural selection of the complete *SSU rRNA* gene was not possible. Additionally, this study exclusively focuses on *P. knowlesi* isolates from Sabah, Malaysian Borneo, and Peninsular Malaysia. Notably, no comparison of genetic diversity and natural selection of the *SSU rRNA* gene with other countries (such as Thailand and Indonesia), which are also experiencing a high number of *P. knowlesi* incidences, was conducted. Therefore, future studies should consider these limitations in their study design.

In conclusion, this study highlights that the genetic diversity of the S-type *SSU rRNA* gene is conserved among the *P. knowesi* isolates in Sabah, Malaysian Borneo and Peninsular Malaysia, and with purifying effect. This finding establishes it as a promising target for comparative studies to determine the evolutionary relationships and common ancestry of *P. knowlesi* species. The data of this study is beneficial for conservation and environmental management and public health officials, especially in understanding the transmission of *P. knowlesi* in Malaysia.

Conflict of interest statement

The authors declare no conflict of interests.

Funding

This study was supported by the Ministry of Higher Education, Malaysia (FRGS0322-SG-1/2013) and Universiti Malaysia Sabah (GUG0521-2/2020).

Authors' contributions

TYL, KHC, YALL, and PCL contributed to the study's concepts and design. JWFN performed the literature search. JWFN and TYL conducted experimental studies. Data acquisition involved JWFN, TYL, KHC, YALL, and PCL. Data analysis was conducted by ETJC and JWFN. ETJC was involved in statistical analysis and manuscript preparation. Manuscript editing and review were conducted by ETJC, KHC, YALL, and PCL. PCL is the guarantor of this study. ETJC and JWFN contributed equally to this study. All authors approved the final version of this manuscript for submission.

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Publisher's note

Edited by Lei Y, Zhang Q, Pan Y