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Investigation of genetic diversity of *Plasmodium knowlesi kelch13*-propeller region in Sabah, Malaysia

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ABSTRACT

Aims: The *kelch13* gene mutations of *Plasmodium falciparum* is associated with delayed parasite clearance after artemisinin-based combination therapy (ACT). It is unclear for *P. knowlesi* that is predominantly reported in Sabah. Therefore, this study aims to analyse the diversity of the *P. knowlesi kelch13* gene in five divisions of Sabah.
Methodology and results: Ninety-five blood samples infected with *P. knowlesi* were obtained. The DNA of *P. knowlesi* samples was extracted and the *kelch13* gene was amplified. The amplicons were cloned and sequenced. The sequencing data were aligned and analysed using MEGA 11 and DnaSP v6 software. A phylogenetic tree was constructed using the Neighbour-joining approach, which showed a diverse clade of *P. knowlesi* in Sabah, with a nucleotide diversity (π) of 0.451 and a haplotype diversity of 0.947. The deduced amino acid sequences were classified into 14 haplotypes, providing evidence of distinct *P. knowlesi* lineages in Sabah. When compared to *P. falciparum*, the *kelch13* sequences of *P. knowlesi* exhibited a higher π of 0.490 and haplotype diversity of 1.000, and similar mutations that conferred drug resistance to ACT in *P. falciparum* were detected in *P. knowlesi* in this study.
Conclusion, significance and impact of study: The *kelch13* gene of *P. knowlesi* isolates in Sabah has high nucleotide and haplotype diversities. Additionally, mutations conferring drug resistance to ACT in *P. falciparum* were identified in *P. knowlesi* in Sabah. The findings in this study can be used to better understand the emergence of drug resistance of *P. knowlesi* in Sabah.

Keywords: Artemisinin-based combination therapy, genetic diversity, *kelch13*, *Plasmodium knowlesi*, Sabah

INTRODUCTION

Malaria in humans is initially caused by four *Plasmodium* species: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The discovery of the zoonotic *P. knowlesi* species through polymerase chain reaction (PCR) detection in 2004 made it the fifth species contributing to the malaria mortality rate in Malaysia (Singh *et al*., 2004; Sabbatani *et al.*, 2010; Singh and Daneshvar, 2010). Between 2013 and 2017, about 16,500 malaria cases were reported in Malaysia, with the majority of cases from Sabah and Sarawak (Ang *et al*., 2020).

The epidemiological changes in *Plasmodium* species distribution in Malaysia's malaria cases shifted from being predominantly caused by human malaria parasites to simian malaria parasites (Rahim *et al*., 2020). As a result, the zoonotic *P. knowlesi* appears to be the most prevalent cause of malaria and the most extensively dispersed species in Malaysia (Brock *et al*., 2016). In 2008, Sabah reported over 80.6% of all malaria cases were naturally acquired *P. knowlesi* infections (Anderios *et al*., 2008). Since then, *P. knowlesi* cases have been increasing (Chin *et al*., 2021). Nevertheless, Malaysia has achieved zero indigenous human malaria cases since 2018. However, imported human malaria has increased (Ooi *et al*., 2023). By analysing the genetic diversity of *P. knowlesi* isolates, a recent study has revealed the distribution of *P. knowlesi* subpopulations in East and West Malaysia (Scott, 2020). This raises concerns about the prevalence of *P. knowlesi* with unique genetic variations in the country.

Early case diagnosis and treatment, which supports the use of artemisinin-based combination therapy (ACT) as a first-line treatment for all *Plasmodium* species and ACT therapies coupled with chloroquine to target *P. knowlesi* (Millar and Cox-Singh, 2015; Ahmed and Quan, 2019), is a fundamental strategy for malaria treatment (Ministry of Health Malaysia, 2014). It is worth noting that artemisinin is a therapy for *P. falciparum* infection used worldwide and approved by the World Health Organization. However, in recent years, frontline treatments for *P. falciparum* malaria have failed in parts of Southeast Asia, a historic epicentre for the emergence and spread of a multidrug-resistant co-lineage. Haplotypic analysis of the *kelch13* artemisinin resistance locus and

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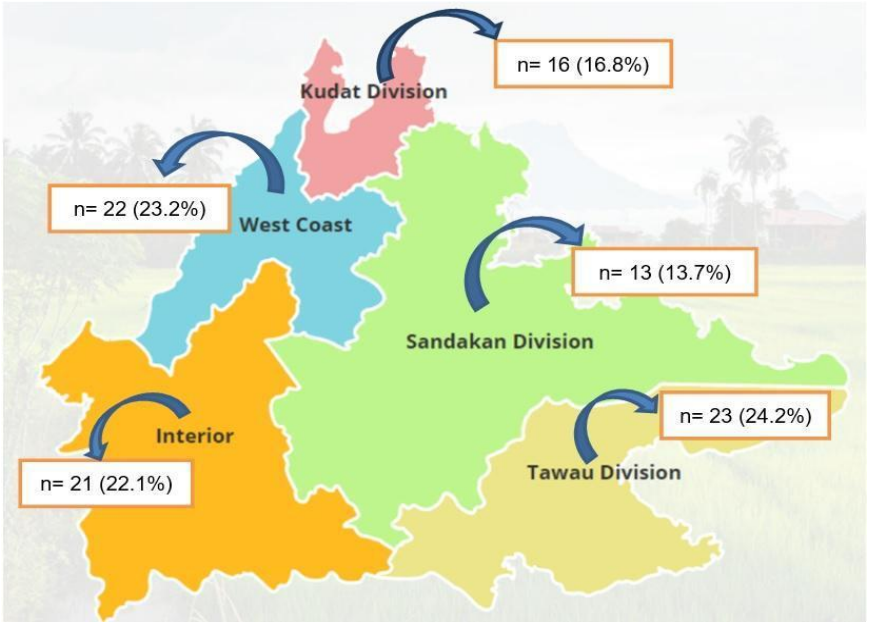

Figure 1: Number of samples collected from different divisions in Sabah for this study.

the plasmepsin 2-3 piperaquine resistance locus, named KEL1/PLA1, contribute to dihydroartemisinin-piperaquine failure (Hamilton *et al*., 2019). Artemisinin resistance has been documented throughout Myanmar, with a high prevalence of *P. falciparum* parasites carrying the *kelch13*-propeller mutations (Nyunt *et al*., 2015; Imwong *et al*., 2017).

ACT remains the most suitable first-line therapy for uncomplicated malaria caused by *P. falciparum* in the pre-elimination stage of malaria in Sabah, Malaysia. However, there is a lack of suspected or proven evidence of endemic artemisinin-resistant *P. falciparum* in Sabah (Grigg *et al*., 2018). An earlier study on the prevalence of *P. falciparum* molecular markers of anti-malarial drug resistance in a residual malaria focus area in Sabah also revealed no detected polymorphism in the propeller region of *kelch13* (Razak *et al*., 2016). Nevertheless, studies on *kelch13* associated with ACT susceptibility resistance have been previously documented in *P. falciparum* (Veiga *et al*., 2016; Miotto *et al*., 2020) and *P. vivax* (Faway *et al*., 2016).

In Sabah, *P. knowlesi* is recognized as a common cause of severe and fatal malaria (Flannery *et al*., 2018). As a result, the development of artemisinin resistance may lead to increased morbidity and mortality in Sabah, Malaysia (Rajahram *et al*., 2012). Therefore, this study investigated the genetic diversity of the *kelch13* gene of *P. knowlesi* in Sabah. This is the first study to analyse the prevalence of genetic variants of the *P. knowlesi kelch13* gene in Sabah, where malaria is endemic. The results from this study allow early detection of genetic variations in the *P. knowlesi kelch13* gene to anticipate possible drug resistance development and aid in developing new drugs or vaccines.

MATERIALS AND METHODS

Blood samples collection

A total of 95 blood samples infected with *P. knowlesi* were obtained from five divisions in Sabah: Tawau Division, Sandakan Division, Kudat Division, West Coast Division, and Interior Division (Figure 1). This study was conducted based on a retrospective data analysis of samples collected from June 2019 to June 2020. Ethical approval for this study was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-20-437-53883).

DNA extraction from blood samples

Following the manufacturer's instructions, the QIAamp® DNA Mini Kit (QIAGEN, Valencia, CA, USA) was used to extract DNA from 200 µL of blood samples. The DNA was resuspended in 50 µL of elution buffer and stored at -80 °C for further analysis.

Amplification of *kelch13* gene using nested PCR

Nested PCR was performed to amplify the *P. knowlesi's kelch13* gene using specifically designed primers (Integrated DNA Technologies, San Diego, CA). The primer design was based on the *P. knowlesi* sequence in the GenBank Database (accession number: XM_002259882). In the first round of nested PCR, 0.4 µM of *kelch13* forward primer (5'-GACCCCTCCACAAAAGTGAA3-3') and reverse primer (5'-ACCCCAGCTTCTCTTCTTCC-3') were used to amplify the full-length *kelch13* gene, which is 2139 bp. A

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total volume of 25 µL, containing 1× PCR buffer (Invitrogen, USA), 0.2 mM of dNTP, 3 mM of $MgCl_2$, 1 unit of *Taq* polymerase (Invitrogen, USA), 9.6 µL of distilled water and 5 µL of extracted DNA, was prepared. The first round of nested PCR was carried out under the following conditions: initial denaturation at 94 °C for 4 min, followed by 20 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 1 min and 30 sec, extension at 72 °C for 2 min and 30 sec and a final extension at 72 °C for 1 min. In the subsequent round of nested PCR, 0.3 µM of the *kelch13* forward primer (5'-CAAAAGTGAACGCTGCAAGA-3') and reverse primer (5'-CACAACCCCAGCTTCTCTTC-3') were used. A volume of 50 µL reaction mixture, consisting of 1× PCR Buffer (Invitrogen, USA), 0.2 mM of dNTP, 2 mM of $MgCl_2$, 1 unit of *Taq* polymerase (Invitrogen, USA), 33.6 µL of distilled water and 2.5 µL PCR amplicons from the first round of nested PCR, was prepared for the second round of nested PCR. The second round of nested PCR was carried out under the following condition: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 1 min, extension at 72 °C for 2 min and 15 sec and a final extension at 72 °C for 1 min. The presence of PCR amplicons was observed using 1% agarose gel electrophoresis.

Gel purification, cloning and direct sequencing

The PCR amplicons were purified from the gel using the GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia). The purified amplicons were ligated into the pEASY®-T1 cloning vector (Transgen Biotech, China) following the manufacturer's protocols. The ligated vector was then transformed into Trans1-T1 competent E. coli and blue-white screening was employed to select E. coli cells with the transformed vector. The transformed plasmids were isolated using the GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia) and subjected to direct sequencing.

kelch13 sequences alignment and analysis

Sequence alignment and analysis were performed as previously described (Chong *et al*., 2018; Chong *et al*., 2020). The *kelch13* sequences were aligned using the CLUSTAL-W tool in Molecular Evolutionary Genetic Analysis 11 (MEGA11) software (Tamura *et al*., 2021). The phylogenetic tree was constructed using the Neighbour-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates to assess the robustness and reliability of the tree. Sequence diversity (π) was determined by DnaSP v5.10 software (Librado and Rozas, 2009). The number of parsimony informative sites, polymorphic sites, synonymous and non-synonymous substitution, singletons, the number of haplotypes (H), and nucleotide diversity of the *kelch13* gene were also determined by comparing to *P. knowlesi* strain H (accession ID: XM_002259882) using the same software. Furthermore, Tajima's D test (Tajima, 1989) and Fu and Li's D* and F* neutrality tests (Fu and Li, 1993), implemented in the DnaSP v5.10 software, were used to investigate natural selection.

Comparison of the *kelch13* propeller domain between *P. falciparum* and *P. knowlesi*

The *kelch13* propeller domain was compared between *P. falciparum* (PF3D7_1343700 and PF3D7_0726400) and *P. knowlesi*. The *kelch13* sequence of the PF3D7_1343700 strain contains mutations previously reported to confer resistance to artemisinin (at nucleotide positions 1314, 1345, 1407, 1669, 1839 and 2082) (Ajogbasile *et al*., 2022) and an additional mutation at locus 1739 of the gene that was found in the PF3D7_0726400 strain (Dong *et al*., 2018; Zaw *et al*., 2020). The presence of these mutations was compared to the *kelch13* sequences of *P. knowlesi* in this study.

RESULTS

Genetic diversity of the *kelch13* gene of *P. knowlesi* in Sabah

The genetic diversity of the *kelch13* gene in this study is tabulated in Table 1. *P. knowlesi* from the Tawau division showed the highest number of variants based on the *kelch13* gene, with a total of 95 variations identified when compared to the *kelch13* gene of the reference *P. knowlesi* strain H. Testing of non-directional and directional pairs frequencies revealed an average of 94.2% nucleotide pair frequencies across all taxa, including 80 identical pairs, 7 transitional pairs, 6 transversional pairs and a 1.2 ratio of transitional pairs/ transversional pairs. The average nucleotide composition of T(U) = 30.2%, C = 26.3%, A = 23.2% and G = 17.9%. On the other hand, the Interior Division showed the lowest number of variants in the *kelch13* gene, with a total of 33 variations. The test of non-directional and directional pairs frequencies revealed an overall average nucleotide pair frequency of 27 identical pairs, 4 transitional pairs, 2 transversional pairs and a 2.5 ratio of transitional/transversional pairs. The average nucleotide composition of T(U) = 27.5%, C = 34.0%, A = 25.3% and G = 13.3%.

Phylogenetic tree of *kelch13* gene of *P. knowlesi* in Sabah

Figure 2 displays the topology of the phylogenetic tree, which is divided into 6 branches. The *P. knowlesi* strain H, marked as diamond red, grouped with 27 samples from different divisions in the same clade. Among these 27 samples, 9 were from the Tawau division, 6 from the Kudat division, 5 from the Interior division, 4 from the Sandakan division and 3 from the West Coast division. The tree topology suggests a close relationship among the *kelch13* gene distribution of *P. knowlesi* in Sabah.

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Table 1: The genetic diversity of the *kelch13* gene in five divisions in Sabah.

Division	No. of sequences	Site use of variable	Nucleotide pair frequency			Nucleotide composition				
			Identical pair	Transitional pair	Transversional pair	R	T(U)	C	A	G
Tawau	24	95	80	7	6	1.2	32.6	26.3	23.2	17.9
Westcoast	23	90	79	6	5	1.3	35.2	31.5	17.7	15.6
Sandakan	14	49	36	8	5	1.7	34.3	27.6	20.1	17.9
Kudat	17	46	38	4	3	1.2	28.8	32.2	18.4	20.7
Interior	22	33	27	4	2	2.5	27.5	34.0	25.3	13.3

Note: All frequencies are averages (rounded) over all taxa.

Table 2: Comparison of the reported nucleotide mutations in *P. falciparum* with *P. knowlesi* from five divisions in Sabah.

P. falciparum PF3D7_1343700 (references)	Sample of *P. knowlesi* from five divisions of Sabah	Sample *P. knowlesi* (Strain H)
K438N (nucloetide 1314) AAA Lysine (Lys) to AAT Asparagine (Asn)	Not detected in this locus	Not detected in this locus
G449S (nucleotide 1345) GGT Glycine (Gly) to AGT Serine (Ser)	Not detected in this locus	Not detected in this locus
C469F (nucleotide 1407) TGC Cysteine (cys) to TTC Phenylalanine (Phe)	ID23 TGC to TTC	Not detected in this locus
A557S (nucleotide 1669) GCA Alanine(Ala) to TCA Serine(Ser)	ID57 GCA to TCA ID560-3 GCA to TCA	Not detected in this locus
Q613H (nucleotide 1839) CAA Glutamine (Gln) to CAT Histidine (His)	KDT 494 CAA to CAC	Not detected in this locus
N694K (nucleotide 2082) AAT Asparagine (Asn) to AAA Lysine (Lys)	ID23 AAT to AAC SDK576 AAT to AAC SDK576-1 AAT to AAC	AAT - AAC
C580Y Locus position: 1739 Cysteine (Cys) TGT to Tyrosine (Tyr) TAT	Not detected in this locus	Not detected in this locus

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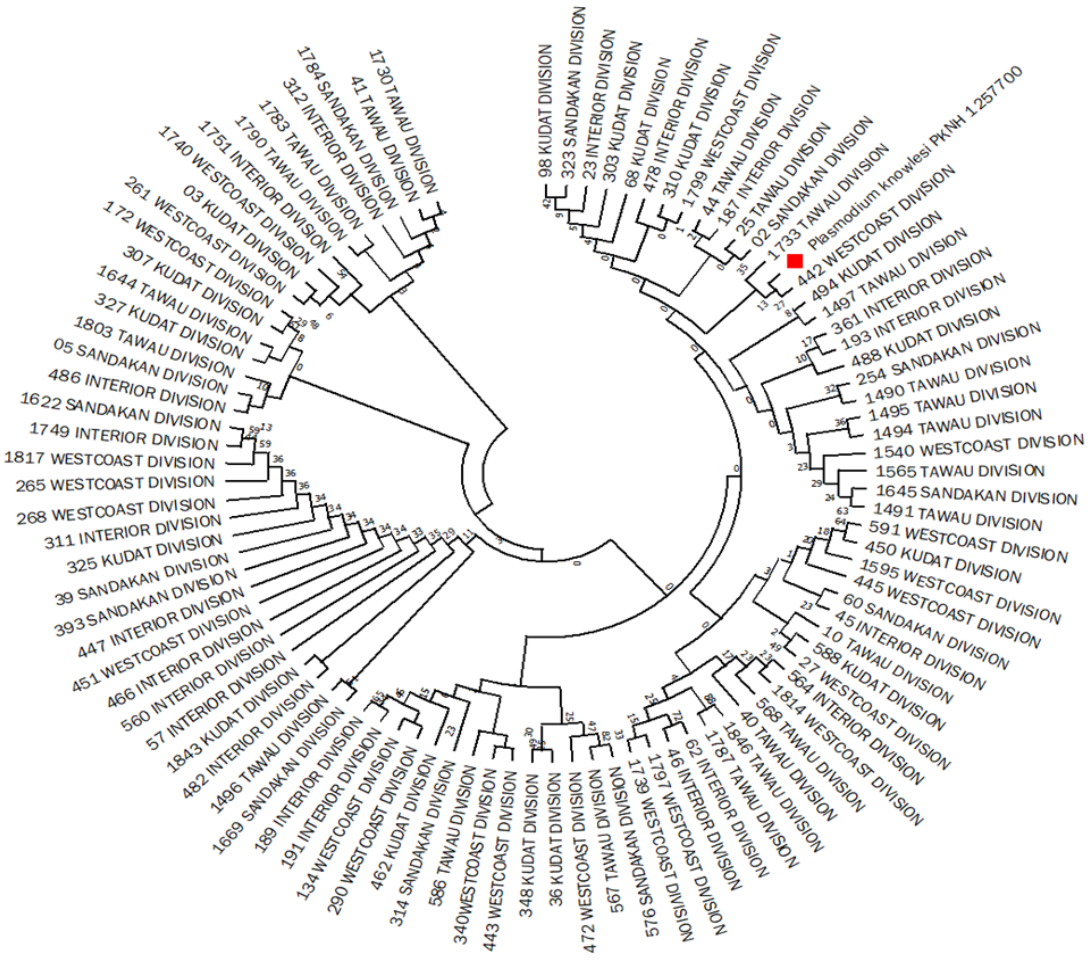

Figure 2: Phylogenetic tree based on the 95 *kelch13* sequences of *P. knowlesi* from five divisions in Sabah. The tree is constructed using a Neighbour-joining approach with 1000 bootstrap replications. The *P. knowlesi* strain H is indicated as diamond in red.

Haplotype-based analyses of genetic variability of the *kelch13* gene

A predominant haplotype with the highest frequency was observed in haplotype 4, which consisted of four sequences. This was followed by haplotype 7 with three sequences and haplotype 6 with two sequences (Figures 3a and 3b). The remaining haplotypes had only one sequence each. Figure 3c shows the genetic distances of the phylogenetic tree with the 20 haplotype sequences of the *kelch13* gene. The haplotype sequences were clustered based on the presence of mutation, with sequences harbouring mutations clustering away from haplotype 1.

Neutrality-based analysis of the *kelch13* gene in *P. knowlesi*

Tajima's D was -0.40 in this study, whereas Fu and Li's D and F were 0.84 and 0.54, respectively. These results suggest an excess of rare variation consistent with population expansion. Analysis of polymorphism-related *kelch13* genes from the five divisions in Sabah showed that Tajima's D became more positive toward the variant distribution.

Variations comparison between the *kelch13*-propeller domain of *P. knowlesi* and *P. falciparum*

Two amino acid changes were identified in the *kelch13* gene of *P. knowlesi* in Sabah when comparing the targeted seven mutations that resulted in changes in amino acids and conferred artemisinin drug resistance in *P. falciparum*. Table 2 shows the presence of mutations in *P. knowlesi* samples collected from the five divisions of Sabah. Two samples from the Interior Division exhibited mutations at nucleotide position 1669 (A557S, a non-synonymous mutation) and 1407 (C469F, a synonymous mutation). These mutations led to amino acid alterations from alanine (GCA) to serine (TCA) and cysteine (TGC)

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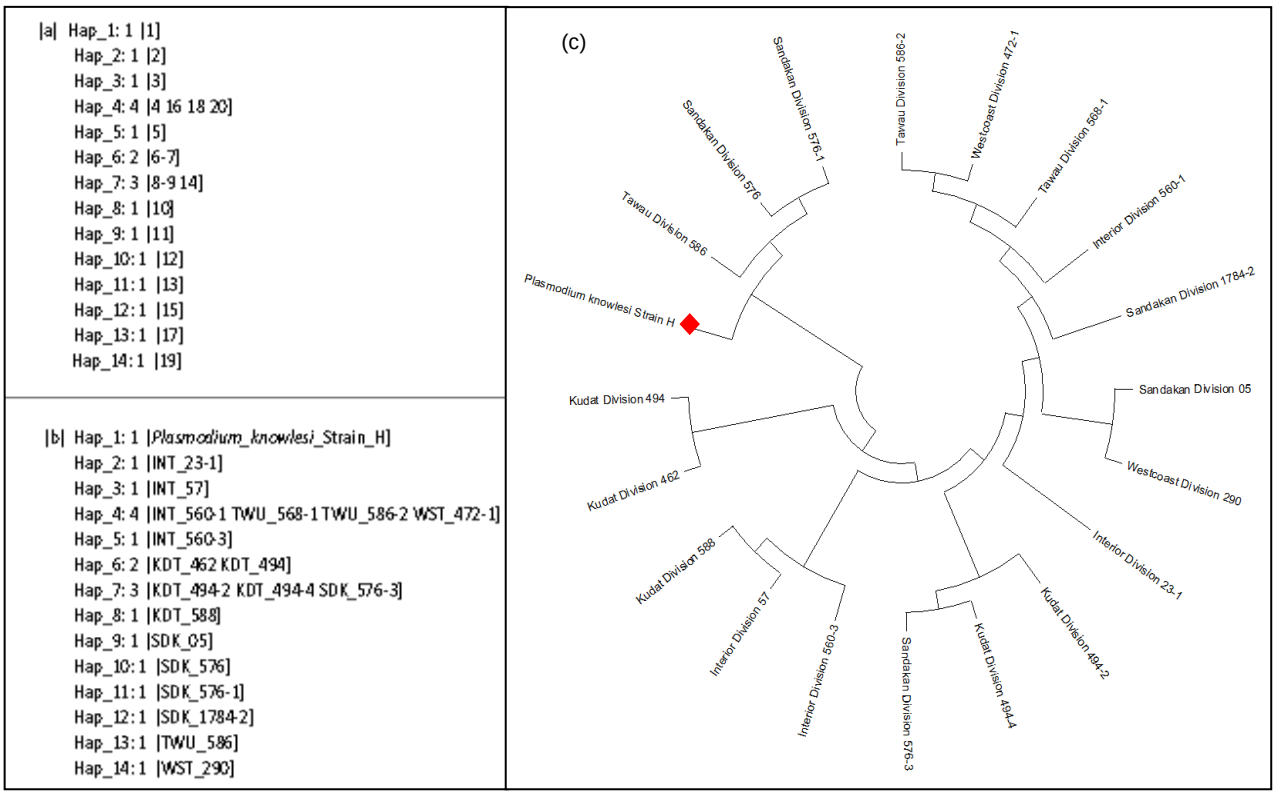

Figure 3: Genetic variability of *kelch13* gene. (a) Summary of haplotype frequencies; (b) Haplotype frequencies based on different divisions; (c) A Neighbour-joining phylogenetic tree of the *kelch13* haplotype sequences of *P. knowlesi* in this study with the *P. knowlesi* strain H indicated as red diamond as the reference.

to phenylalanine (TTC), respectively. Further analysis identified nucleotide changes at position 1839 (Q613H), resulting in a non-synonymous mutation from glutamine (CAA) to histidine (CAC) in a sample from the Kudat Division (sample ID: KDT494). Surprisingly, these changes were not detected in the reference strain *P. knowlesi* strain H. This indicates a potential risk of drug resistance development in *P. knowlesi* strains from the Interior and the Kudat Division of Sabah. Meanwhile, changes in the nucleotides AAT to AAC were observed in samples ID23, SDK576, and SDK576-1, but did not lead to any changes in amino acids.

DISCUSSION

Malaysia has eliminated human malaria transmission since 2018. However, reports on zoonotic *P. knowlesi* infection have increased significantly in 2019 and 2020 (Dian *et al*., 2022). The number of *P. knowlesi* notifications in the five divisions of Sabah doubled from 309 in 2019 to 649 in 2020 (unpublished data, Department of Health Sabah). Although the increased prevalence of *P. knowlesi* infections can be linked to several factors, such as improved diagnostic capacity, a decline in human malaria cases and increased knowledge of *P. knowlesi*, the exact causes remain unclear. The phylogenetic tree in this study included samples from all five divisions in Sabah, depicting the relationship between the *kelch13* gene in *P. knowlesi* samples with *P. knowlesi* strain H as the reference. The tree's topology structure reveals that all *kelch13* genes of *P. knowlesi* samples found in Sabah are closely related to each other but are grouped into different clades. This suggests the presence of genetic variations within the *kelch13* gene of *P. knowlesi* isolates in this study.

The current observations of diversity and mutations in the *kelch13* gene in *P. knowlesi* might lead to future drug resistance to artemisinin, which is the first-line treatment for malaria infection in Malaysia (Ministry of Health Malaysia, 2014). A previous study on the surveillance of artemisinin resistance and therapeutic efficacy *in vivo* identified no endemic artemisinin resistance in *P. falciparum* (Grigg *et al*., 2018). Hence, for uncomplicated malaria in Sabah, ACT remains the first line of treatment. However, this study revealed high variability in the *kelch13* gene from all the divisions investigated, especially in the Tawau Division. Tawau served as the focal point of malaria infections during the 1970s and 1990s, a period marked by industrialization across the state of Sabah in Malaysia (Jayaraj *et al*., 2017). The deforestation efforts aimed at transforming the region into oil palm plantations indirectly facilitate interactions between *P. knowlesi* and its hosts, consequently leading to the expansion of malaria infections within the division.

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In contrast to the Tawau Division, the Interior Division exhibits a low degree of variability in the *kelch13* gene. This could be attributed to the relatively small percentage of land undergoing deforestation and agricultural expansion, which is closely associated with global changes in the dynamics and geographic distribution of malaria and other vector-borne diseases (Joveen-Neoh *et al*., 2011; Hu *et al*., 2021). The *kelch13* 3' terminal propeller region showed significantly higher haplotype and nucleotide variabilities, particularly at synonymous sites compared to non-synonymous sites. This suggests that the observed variants in the *kelch13* gene may be the result of random and neutral evolutionary processes. The Tajima's D test in this study yielded a negative value, further confirming that there was no evidence of an excess of uncommon variation inconsistent with either population expansion or positive selection. However, interpreting the observed trends in *kelch13* requires a deeper understanding of the genetic diversity across the *P. knowlesi* genome (Escalante and Pacheco, 2019).

To gain a better understanding of the selective forces acting on *kelch13* in various *Plasmodium* species, including both human and simian malaria parasites, further research is necessary to investigate the patterns of variation in *kelch13* across a broader range of species (Grigg *et al*., 2014). Despite the mutations detected in the *kelch13* gene of *P. knowlesi* in this study that might confer resistance to ACT, no widespread artemisinin-resistant variants were reported in the five divisions in Sabah. This may be explained by host immune pressure, as a similar study reported no resistance despite the high genetic diversity and significant indications of positive natural selection in the core region of MSP7D (Ahmed and Quan, 2019). There is no evidence of endemic artemisinin-resistant *P. falciparum* in the pre-elimination setting in Sabah, Malaysia to date (Grigg *et al*., 2018).

CONCLUSION

This study identified two mutations previously reported to confer resistance to ACT. The discovery of high diversity and mutations in the *kelch13* gene of *P. knowlesi* in Sabah raises concerns about the potential development of drug resistance towards ACT. The genetic diversity information obtained from this study can significantly benefit epidemiology researchers by shedding light on new targets for intervention or biomarkers that can enhance the understanding of malaria disease control efforts.

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