Original Article

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Mutations in *Plasmodium knowlesi* Kelch protein 13 and the dihydropteroate synthase gene in clinical samples

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ABSTRACT

Objective: To determine the genetic diversity, natural selection and mutations in *Plasmodium* (*P.*) *knowlesi* drug resistant molecular markers *Kelch 13* and *dhps* gene in clinical samples of Malaysia.

Methods: *P. knowlesi* full-length gene sequences *Kelch 13* gene (*PkK13*) from 40 samples and *dhps* gene from 30 samples originating from Malaysian Borneo were retrieved from public databases. Genetic diversity, natural selection, and phylogenetic analysis of gene sequences were analysed using DNAsp v5.10 and MEGA v5.2.

Results: Seventy-two single nucleotide polymorphic sites (SNPs) across the full-length *PkK13* gene (63 synonymous substitutions and 9 non-synonymous substitutions) with nucleotide diversity of $\pi\sim0.005$ was observed. Analysis of the full-length *Pkdhps* gene revealed 73 SNPs and $\pi\sim0.006$ (44 synonymous substitutions and 29 non-synonymous substitutions). A high number of haplotypes (*PkK13*; H=37 and *Pkdhps*; H=29) with haplotype diversity of Hd ~0.99 were found in both genes, indicating population expansion. Nine mutant alleles were identified in PkK13 amino acid alignment of which, 7 (Asp^{3}Glu, Lys^{50}Gln, Lys^{53}Glu, Ser^{123}Thr, Ser^{127}Pro, Ser^{149}Thr and Ala^{169}Thr) were within the *Plasmodium* specific domain, 2 (Val^{372}Ile and Lys^{424}Asn) were in the BTB/POZ domain and no mutation was observed within the kelch propeller domain. The 29 non-synonymous mutations in the *Pkdhps* gene were novel and only presented in exon 1 and 2.

Conclusions: Monitoring the mutations from clinical samples collected from all states of Malaysia along with clinical efficacy studies will be necessary to determine the drug resistance in *P. knowlesi*.

KEYWORDS: *Plasmodium knowlesi*; *Kelch 13*; *dhps*; Dihydropteroate synthase; Drug resistance; Mutation

1. Introduction

Emerging infectious diseases originating in wildlife pose a serious threat to the world health, security, and economic growth, so preventing their spread is a top concern for public health agencies. Humans and non-human primates share a high degree of genetic and physiological similarities, which makes them vulnerable to the majority of infections that can cross primate species boundaries. One such example is *Plasmodium* (*P.*) *knowlesi* (Pk) which crossed the species boundaries and started infecting humans in Southeast Asian countries and is now the most common malaria parasite in the region[1]. It poses a challenge to malaria elimination efforts in Southeast Asia. Infection with *P. knowlesi* can quickly lead to high parasitaemia, and the risk of severe disease in adults is as high as that of falciparum malaria[2]. Thus, early diagnosis and treatment are critical in terms of reducing morbidity and mortality.

Significance

Mutations in the *K13* and *dhps* genes are the key predictors for anti-malarial drug resistance associated with artemisinin and sulfadoxine in the field. This study gives first-hand information on the genetic diversity, natural selection, and mutations within these two genes from patients infected with *Plasmodium knowlesi* malaria.

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Proguanil was used in Malaysia in the late 1940s, however, resistance developed quickly. Pyrimethamine resistance was noted in the middle of the 1970s[3], although sulfadoxine-pyrimethamine (SP) was used as a first-line drug for more than 30 years until artemisinin-combination therapy (ACT) was launched in 2009. As per the Ministry of Health, Malaysia, the first-line treatment for *P. knowlesi* and *P. malariae* suspected cases is ACT (artemether-lumefantrine or artesunate-mefloquine), for the states of Sabah and Sarawak in Peninsular Malaysia. However, in remote regions of Sabah and Sarawak in Malaysian Borneo, single dose pre-referral SP for falciparum malaria is still being used[4]. Since *P. knowlesi* and *P. falciparum* are morphologically indistinguishable microscopically during the early ring stages and because the two parasites can co-infect, it is quite possible that *P. knowlesi* parasite populations in Malaysia were exposed to SP. The enzyme dihydropteroate synthase is the target of sulfadoxine, and sequencing of the dihydropteroate synthase gene has shown amino acid mutations in codons 436, 437, 540, 581 and 613 confer drug resistance in *P. falciparum*[5,6]. A triple dihydropholate reductase mutation (N51I/C59R/S108N) combined with a double dhps mutation (A437G/K540E) is a useful predictor of clinical SP therapy failure in *P. falciparum*[7].

Over the last two decades, ACTs have made a considerable contribution to lowering malaria's global impact[8,9]. The first synthetic antimalarial, chloroquine, was used to treat malaria on a global scale. *P. falciparum*, the parasite that causes the most lethal type of human malaria, has developed resistance to chloroquine and, other antimalarial drugs such as sulfadoxine and pyrimethamine, mefloquine, and quinine became resistant in various malaria endemic countries[10]. Parasite resistance to artemisinin, the most effective and novel drug used against malaria infection worldwide, is now being reported in many research studies; as a result, first-line treatment strategies, including ACT, are becoming less effective in areas where drug resistance is widespread[11,12]. Artemisinin resistance has been found in Cambodia, Lao People's Democratic Republic, Myanmar, Thailand, and Vietnam[15]. The use of artemisinin in monotherapy and in combination (ACT), the most effective and newest drug in use, is being reported in many research studies. It should be noted that the WHO advises against the use of artemisinins in monotherapy, as it is one of the causes of increasing resistance to this drug, and may jeopardise the use of ACTs[13,14]. Artemisinin resistance has been found in Cambodia, Lao People's Democratic Republic, Myanmar, Thailand, and Vietnam[15]. Recent research has revealed that mutations in the *P. falciparum Kelch 13* propeller region as a molecular marker of ACT resistance, which is associated with delayed parasite clearance *in vitro* and *in vivo*[13]. The *kelch* gene is composed of three domains: a *Plasmodium*-specific domain, a BTB/POZ (Broad-complex, tramtrack and bric-à-brac) domain, and the kelch propeller domain[16]. Researches have reported several

SNPs across the full-length *P. falciparum K13* gene, however, only 20 of the 124 non-synonymous *Kelch 13* propeller mutations have been linked to resistance to artemisinin so far[17–20]. Thus, mutations within the propeller region are established as a strong and reliable marker for *P. falciparum* artemisinin resistance in field isolates. In the past 9 years (2011-2019), 8 473 indigenous *P. knowlesi* malaria cases have been reported from Sarawak, Malaysian Borneo which accounted for more than 60%-70% malaria cases in Malaysia[21–23]. Previous drug resistance studies on *P. knowlesi* were limited to clinical efficacy studies and antimalarial drugs such as artemisinins (Artesunate-mefloquine) and chloroquine were found to be effective for uncomplicated knowlesi cases[24,25]. A study on *Pkdhfr* gene polymorphism from Sabah identified 14 non-synonymous mutations across the full-length gene along with a moderate level of genetic diversity and no selection pressure[4]. Given the importance of studying the *Kelch 13* and *dhps* gene polymorphisms in other *Plasmodium* species, this study was conducted to report on the pattern of polymorphisms across the full-length gene and determine genetic diversity and natural selection in *P. knowlesi* clinical samples. The study provides new knowledge and information crucial for malaria control strategies in Southeast Asian countries.

2. Subjects and methods

2.1. Sequence data

Forty full-length *PkK13* gene (2 139 bp) sequences and 30 *Pkdhps* (2 696 bp) sequences were retrieved from public genomic databases (https://www.ebi.ac.uk/ena/browser/home), with majority of them originating from clinical isolates from Malaysian Borneo (*n*=36) and long-time isolated parasite lines from Peninsular Malaysia (*n*=4) including the reference sequence *PkK13*; PKNH_1257700 and *Pkdhps*; PKNH_1429900) for H-strain[26]. Only high quality DNA sequences were retrieved for analysis. Accession numbers of sequences used for this study are listed in Supplementary Table 1.

2.2. Data analysis

The sequence data were aligned by using the CLUSTAL-W program in MegAlign Lasergene v 7.0 (DNASTAR) and converted to FASTA format for further analysis. Sequence diversity (π) was determined using the DnaSP v5.0 software[27], as a measure of genetic diversity. DnaSP software was also used to determine the number of polymorphic sites, synonymous substitutions (silent mutations), non-synonymous substitutions (replacement changes), singleton sites, haplotypes (H), and the haplotype diversity in *P. knowlesi Kech13* and *dhps* gene using DNAsp software. The

Table 1. Estimates of nucleotide diversity, haplotype diversity and neutrality indices of *PkK13* and *Pkdhps* gene.

No. of samples	SNPs	Syn	NonSyn	No. of haplotypes	Mean diversity±SD		Codon based Z-test dS-dN	Fu & Li's D*	Fu & Li's F*	Taj D
					Haplotype	Nucleotide				
40	72	63	9	37	0.995±0.007	0.005 02±0.000 24	6.08*	-1.89	-2.01	-1.32
30	73	44	29	29	0.998±0.009	0.006 73±0.003 60	4.60*	-1.86	-1.86	-1.01

*P<0.05. Syn: synonymous substitution. NonSyn: non-synonymous substitution; SD: Standard deviation; Taj: Tajima's.
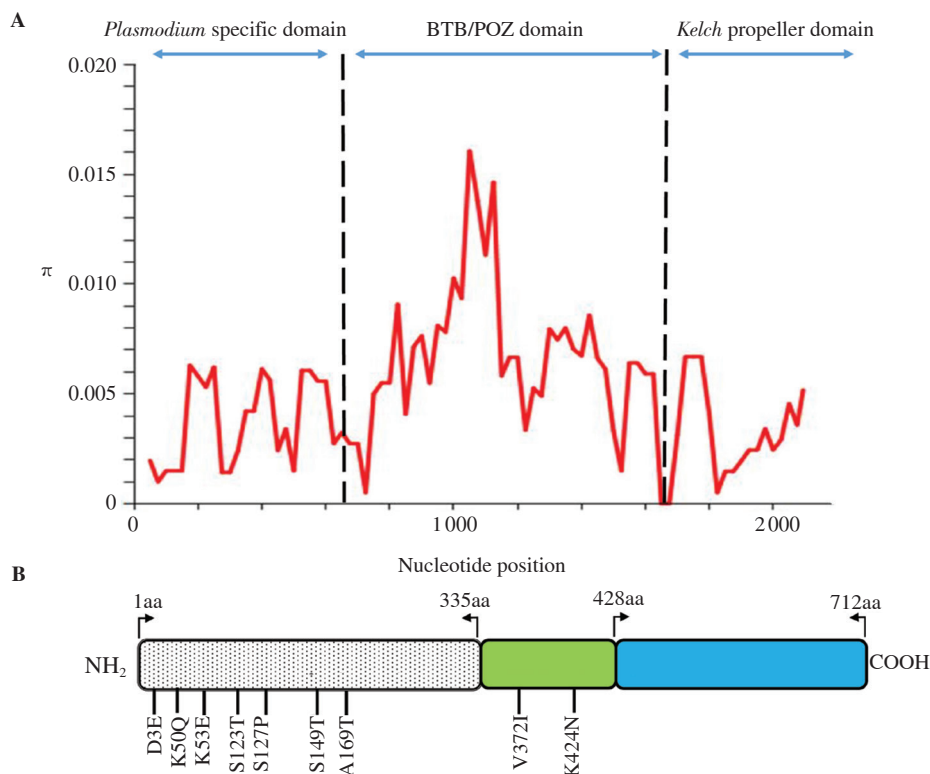

Figure 1. Graphical representation of nucleotide diversity across the *Plasmodium knowlesi kelch* gene. (A) The three domains: *Plasmodium* specific domain, BTB/POZ domain and *Kelch* propeller domain. (B) Schematic representation of *Plasmodium knowlesi K13* gene mutations from 40 samples.

same software was used to generate a graphical representation of nucleotide diversity, with a window length of 100 bp and a step size of 50 bp.

Natural selection was determined by calculating the rates of synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) as computed by using Nei and Gojobori's method and robustness were estimated by the bootstrap method with 1 000 pseudo replicates as implemented in the MEGA 5.0 software[28]. The difference between dN and dS was determined by applying codon-based *Z*-test (*P*<0.05) in MEGA software v.5 with 1 000 bootstrap replications[28]. The Tajima's D, Fu & Li's D* and F* neutrality tests were performed as implemented in DnaSP v5.10 software. Tajima's D is expected to be zero under neutrality. When Tajima's D values are positive and statistically significant, which indicates positive/balancing selection, whereas negative values indicate negative selection or population expansion. For domain-wise analysis of *P. knowlesi Kelch 13* mutations within the clinical isolates, deduced amino acid from all 40 sequences were aligned along with the *P. falciparum Kelch 13*

sequence (PF3D7_1343700), and amino acid changes observed in each domain are shown as a schematic diagram. A similar analysis was conducted for 30 amino acid sequences of *Pkdhps*.

2.3. Phylogenetic analyses

Phylogenetic analyses of the *P. knowlesi Kelch 13* were performed using deduced amino acid sequences using maximum likelihood approach based on the Poisson correction model as in MEGA 5.0.[28] along with the closest ortholog sequences of *P. coatneyi*, *P. cynomolgi*, *P. vivax* and *P. falciparum*.

3. Results

3.1. Sequence alignment, polymorphism and genetic diversity analysis of PkK13 and Pkdhps

Seventy-two SNPs (63 synonymous and 9 nonsynonymous

Table 2. Domain wise alleles observed within clinical isolates of *Plasmodium knowlesi* Kelch 13 protein.

Domain	Amino acid position	Allelic variant (wild/mutant)	Amino acid full forms	Number (%) of isolates (*n*=40)
Plasmodium specific domain	3	D (wild)	Aspartic acid	38 (95)
		E (mutant)	Glutamic acid	2 (5)
	50	K (wild)	Lysine	39 (98)
		Q (mutant)	Glutamine	1 (2)
	53	K (wild)	Lysine	39 (98)
		E (mutant)	Glutamic acid	1 (2)
	123	S (wild)	Serine	39 (98)
		T (mutant)	Threonine	1 (2)
	127	S (wild)	Serine	38 (95)
		P (mutant)	Proline	2 (5)
	149	S (wild)	Serine	39 (98)
		T (mutant)	Threonine	1 (2)
	169	A (wild)	Alanine	39 (98)
		T (mutant)	Threonine	1 (2)
BTB/POZ Domain	372	V (wild)	Valine	39 (98)
		I (mutant)	Isoleucine	1 (2)
	424	K (wild)	Lysine	39 (98)
		N (mutant)	Aspargine	1 (2)

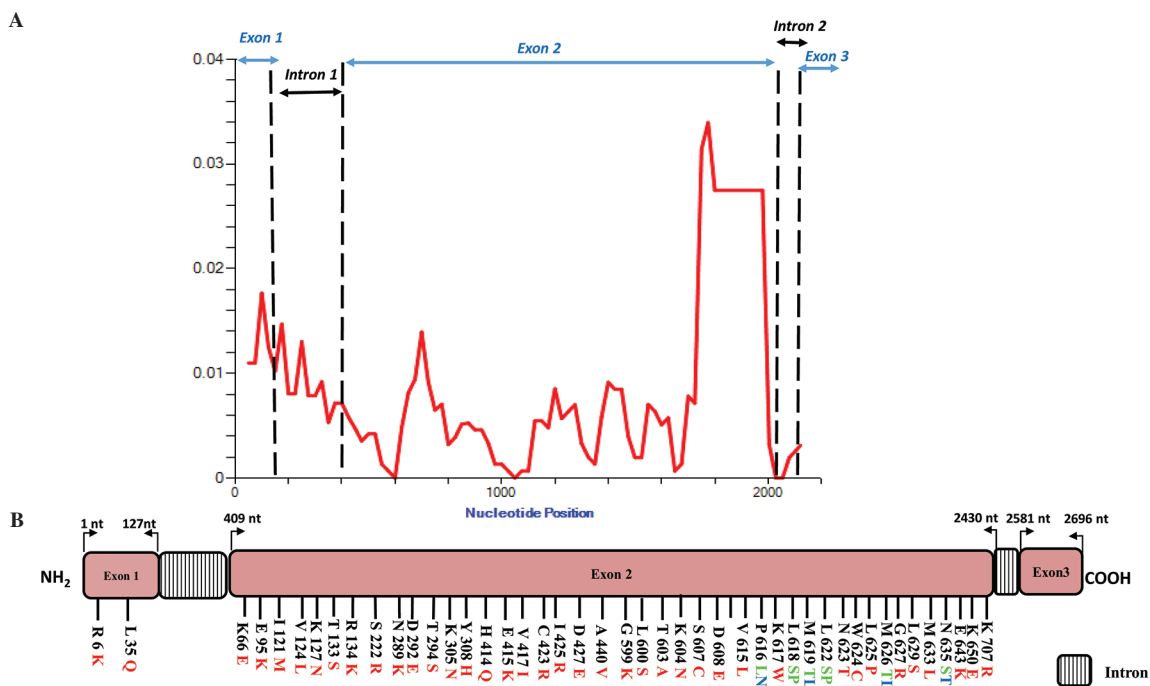The wild type alleles were considered based on the reference line *i.e. Plasmodium knowlesi* H-strain.

Figure 2. Graphical representation of nucleotide diversity across the *Plasmodium knowlesi dhps* gene. (A) The diversity within the three exons: exon 1, exon 2 and exon 3. (B) Schematic representation of *Plasmodium knowlesi dhps* gene mutations from 30 samples. Amino acid with tri-variants or triple mutants are shown in blue.

mutations) were identified in DNA sequence alignment of 40 full-length *P. knowlesi* Kelch with 13 sequences (Table 1). Thirty-three singleton sites (site having only one nucleotide in among the sequences) were found in 38 parsimony informative sites (sites that have a minimum of two nucleotides that are present at least twice), and among them, 32 had two variants and 1 had three variants (C573A/T). The overall nucleotide diversity was found to be $\pi=0.005\,02$. The graphical representation of nucleotide diversity indicated that SNP variations were throughout the gene; however, the central BTB/POZ domain had higher number of SNPs (Figure 1A).

The number of haplotypes was found to be high (H=37), leading to higher haplotype diversity (Hd=0.995) (Table 1). Alignment and comparison of 40 full-length deduced amino acid sequences of *P. knowlesi* Kelch 13 protein revealed that there were 9 mutations (due to 9 non-synonymous substitutions). Domain-wise analysis showed that out of the 9 mutations, 7 were at the *Plasmodium* specific region (Asp³Glu, Lys⁵⁰Gln, Lys⁵³Glu, Ser¹²³Thr, Ser¹²⁷Pro, Ser¹⁴⁹Thr and Ala¹⁶⁹Thr) and 2 mutations (Val³⁷²Ile and Lys⁴²⁴Asn) at the BTB/POZ domain (Figure 1B). The frequency of each allele was observed within the 40 clinical samples (Table 2).
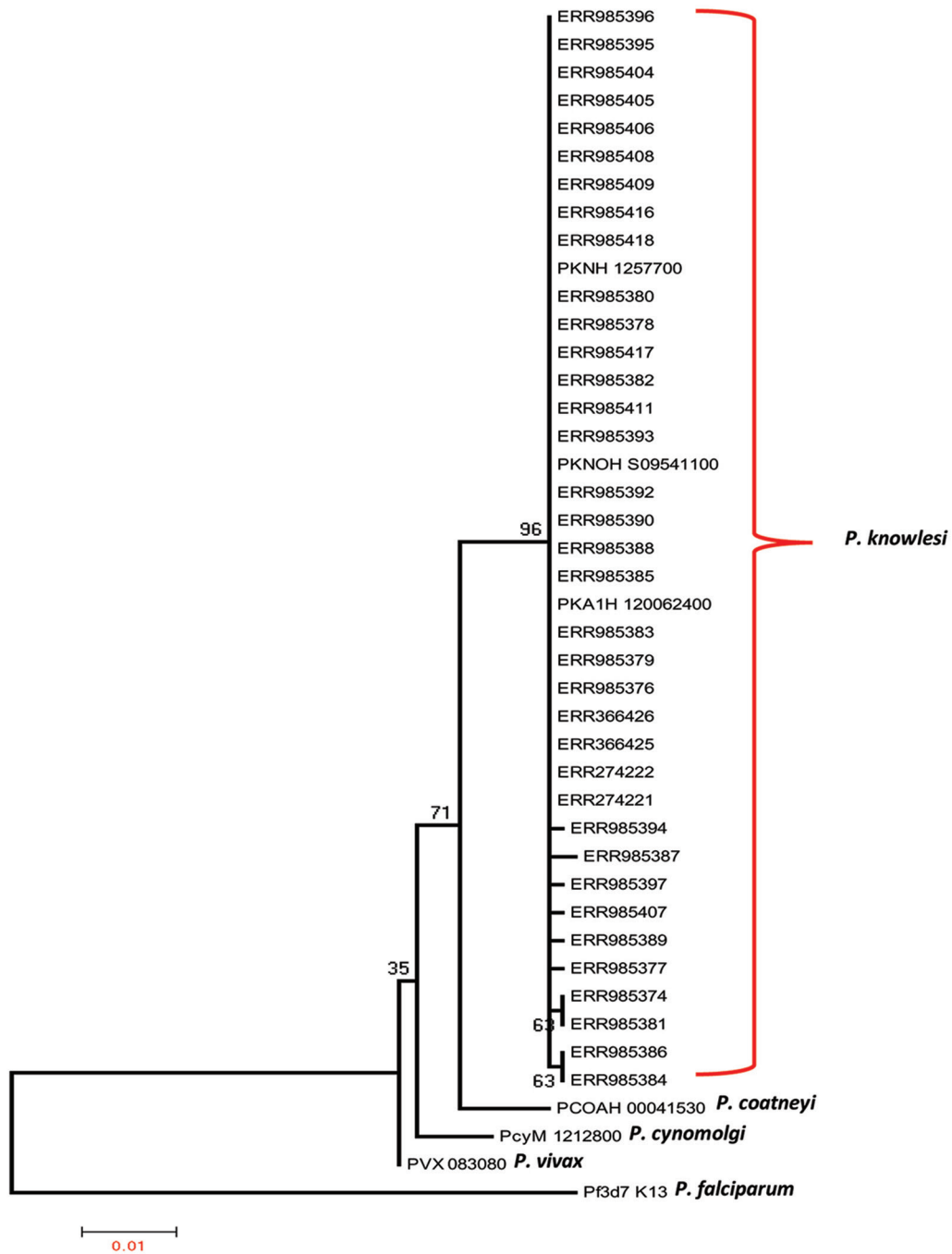
Figure 3. Phylogenetic relationship of *Plasmodium knowlesi Kelch* amino acid sequences from clinical isolates of Malaysia and its ortholog in other *Plasmodium* species using maximum likelihood method.

DNA sequence alignment of 30 full-length *P. knowlesi* dhps sequences revealed 73 SNPs (44 synonymous and 29 nonsynonymous mutations) (Table 1), which was higher compared to the *Kelch 13* gene, 35 parsimony informative sites and 37 were singleton sites. Among the 37 singleton sites, 35 were two variants and 2 were three variants (A1886G/C). The overall nucleotide diversity was found to be π=0.006 73. The graphical representation of nucleotide diversity indicated that SNP variations were found

across the full-length *dhps* gene (Figure 2A). The graph indicated that nucleotide diversity was higher towards the 3' end of the gene owing to the presence of a repeat region comprising the peptide sequence GSLNWLM. The non-synonymous substitutions (mutations) in each exon were shown in Figure 2B. No non-synonymous substitutions were noted in exon 3. The DNA polymorphism and amino acid polymorphism across the full-length gene were shown in Supplementary Figures 1 and 2, respectively. The number of

haplotypes was found to be high (H=29), leading to higher haplotype diversity (Hd=0.998) which was similar to the *PkK13* gene (Table 1).

3.2. Natural selection in PkK13 and Pkdhps

Natural selection analysis indicated strong purifying selection for both of the genes. dS-dN, Tajima's D as well as Li and Fu's D* and F* values were negative, indicative of negative/purifying selection (Table 1). Li and Fu's D* and F* and the high number of haplotypes due to the presence of high singleton sites were indicative of population expansion.

3.3. Phylogenetic analyses

Phylogenetic analysis of 40 deduced amino acid sequences of *P. knowlesi* K13 clinical isolates with other ortholog species using maximum likelihood indicated that they formed a single clade with closest ortholog in *P. coatneyi*, followed by *P. cynomolgi*, *P. vivax* and *P. falciparum* (Figure 3).

4. Discussion

P. knowlesi infections cases are increasing in Southeast Asian countries and multiple drug combinations have been reported to be effective such as artemether-lumefantrine, artesunate-mefloquine and chloroquine plus primaquine[25,29,30]. In the present study *Kelch 13* and *dhps* gene diversity and mutations associated with artemisinin and sulphadoxine resistance were studied from clinical samples of Malaysia. Though no mutations (non-synonymous substitutions) were observed with the Kelch propeller domain, indicating the ACT-therapy is still an effective treatment for *P. knowlesi* infections. All 29 mutations (non-synonymous substitutions) identified within the *Pkdhps* gene were novel and may indicate drug failure due to previous uncontrolled use of SP in remote areas of Malaysia. However, clinical efficacy study along with monitoring of mutations in *dhfr* and *dhps* genes would be necessary to confirm the association of these mutations with sulphadoxine and pyremethamine failure.

High levels of SNPs across the *Kelch 13* and *dhps* gene was indicative of recent population expansion (negative values for Tajima's D, Li and Fu's D* and F* values). Similar observation on recent population expansion was also seen in *P. knowlesi* dhfr gene which is a molecular marker of anti-folate drug resistance to pyremethamine[4]. The genetic diversity observed across the full-length *PkK13* and *Pkdhps* was similar to the *Pkdhfr*, indicating that both anti-folate as well as artemisinin derivatives could be effective antimalarials against knowlesi infections[4]. The absence of strong positive selection within the *PkK13* gene reflected the same

effectiveness. It is worth noting that despite strong negative selection within the *PkK13* sequences, we did not find geographical clustering across sequences from Malaysian Borneo and Peninsular Malaysia in maximum likelihood based phylogeny. Geographical clustering of gene sequences was observed in multiple genes of *P. knowlesi*[31–38]. Since this is the first study to report on the polymorphism across the full-length *Kelch 13* and *dhps* gene, the mutations observed would serve as a starting point for future surveillance studies with a higher number of clinical samples in all *P. knowlesi* affected countries of Southeast Asia. And since the data is from clinical samples from an endemic region (Sarawak, Malaysian Borneo), these polymorphisms would also serve as a baseline data for future interventions. Though no mutations were noted within the *Kelch* propeller domain, indicating ACT is still an effective drug for *P. knowlesi* infections, and periodic monitoring of mutations from clinical samples will be necessary to ascertain artemisinin and SP resistance in *P. knowlesi*.

In conclusion, moderate levels of diversity were observed for both *PkK13* and *Pkdhps* genes, and strong purifying/negative natural selection was observed for both the genes, indicative of population expansion. Future studies should focus on time to time monitoring of point mutations on these drug resistance molecular markers with samples originating throughout *P. knowlesi* endemic regions.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Author's contributions

AS contributed to the study conception, design, implemented the study, analyzed the data, interpreted the data and wrote the manuscript.

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