ORIGINAL ARTICLE

Analysis of *Plasmodium knowlesi* Mitochondrial DNA Aiming to Identify Molecular Markers Useful for Epidemiological Analysis of the Zoonotic Malaria in Sabah, Malaysia

*Shigeharu SATO[1,2], Nelbon Bin GILOI[3], Naziatul Azlin IBRAHIM[4], Jiloris Frederick DONY[4], Kamruddin AHMED[1,5,6] and Mohammad Saffree JEFFREE[3]

[1] Department of Pathology and Microbiology, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.
[2] School of Tropical Medicine and Global Health, Nagasaki University, Nagasaki 852-8523, Japan
[3] Department of Public Health Medicine, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.
[4] Kota Kinabalu Public Health Laboratory, Ministry of Health Malaysia, Jalan Kolam, Bukit Padang, 88850 Kota Kinabalu, Sabah, Malaysia
[5] Borneo Medical and Health Research Centre, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.
[6] Research Center for Global and Local Infectious Diseases, Oita University, Oita 879-5593, Japan

ABSTRACT

Introduction: Although no indigenous human malaria cases have been recorded since 2018 in Malaysia, people in the country, especially those in the two states in Borneo, are still at the risk of zoonotic malaria caused by *Plasmodium knowlesi*. For better control of *P. knowlesi* malaria, epidemiological understanding of the disease is essential. Molecular markers in the mitochondrial DNA are expected to be useful, but characterisation of the parasite's mitochondrial genome remains limited. **Methods:** From the total DNA extracted from *P. knowlesi* malaria blood samples collected in various districts in Sabah in 2020 and 2022, two overlapping DNA fragments covering a 2.3 kb region containing the parasite's entire *cox3* coding sequence were PCR amplified. Fragments of 12 different parasite strains were sequenced by direct Sanger sequencing and variations in the 2.3 kb region were identified by comparison with the reference sequence in the database. **Results:** Nine single nucleotide polymorphisms (SNPs)were identified in 10 strains investigated in this study. Seven of these SNPs were strain-specific, while remaining 2 were shared by 3 different strains each. In addition, one of the A clusters present in the reference sequence was extended in all the 12 strains investigated. **Conclusion:** The SNPs identified here will probably serve as useful molecular markers in epidemiological study of *P. knowlesi* in Sabah. The fact that 9 SNPs were identified in the 2.3 kb region of only 12 strains analysed proves that the mitochondrial DNA is a good source of molecular markers useful in *P. knowlesi* research.

Keywords: Genetic variation; Malaria; Mitochondrion; Molecular marker; *Plasmodium knowlesi*

Corresponding Author:
Shigeharu Sato, PhD
Email: ukssato@gmail.com
Tel: +608-8320000

INTRODUCTION

Malaria has been one of the most harmful infectious diseases in human history. This disease is caused by protozoan parasites classified to the genus *Plasmodium* and its transmission is mediated by female anopheline mosquitoes. Four *Plasmodium* species - *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* - cause most malaria cases in the world, and each species cannot survive without infecting humans. Thus, traditional malaria caused by these four parasite species is supposed to be controllable by combining vector control and patient control. Indeed, thanks to the national anti-malaria programs rolled out since 1960s, malaria burden in Malaysia has been drastically reduced. There were as many as 243,870 malaria cases reported in 1931, but the annual case number was reduced to 44,226 by 1980 (1). The reducing trend continued further, and no indigenous malaria cases caused by any of the 4 *Plasmodium* species above have been recorded since 2018 (2).

As the number of traditional human malaria cases was reduced, it became clear that another, zoonotic malaria caused by *P. knowlesi* is spreading in Malaysia, especially in the two states in Borneo (3-5). All of the 4131 non-imported malaria cases reported in Malaysia

in 2018 were of *P. knowlesi* malaria (2). Unlike the 4 *Plasmodium* species causing traditional malaria, *P. knowlesi* is naturally maintained in wild macaque populations. Evidence reported in literature suggest that human-mosquito-human transmission of *P. knowlesi* is not impossible, though such events seem to occur only extremely rarely if they do (6). As a result, the parasites infecting human patients likely contribute little on parasite population maintenance. Control of *P. knowlesi* malaria is expected to be much more difficult to achieve than control of traditional human-specific malaria. Therefore, better understanding of the epidemiology of the disease is critically important for effective control of the zoonotic disease.

The entire genome of *P. knowlesi* has been already sequenced (7), and these days, genomic information of the entire genome of the parasite determined with the whole genome sequencing technology have been often used for population genetics analysis of *P. knowlesi* isolated from human malaria patients and wild macaques (8-11). Sequence information of the entire genome is definitely useful for detailed epidemiological analysis, but much lighter information focusing on a selected set of molecular markers in the genome is sufficient for the analysis. For example, for developing a LAMP system to rapidly detect parasites of a specific genotype, finding suitable molecular markers is critically important. In addition, analysis focusing on molecular marker is generally much less costly, quicker, and less tedious compared to the whole genome analysis.

The cell of *Plasmodium* species including *P. knowlesi* has the mitochondrion that contains its own genomic DNA. The mitochondrial DNA of *Plasmodium* species commonly consists of multiple copies of a 6 kb unit that form tandem repeat. Each 6 kb unit encodes 3 proteins (Cytochrome oxidase subunits 1 and 3, and cytochrome b) and fragmented large and small subunit rRNAs. It has been estimated that *P. falciparum* has on average 20 copies of the 6 kb mitochondrial DNA unit in a cell (12) while only one copy of the nuclear genome is expected to present in the haploid cell. By analogy, it is presumed that *P. knowlesi* cells have more copies of the mitochondrial DNA unit compared to the nuclear DNA. Thus, molecular markers present in the mitochondrial genome are expected to be detectable with a higher sensitivity compared to those in the nuclear genome.

Patients with *P. knowlesi* malaria may develop severe malaria and die when appropriate treatment is not given in time (13). It is not known if the parasite's genetics have anything to do with the severity of malaria the patient potentially develops. Some variations present in the mitochondrial DNA might be associated with the severity.

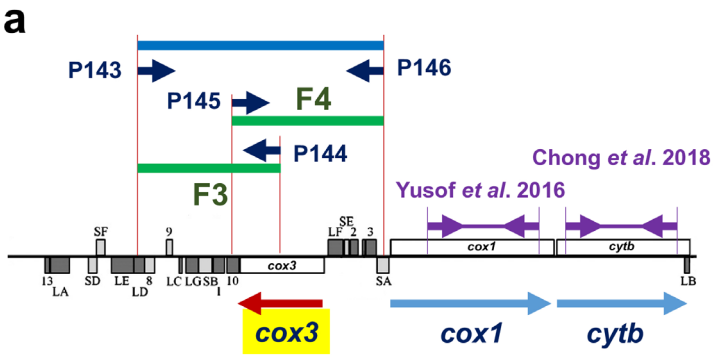
There was a study in which variations in the nucleotide sequence of the mitochondrial genome of *P. knowlesi* was analysed (14), though no *P. knowlesi* isolated from human patients in Sabah were included in the research. Next, sequence variations within the coding sequence of *cox1* and those within the A-type 18S rRNA gene in the nuclear genome were identified in *P. knowlesi* strains isolated in Peninsular Malaysia and in Borneo including Sabah (15). This work revealed that the *cox1* coding sequence contains two single nucleotide polymorphisms (SNPs) that distinguish Borneo strains from Peninsular strains. Following that, variations in part of *cytb* coding sequence of parasite strains isolated in several different districts in Sabah were identified (16). The study revealed that the investigated part of the *cytb* gene is fairly well conserved between strains collected in Sabah and those collected elsewhere (Peninsular Malaysia and Sarawak). No clear correspondence between the parasite's collection site and the pattern of variations in the *cytb* gene were detected in those Sabahan strains analysed.

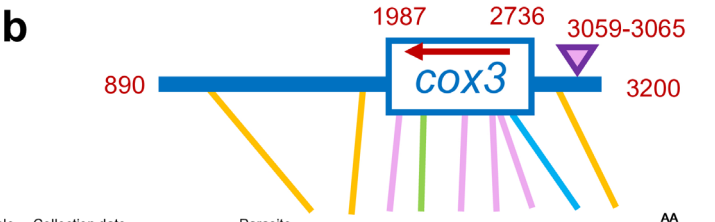
Sequence variations in the mitochondrial DNA outside the two region mentioned above have not been characterised in *P. knowlesi* strains distributing in Sabah yet. There might be variations which are useful as molecular markers in molecular epidemiological study. Thus, aiming to find new molecular markers useful in molecular epidemiological analysis, we carried out search for sequence variations in a 2.3 kb region containing the *cox3* gene for analysis.

MATERIALS AND METHODS

Kota Kinabalu Public Health Laboratory (KKPHL) is routinely analysing malaria patients' blood samples sent from hospitals and clinics around Sabah to identify the *Plasmodium* species in each specimen. Of the blood samples analysed by KKPHL, 35 samples which they confirmed to be *P. knowlesi*-positive between August and September in 2000 (20 samples) and in June 2022 (15 samples) were provided to this research for analysis.

From each *P. knowlesi*-positive blood sample, total DNA was extracted using NucleoSpin Blood QuickPure kit (Macherey-Nagel) following the standard protocol provided with the kit. By semi-nested PCR with a conventional Taq DNA polymerase, two overlapping DNA fragments (F3, F4) that cover a 2.3 kb region of the mitochondrial genome containing the entire coding sequence of the *cox3* gene were specifically amplified from 12 DNA preparations extracted from the blood samples (Fig. 1a). Each 1st PCR (10 µL reaction) was done with: 0.5 µL of the total genomic DNA; primers (0.25 µM each), P143 (5′-GTTAAGGTGCTCAGGGTCTTAC-3′) and P146 (5′-GTCAGGCGTTAAAAGCGTTC-3′); reaction conditions, initial denaturation at 89°C for 1 min





Sample name	Collection date (YYYY-MM-DD)	District	Parasite count	A_{1161}	C_{1868}	T_{2082}	A_{2176}	G_{2437}	C_{2453}	C_{2568}	A_{2593}	A_{2944}	AA change in Cox3
20-1060	2020-09-04	Tuaran	154	A	C	T	A	G	C	C	A	A	None
20-1038	2020-08-01	Tawau	117765	A	C	T	A	G	C	C	A	A	None
20-0997	2020-08-27	Beluran	38400	A	C	C	A	G	C	C	A	A	$I_{219}V$
20-0983	2020-08-25	Pitas	32238	A	C	T	A	C	C	C	A	A	$F_{100}L$
20-1015	2020-08-31	Kudat	43960	A	C	T	A	G	C	T	A	A	$V_{57}I$
22-0735	2022-06-30	Lahad Datu	12746	A	C	T	A	G	T	C	A	T	$S_{95}N$
20-1052	2020-09-05	Tambunan	13544	A	C	T	A	G	C	C	G	A	None
22-0696	No record	Lahad Datu	11339	A	C	T	A	G	C	C	G	A	None
22-0726	2022-06-29	Beluran	4876	C	T	T	A	G	C	C	G	A	None
20-1031	No record	Lahad Datu	67502	A	C	T	T	G	C	C	A	A	None
20-1051	2020-09-21	Kinabatangan	6168	A	C	T	T	G	C	C	A	A	None
22-0697	2022-06-25	Lahad Datu	168	A	C	T	T	ND	ND	ND	ND	ND	-

Figure 1 : Sequence variations identified in the 2.3 Kb region of the mitochondrial genome of *Plasmodium knowlesi* strains collected in Sabah, Malaysia. **a.** Location of the 2.3 kb region in the mitochondrial genome. The mitochondrial genome of the parasite consists of a 6 kb unit that form tandem repeats, and one unit of the reference strain Malayan MRA-487 (accession number NC_007232) is presented as a black horizontal bar. Boxes above or below the bar indicate locations of the 3 protein-coding genes (*cox1*, *cox3*, *cytb*; open box) and regions encoding fragments of LSU and SSU rRNA (dark and light grey boxes, respectively; inferred from *P. falciparum* sequence data M76611). Each region shown with a box above the bar is transcribed from left to right, while the one with a box below the bar is from right to left. For each protein-coding gene, the direction of transcription is indicated with an arrow with the gene name at the bottom. The 2.3 kb region analysed in this research is shown with the blue line at the top, and the 2 PCR fragments (F3 and F4) whose nucleotide sequence was determined are indicated with green lines below the blue bar. The position and the direction of each PCR primer used in this research are indicted with a black arrow. In addition, the regions which investigated in preceding studies (15, 16) are indicated with purple lines and arrows. **b.** Sequence variations identified in the 2.3 kb region. The blue bar at the top and the box on it respectively indicate the 2.3 kb region and the coding region of *cox3* in the mitochondrial DNA of the reference Malayan MRA-487 strain. Each number indicates the coordinate in the sequence data NC_007232. Nine residues of the reference sequence to which SNPs were identified in the 12 strains analysed in this research are shown below, and the position of each in the 2.3 kb region is indicated with a line. The lines are colour-coded to indicate outside the coding sequence (orange), non-synonymous (pink) or synonymous (green and cyan), respectively. In addition, the location of the A cluster extended in all the 12 strains compared to the reference strain is indicated with a triangle. Below, the residues of the 12 isolates analysed are shown in the form of a sequence alignment, together with information of collection date, district of collection, parasite count per microlitre determined at Kota Kinabalu Public Health Laboratory, and the amino acid changes in the *Cox3* product caused by nucleotide change. Each SNP is highlighted with a coloured background. Collection date information was missing from blood samples 20-1031 and 22-0726, as it was not informed to Kota Kinabalu Public Health Laboratory. ND: not determined yet.

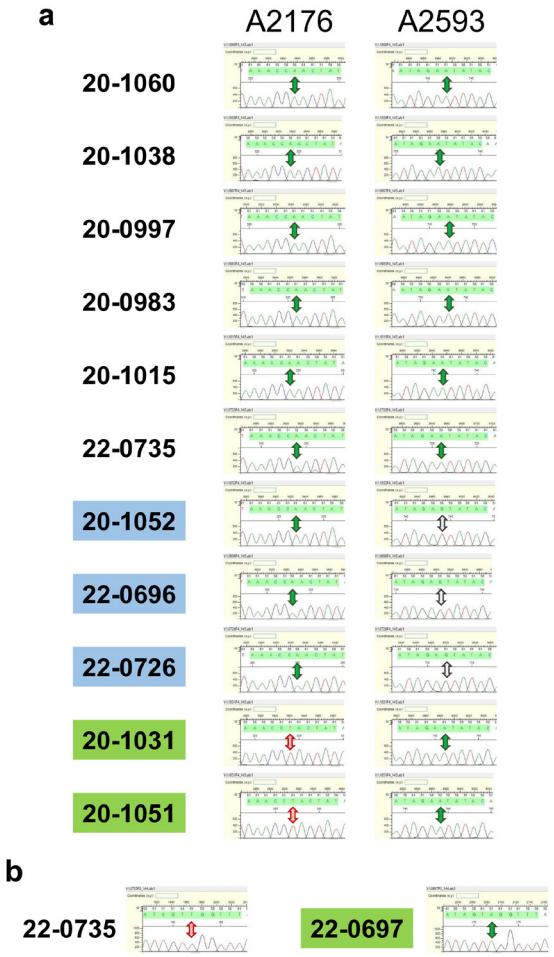


Figure 2 : Sequencing charts of the 12 *Plasmodium knowlesi* strains at regions corresponding to A2176 or A2593 of the reference sequence. **a.** Charts of the 11 strains obtained in the sequencing reactions of the F4 fragment with primer P145. **b.** Charts of the opposite strand of the F3 fragment of 22-0735 and 22-0697 obtained in the sequencing reaction with primer P144. Chart of the region containing the residue corresponding to A2176 or A2593 of the reference sequence plus 5 residues each preceding and following it (total 11 residues; highlighted with light green) was developed from the sequencing data using Sequence Scanner Software 2 (Applied Biosystems), and the peak corresponding to A2176 or A2593 is indicated with a colour-coded bidirectional arrow (A, green; G, grey; T, red). The number shown below each determined residue is a Phred Quality Score determined by the ABI Sanger sequencing analysis system in each experiment. Note that the Phred Scores shown in these panels are in most cases >50 which suggests that the credibility of sequencing data is very high. The chart of A2176 region of 22-0735 in a suggests this strain has both A and T in the A2176 region. However, no peak other than T and A (complimentary to A and T, respectively) appeared in the chart of 22-0735 and 22-0697 in **b.** The Phred Scores of the A2176- corresponding residue was 61 in both experiments. These strongly suggest that the A2176-corresponding residue in 22-0735 and 22-0697 is exclusively A and T, respectively.

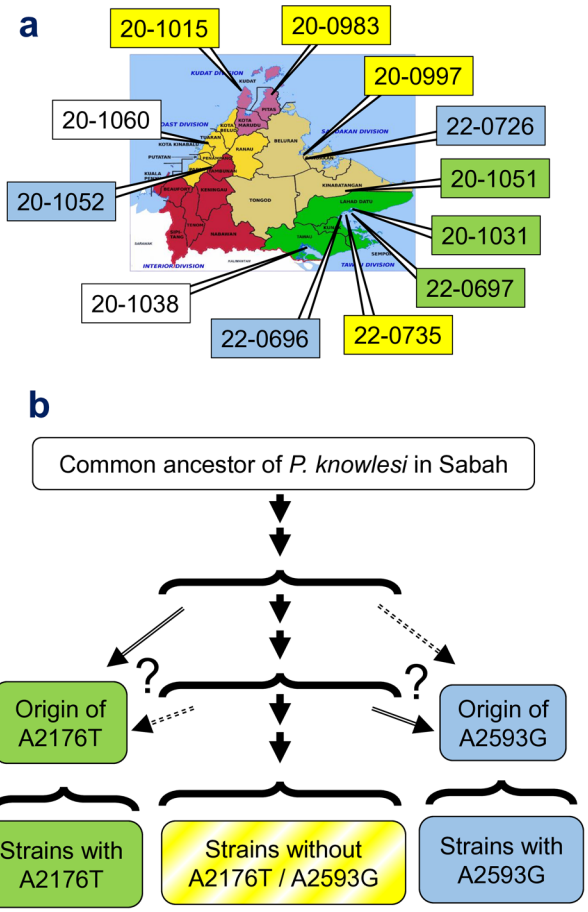


Figure 3 : a. Sources of the 12 blood samples containing *Plasmodium knowlesi* strains analysed in this research. Samples whose name starts with 20- and 22- are collected in 2020 and 2022, respectively, and those containing a parasite strain without any SNP compared to the reference Malayan strain in the 2.3 kb region of the mitochondrial genome, with A2176T, with A2593G, and with SNP(s) but free from A2176T and A2593G are colour coded with a white, green, blue, and yellow background, respectively. **b.** Proposed diversification of *P. knowlesi* that took place in Sabah.

followed by 30 cycles of (89°C for 10 sec, 60°C for 4 min). Each 2nd PCR was done with: template, 0.5 µL of 1/50 diluted 1st PCR reaction; primers (0.25 µM), P143 and P144 (5'-GCAAGTGCATCATGTATGAC-3') or P145 (5'-ATCATTACTCAAGTCAGCATAGT-3') and P146; reaction conditions, initial denaturation at 89°C for 1 min followed by 40 cycles of (89°C for 10 sec, 60°C for 2 min). After the completion of the 2nd PCR, 1 uL of each reaction was analysed by agarose gel electrophoresis. For each sample, 30 to 40 uL of the reactions which contained PCR products forming a single band in the gel were combined, processed with *E. coli* exonuclease I (NEB) and rSAP shrimp alkaline phosphatase (NEB), and sequenced by Sanger sequencing from both ends without subcloning. After manual curation using Applied Biosystems Sequence

Scanner Software 2 (Thermo Fisher Scientific), obtained nucleotide sequences were aligned with the reference sequence in the database (accession no. NC_007232) using Clustal X2 (17) and UGENE (18).

This research was ethically approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMRR 18-1619-41192).

RESULTS

The nucleotide sequence of the entire mitochondrial DNA of *P. knowlesi* has been determined (19) and it has been known that there are only 3 protein coding genes encoded in it like in the mitochondrial DNA of other *Plasmodium* species. Sequence variations between different *P. knowlesi* isolates in regions corresponding to *cox1* and *cytb* genes have been studied previously (15, 16), but those in other region have not been characterised well. So we selected a 2.3 kb region containing the *cox3* gene - a new region in the parasite's mitochondrial DNAs - for analysis (Figure 1a).

Total DNA was extracted from *P. knowlesi*-positive blood samples of malaria patients identified in various districts of Sabah, and two overlapping fragments covering the 2.3 kb region were amplified by nested PCR from the DNA preparations. Aimed PCR fragments were obtained from 8 DNA preparation extracted from the blood samples collected in 2020 (20-0983, 20-0997, 20-1015, 20-1031, 20-1038, 20-1051, 20-1052, 20-1060), and the nucleotide sequence of each was determined by direct Sanger sequencing. Although these fragments were not cloned by subcloning before sequencing, none of the residues in the determined sequences were suggested to consist of mixed different bases (Figure 2). This suggests that each patient who provided corresponding blood sample was infected by a single strain of *P. knowlesi* and that the determined nucleotide sequences are free from errors caused by PCR.

The determined nucleotide sequences were aligned with the reference sequence Malayan strain MRA-487 of *P. knowlesi*, which was isolated in Peninsular Malaysia before 1960 and maintained in the laboratory since then (20). There were 5 SNPs (T2082C, A2176T, G2437C, C2568T, A2593G) identified in the 2.3 kb part of mitochondria genome of the 8 *P. knowlesi* strains (Figure 1b). All these 5 SNPs were localised within the coding sequence of *cox3*. T2082C, G2437C, and C2568T cause amino acid substitutions I219V, F100L, and V57I, respectively, in the Cox3 protein, while the remaining two are synonymous SNPs which do not affect the amino acid residue encoding. Distribution of the 5 SNPs looked isolate specific, though A2176T, one of the two synonymous SNPs, was identified in the parasites isolated from two different patients living in Lahad Datu and Kinabatangan districts.

To characterise the presence and distribution of SNPs in this region further, we included 4 additional *P. knowlesi*-positive blood samples of malaria patients in Sabah collected in June 2022 (22-0696, 22-0697, 22-0726, 22-0735) to our analysis. So far, we have completed sequencing of both F3 and F4 PCR fragments amplified from 3 blood samples (22-0696, 22-0697, 22-0726) while the sequence of only F3 has been determined for 22-0735. Judging from the results of the sequencing, each malaria patient who provided blood sample was likely infected by only one strain of *P. knowlesi*, like those who provided the 2020 samples in this analysis (Figure 2).

Each of the two synonymous SNPs identified in the 2020 parasite strains were also identified in the 2022 strains, and one of the parasite strains with A2593G also had two new SNPs outside the *cox3* coding region. By contrast, none of the 3 non-synonymous SNPs present in 2020 strains were identified in the sequences of these 4 strains determined. Instead, another non-synonymous SNP, C2453T, was identified in the *cox3* coding sequence of the parasite in the blood sample 22-0735 together with another SNP present outside the coding sequence.

Combined together, 9 SNPs were identified in the 2.3 kb region of the mitochondrial DNA of the parasites that caused 12 malaria cases in 2020 and 2022 in Sabah (Figure 1b). Two parasite strains in samples 20-1038 and 20-1060 lacked any of these 9 SNPs. However, all the 11 parasite strains whose F4 sequence has been determined, including these two SNP-free ones, shared the same feature that the A cluster corresponding to the one between A3059 and A3065 of MRA-487 is extended.

DISCUSSION

As suggested in *P. falciparum* (21), the mitochondrial genome is probably inherited through female gamete only even in *P. knowlesi*. Therefore, acquisition and loss of each SNP is expected to be an independent event, solely depending on spontaneous point mutation. This means that a set of SNPs present in the parasite's mitochondrial genome are useful as for both phylogenetic and epidemiological analyses of the parasite. Although the number of the mitochondrial genome with each SNP apart from the two synonymous SNPs (A2176T and A2593G) is still only one, A2176T and A2593G were identified in three different parasite strains each. The three parasite strains with A2176T were present in the blood samples collected from malaria patients who lived in Lahad Datu and Kinabatangan, two districts next to each other in eastern Sabah (20-1031, 20-1051 and 22-0697; highlighted with

green background in Figure 3a). This may suggest that parasites with A2176T are specifically distributed in an area containing these two districts, while it is also possible that those parasites are prevalent in a wider area. On the other hand, the patients who were infected by the three strains with A2593G were living in Tambunan, Lahad Datu and Beluran, which were separated apart from each other (20-1052, 22-0696 and 22-0726; highlighted with blue background in Figure 3a). It must be noted that no sequence data of parasites in the samples collected in the districts in the central to southwestern Sabah have been obtained in either any preceding studies or this research. It is possible that the strains with A2593G are actually distributed in those districts of no data and form a continuous distribution which is much wider than where parasites with A2176T are distributed. To grasp accurate and precise distribution area/pattern of parasites with each identified SNP including A2176T and A2593G, it is required to collect more parasites at more various collection points and accumulate more analysed data.

A preceding study done by Yusof et al identified two SNPs in the mitochondrial *cox1* coding sequence that is universally conserved in *P. knowlesi* strains isolated in Borneo but missing in strains isolated in Peninsular Malaysia (15). This suggests that the *P. knowlesi* strains in Borneo (including both Sabah and Sarawak) share the common ancestor in their evolutionary history. The ancestor was probably similar to parasite strains in the samples 20-1038 and 20-1060 which were free from any SNP in the 2.3kb region. From the ancestor, various descendants must have been generated due to spontaneous point mutation which can be introduced at each generation of the parasite (Figure 3b). The fact that multiple strains that share either A2176T or A2593G were isolated in such a small number of parasites analysed in this study suggests that each group of strains share one common ancestor. The order of the emergence of the common ancestors of the two groups is unknown. The parasite strain in sample 22-0697 had two additional SNPs in addition to A2176T, while no strain with A5293G and another SNP was identified in this study. This may suggest that the emergence of the common ancestor of the A2176T strains predated that of the A5293G common ancestor, though more parasites with each SNP should be analysed to make this speculation credible.

The number of samples analysed is too low to tell if any of the variations identified in the 2.3 kb region of mitochondrial DNA in this research critically affect the severity of malaria caused in humans or not. All the blood samples analysed were collected from symptomatic malaria patients but there were no SNPs identified in all *P. knowlesi* strains that caused those malaria in humans. It is interesting that all parasite strains had an extended A cluster corresponding to A3059 and A3065 of MRA-487 in this 2.3 kb region. Future analysis with a larger number of samples would solve the question of whether the length of this A cluster has anything to do with the severity of malaria in humans.

CONCLUSION

In this research, by analysing a 2.3 kb region of the mitochondrial DNA which covers the *cox3* coding sequence, we identified as many as 9 SNPs in only 12 parasite strains, and 7 of the 9 SNPs identified were strain specific. This proves that the mitochondrial DNA is a good source of molecular markers, which tell the parasite's identity and are useful for studying the parasite's epidemiology. By the presence/absence of the two synonymous SNPs, the 12 parasite strains analysed were classified into three groups (with A2176T, with A2593G, and without A2176T/A2593G). Each of these three groups may be distributed in a vast area spreading across central to southern and eastern Sabah. Human malaria cases have been seldom reported in this area possibly mainly because of the low population density. *P. knowlesi* malaria is a zoonotic disease and the parasite is expected to be maintained in wild macaque populations there. Therefore it is required to investigate the distribution of the parasite in macaques to understand epidemiology of *P. knowlesi* malaria in Sabah sufficiently. Because analysis of the molecular markers in the parasite's mitochondrial DNA does not require a large amount of blood specimen, it is an ideal way for analysing not only blood samples collected from human malaria patients but also wild samples of which availability is limited.

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