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Genetic and haplotype analyses targeting cytochrome b gene of *Plasmodium knowlesi* isolates of Malaysian Borneo and Peninsular Malaysia

Eric Tzyy Jiann Chong[a], Joveen Wan Fen Neoh[b], Tiek Ying Lau[b], Yvonne Ai-Lian Lim[c,d], Kek Heng Chua[e], Ping-Chin Lee[a,b,*]

[a] Biotechnology Programme, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[b] Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[c] Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[d] Centre of Excellence for Research in AIDS (CERiA), University of Malaya, 50603 Kuala Lumpur, Malaysia
[e] Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

Malaria is a notorious disease which causes major global morbidity and mortality. This study aims to investigate the genetic and haplotype differences of *Plasmodium knowlesi* (*P. knowlesi*) isolates in Malaysian Borneo and Peninsular Malaysia based on the molecular analysis of the cytochrome b (*cyt b*) gene. The *cyt b* gene of 49 *P. knowlesi* isolates collected from Sabah, Malaysian Borneo and Peninsular Malaysia was amplified using PCR, cloned into a commercialized vector and sequenced. In addition, 45 *cyt b* sequences were retrieved from humans and macaques bringing to a total of 94 *cyt b* gene nucleotide sequences for phylogenetic analysis. Genetic and haplotype analyses of the *cyt b* were analyzed using MEGA6 and DnaSP ver. 5.10.01. The haplotype genealogical linkage of *cyt b* was generated using NETWORK ver. 4.6.1.3. Our phylogenetic tree revealed the conservation of the *cyt b* coding sequences with no distinct cluster across different geographic regions. Nucleotide analysis of *cyt b* showed that the *P. knowlesi* isolates underwent purifying selection with population expansion, which was further supported by extensive haplotype sharing between the macaques and humans from Malaysian Borneo and Peninsular Malaysia in the median-joining network analysis. This study expands knowledge on conservation of the zoonotic *P. knowlesi cyt b* gene between Malaysian Borneo and Peninsular Malaysia.

1. Introduction

Malaria is causing major global morbidity and mortality as reported in the World Malaria Report 2015. It was estimated that there were 214 million cases of malaria and occurrence of 438 000 malaria deaths in 2015 (WHO, 2015). Although there are more than 20 *Plasmodium* species known to infect primates, only five of them are responsible for human malaria and these include *P. vivax*, *P. malariae*, *P. falciparum, P. ovale* and *P. knowlesi*. Interestingly, the simian *P. knowlesi* which is previously known to cause malaria only in macaques (Garnham, 1966) has a widespread occurrence in humans and is prevalent in Southeast Asia (Lee and Vythilingam, 2014).

Since the landmark report of *P. knowlesi* infection in humans in Kapit Division, Sarawak in 2004 (Singh et al., 2004), *P. knowlesi* infections have been documented in many Southeast Asian countries except Laos. In Malaysia, human malaria cases due to *P. knowlesi* have been reported numerous times in Peninsular Malaysia (Cox-Singh et al., 2008; Kantele et al., 2008; Vythilingam et al., 2008; Lee et al., 2010) and in Malaysian Borneo including Sabah and Sarawak (Singh et al., 2004; Lau et al., 2011; Goh et al., 2013; William et al., 2013, 2014; Lee et al., 2015). Recent studies have shown marked geographic divergence of *P. knowlesi* isolates between Malaysian Borneo and Peninsular Malaysia using molecular markers such as 18S rRNA, cytochrome c oxidase subunit I and multilocus microsatellites (Yusof et al., 2016; Divis et al., 2017), however genetic and haplotype analyses of *P. knowlesi* isolates between these two regions based on the cytochrome b (*cyt b*) marker remain underreported.

Unlike genomic DNA, mitochondrial DNA is maternal inheritance, absence of recombination and has a simple sequence organization (Harrison, 1989). These characteristics make mitochondrial DNA suitable as a genetic marker for population and evolutionary biology, especially in haplotype analyses. C*yt b* is one of the mitochondrial genes that has been frequently used in investigating the molecular epidemiology of malaria in previous studies (Escalante et al., 1998; Perkins and Schall, 2002; Putaporntip et al., 2010; Muehlenbein et al., 2015). In this study, we further unravel the genetic and haplotype diversity of *P.*

* Corresponding author at: Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia.
E-mail address: leepc@ums.edu.my (P.-C. Lee).

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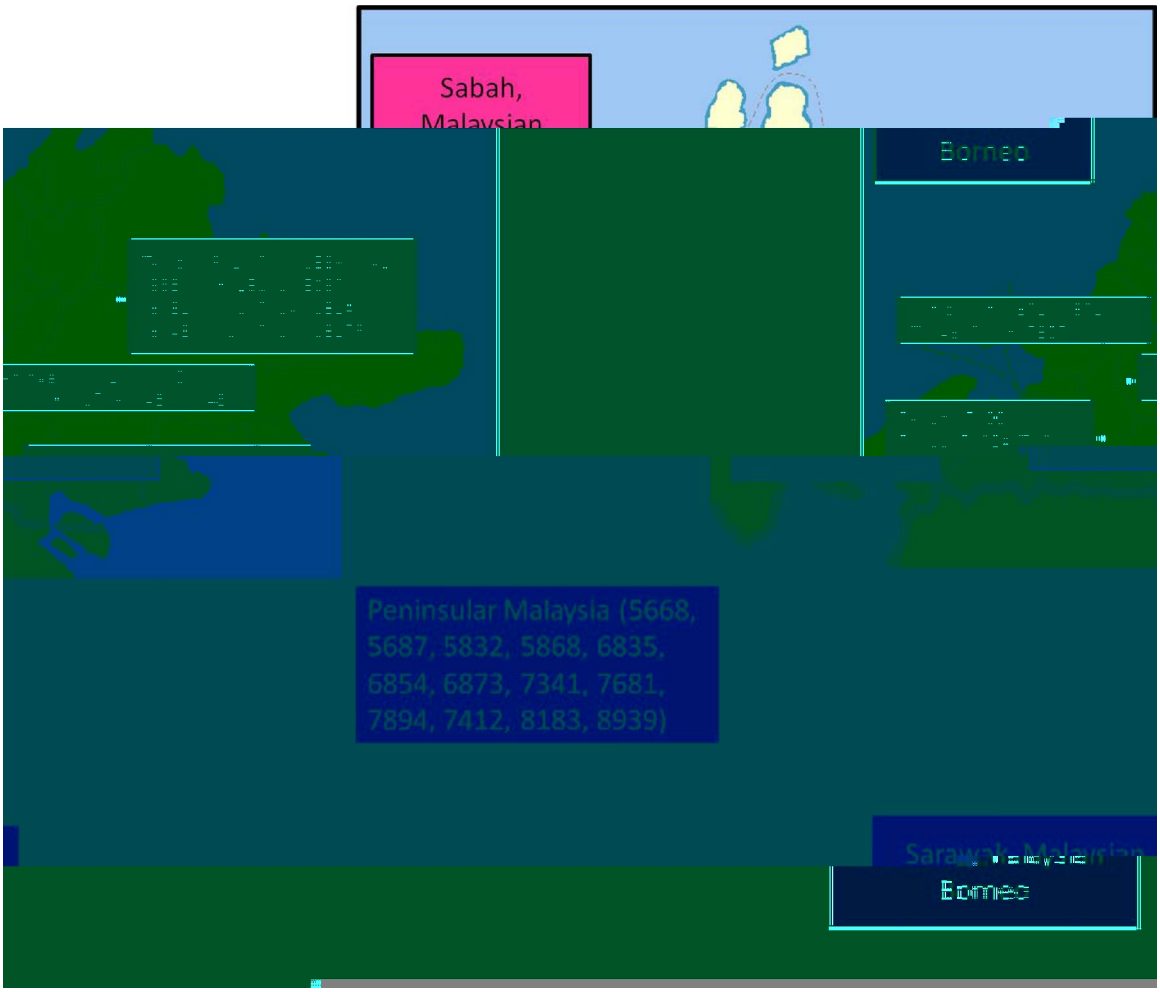

Fig. 1. Map of Malaysia. *P. knowlesi* samples were collected from different areas in Sabah, East Malaysia including Telupid Health Clinic ($N = 16$), Keningau Hospital ($N = 6$), Nabawan Health Clinic ($N = 3$), Tambunan Hospital ($N = 5$) and Tenom Hospital ($N = 6$), and at the University of Malaya Medical Centre in Peninsular Malaysia ($N = 13$). Codes in the brackets are referring to *P. knowlesi* sample labeling in this study.

knowlesi isolates between Malaysia Borneo and Peninsular Malaysia based on the molecular analysis of the *cyt b* gene.

2. Materials and methods

2.1. Malaria blood samples and DNA extraction

Forty-nine samples of *P. knowlesi* were collected from left-over peripheral blood samples of malaria patients from different areas of Sabah, Malaysian Borneo including Telupid Health Clinic ($N = 16$), Keningau Hospital ($N = 6$), Nabawan Health Clinic ($N = 3$), Tambunan Hospital ($N = 5$) and Tenom Hospital ($N = 6$), and at the University of Malaya Medical Centre in Peninsular Malaysia ($N = 13$) from 2008 to 2011 for this study (Fig. 1). Genomic DNA was extracted for all samples using method as previously described (Lim et al., 2010). Ethical approval of this study was approved by the Ethics Committee of University of Malaya Medical Centre (Reference no: 709.2).

2.2. Amplification of cyt b gene using polymerase chain reaction

The *cyt b* gene was amplified using pre-designed primer pairs Pkcyt-F (5′-TACCCATGCCCATTAAATATTA-3′) and Pkcyt-R (5′-CATTATGAT TACAGTCCCAAGC-3′). PCR amplification was carried out in a 20 µL reaction mixture containing 1X PCR buffer (Qiagen, USA), 0.2 mM of dNTPs, 0.2 µM of each primer and 1.25 unit of TopTaq DNA polymerase (Qiagen, USA). PCR condition was set at: 94 °C for 3 min followed by 35 amplification cycles at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. PCR fragment with expected size of 1076 bp was electrophoresized and analyzed in 1% agarose gel stained with ethidium bromide.

2.3. Cloning and bidirectional sequencing

PCR products were purified from gel using QIAquick Gel Extraction Kit (Qiagen, USA) and cloned into pJET1.2/blunt vector using CloneJET PCR Cloning Kit (Thermo Scientific, USA) according to manufacturer's recommendation. The desired plasmid containing the *cyt b* fragment was extracted using QIAprep Spin Miniprep Kit (Qiagen, USA) according to the manufacturer's instruction, and subjected to bi-directional sequencing using pJET1.2 forward and reverse sequencing primers.

2.4. Sequence alignment and phylogenetic analysis

A total of 94 *cyt b* gene nucleotide sequences were aligned using CLUSTAL-W in Molecular Evolutionary Genetic Analysis 6 (MEGA6) Software (Tamura et al., 2013) for phylogenetic analysis including 49 sequences of this study and 45 sequences that were retrieved from the GenBank database. The retrieved *cyt b* sequences comprising of 21 sequences isolated from macaque in Sarawak (EU880471-EU880473, EU880475-EU880478, EU880480, EU880483-EU880484, EU880489-EU880499), 2 sequences isolated from macaque in Peninsular Malaysia

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