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Research paper

Genetic diversity of zoonotic malaria parasites from mosquito vector and vertebrate hosts

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ABSTRACT

We explored and constructed haplotype network for simian malaria species: *Plasmodium knowlesi, P. cynomolgi* and *P. inui* aiming to understand the transmission dynamics between mosquitoes, humans and macaques. Mosquitoes were collected from villages in an area where zoonotic malaria is prevalent. PCR analysis confirmed *Anopheles balabacensis* as the main vector for macaque parasites, moreover nearly 60% of the mosquitoes harboured more than one *Plasmodium* species. Fragments of the A-type small subunit ribosomal RNA (SS rRNA) amplified from salivary gland sporozoites, and equivalent sequences obtained from GenBank were used to construct haplotype networks. The patterns were consistent with the presence of geographically distinct populations for *P. inui* and *P. cynomolgi*, and with three discrete *P. knowlesi* populations. This study provides a preliminary snapshot of the structure of these populations, that was insufficient to answer our aim. Thus, collection of parasites from their various hosts and over time, associated with a systematic analysis of a set of genetical loci is strongly advocated in order to obtain a clear picture of the parasite population and the flow between different hosts. This is important to devise measures that will minimise the risk of transmission to humans, because zoonotic malaria impedes malaria elimination.

1. Introduction

Laboratory studies showed that simian malaria could be transmitted to humans, but proof that simian malaria could constitute a true zoonosis was only obtained with the first case of a naturally-acquired *P. knowlesi* infection by a surveyor working in Pahang State, Peninsular Malaysia (Chin et al., 1965), but no further similar cases were recorded over the following decades.

In 2004 Singh and colleagues identified a large focus of zoonotic *P. knowlesi* malaria in the Kapit District of Sarawak State in Malaysian Borneo (Singh et al., 2004), and over the next years similar cases, albeit comparatively fewer in number, were reported from various Southeast Asian countries where the natural hosts *M. fascicularis* and *M. nemestrina* were endemic (Vythilingam et al., 2016). Malaysia aims to achieve malaria elimination by the year 2020, and indeed the drop between 2008 and 2016 from a total of 4754 to 266 recorded cases for *P. falciparum* and *P. vivax* is highly encouraging. However, over the last decade the number of detected zoonotic *P. knowlesi* infections in Malaysia has steadily increased, and currently represents about 90% of all recorded malaria cases, most of which originating from Malaysian Borneo. Given the clinical severity and potential mortality associated with knowlesi infections (Ahmed and Cox-Singh, 2015; Cox-Singh et al., 2008), and the threat this parasite poses to achieving a malaria-free status, it becomes important to investigate the epidemiology of the *P. knowlesi*, as well as that of the other malaria parasites species of macaques able to infect humans, *P. cynomolgi* and *P. inui* (Coatney et al.,

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1966; Eyles et al., 1960; Schmidt et al., 1961).

Effective control or mitigation of zoonotic malaria would require detailed insights into the prevalence and nature of the parasites in their natural hosts and, crucially, knowledge of the mosquito species responsible for their transmission between the macaques and to the humans. However, to date only four surveys have been conducted to determine the distribution of *Plasmodium* parasites in wild-caught macaques (*M. nemestrina* and *M. fascicularis*): 108 animals from Kapit District in Sarawak (Lee et al., 2011), and 70 animals from Hulu Selangor District in Selangor (Akter et al., 2015), while 99 and 648 animals were surveyed in southern Thailand from two and four provinces, respectively, (Putaporntip et al., 2010; Seethamchai et al., 2008). Whereas a majority of the monkeys from Malaysia were found infected with one or more of five parasite species (*P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. inui*, and *P. knowlesi*), the prevalence and diversity of parasites from Thai animals was substantially lower. Furthermore, investigations on the intra-specific genetic diversity have been primarily confined to *P. knowlesi*, with little known for the other parasites species. Comparison of *P. knowlesi* genotypes present in humans with those found in macaques did not indicate any exclusive association of a sub-population with one or other vertebrate hosts (Lee et al., 2011). Most notably, these studies revealed the presence of genetically distinct clusters across macaque species and geographical sites (Assefa et al., 2015; Divis et al., 2015, 2017; Fong et al., 2015a, 2016, 2015c; Pinheiro et al., 2015; Yusof et al., 2016).

Early entomological studies had incriminated *An. hackeri*, *An. leucosphyrus* and *An. cracens* (then reported as *An. balabacensis*), as the vectors for *P. knowlesi* and all the other simian malaria parasites (Warren and Wharton, 1963). Recent detailed investigations, prompted by the discovery of the natural focus of knowlesi malaria in Malaysian Borneo, further identified *An. latens* (Tan et al., 2008), *An. cracens* (Jiram et al., 2012), *An. introlatus* (Vythilingam et al., 2014), *An. balabacensis* (Wong et al., 2015) and *An. dirus* (in Vietnam) (Marchand et al., 2011) as vectors. To date, all the anopheline species incriminated as transmitters of macaque *Plasmodium* parasites belong to the Leucosphyrus Group, though the particular species implicated varies geographically.

The particularly high prevalence of zoonotic malaria in the Kudat District in northern Sabah State, suggesting a pattern of peri-urban transmission (Barber et al., 2012), prompted epidemiological investigations (Fornace et al., 2016; Grigg et al., 2014; Grigg et al., 2017) that included a molecular survey of the parasite species circulating in the mosquitoes (Chua et al., 2017). The partial sequences obtained for the parasite's small subunit ribosomal RNA (SSU rRNA), were used to confirm the presence of the five species known to infect macaques. We wished to extend this analysis to other sequences obtained from the midgut or salivary glands of dissected infected mosquitoes, and to all equivalent sequence derived from previous investigations. The aim was to construct a haplotype network in an attempt to obtain phylogeographic data for the parasite species, and eventually an insight into the transmission dynamics between macaques and humans.

2. Materials and methods

2.1. Identification of malaria parasites from field collected Anopheles balabacensis

Entomological investigations were carried out from August 2013 to July 2014 in Limbuak Laut (LL) (117°065′75″E, 7°215′84″N) and Timbang Dayang (TD) (117°102′92″E, 7°155′85″N) (three nights/month) on Banggi Island, and Kampung Paradason (KP) (116°786′35″E, 6°768′37″) (two nights/month)on mainland Kudat. Adult mosquitoes were collected using human landing catch (HLC) from 1800 to 0600 h. *Anopheles* mosquitoes that were collected by Wong et al. (2015) was used. Mosquitoes were dissected to obtain midguts and salivary glands, and were examined for oocysts and sporozoites, respectively. All positive midguts and salivary glands were transferred to individual microcentrifuge tubes containing 95% ethanol for subsequent molecular analysis.

2.2. Extraction of DNA

DNA was extracted from individual positive midguts and salivary glands. Ethanol was allowed to evaporate from the specimen tubes, after a brief centrifugation, by incubation in a Thermomixer (Eppendorf, Germany) at 70 °C. Genomic DNA was extracted from positive midguts and salivary glands mosquitoes using DNeasy Blood and Tissue Extraction kit (Qiagen, Germany) according to manufacturer's recommendation. The eluted DNA was kept at −20 °C until required.

2.3. Nested PCR amplification for detection of Plasmodium species and sequencing

All positive midguts and salivary glands samples were subjected to nested PCR assay based on the small subunit ribosomal RNA genes (SSU rRNA) aimed at determining the parasite species present using previously described PCR cycling parameters (Lee et al., 2011; Singh et al., 2004). Positive and negative controls were included in all PCR assays. The PCR primer sequences and annealing temperatures are provided in Table A1. Amplicons from the secondary amplification were analysed on 1.5% agarose gel electrophoresis and visualised under an ultraviolet transilluminator. The amplicons from positive samples were excised from the gel and sent to a commercial laboratory (MyTACG, Malaysia) for sequencing.

2.4. Cloning of Plasmodium SSU rRNA A-type gene fragments

Genomic DNA purified from ten sporozoite-positive *An. balabacensis* sporozoites and three genomic DNA purified from infected macaque blood collected in Hulu Selangor from three macaques (Akter et al., 2015) were analysed. Primary amplification of the A-type SSU rRNA gene was performed with *Plasmodium*-specific primers, rPLU1 and rPLU5 (Singh et al., 1999) in a 50 µL reaction mixture containing 1× reaction buffer (2× Accura HF reaction buffer) (Lucigen Corp, Middleton WI, USA), 0.2 mM of each deoxyribonucleoside triphosphate (dNTP) (Promega Madison WI, USA), 0.5 µM of each primer, 1.5 u of Accura High-Fidelity Polymerase (2 U/µL) (Lucigen), and 5 µL of purified genomic DNA template. PCR amplification parameters were: 94 °C for 5 mins, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension of 72 °C for 10 mins.

The resulting PCR product was subjected to a secondary amplification using modified forward *Plasmodium*-genus primer and reverse *Plasmodium*-species primers and reaction conditions as listed (Chua et al., 2017; Imwong et al., 2009; Lee et al., 2011) as shown in Table A1. Two microliters of the product from the primary PCR were used as the template to initiate the reaction.

The resulting amplified A-type SSU rRNA gene fragment from each isolate was gel-purified using Nucleospin Gel and PCR Clean Up (Machery-Nagel, Germany). An A-tailing procedure was carried out in a 10 µL reaction containing 2.0 µL of 5× Colourless GoTaq Flexi Buffer (Promega Madison WI, USA), 1.0 µL of 25 mM $MgCl_2$ (Promega), 0.2 µL of 10 mM dNTP (Promega), 1.0 µL of GoTaq DNA Polymerase (5 U/µL), 3 µL of purified PCR amplicons and deionized water. The reaction mixture was incubated at 70 °C for 30 min. The A-tailed purified PCR fragment was cloned into pGEMT Easy Vector System I (Promega Madison WI, USA) and transformed into TOP 10 competent *Escherichia coli* cells (Invitrogen, USA) by heat shock. 100 µL of transformants were plated on LB agar plate supplemented with ampicillin and incubated overnight at 37 °C. Positive colonies were picked and expanded for subsequent purification of recombinant plasmid DNA using Nucleospin Plasmid (Machery-Nagel, Germany) according to manufacturer's protocol. Plasmids from at least three positive colonies for each fragment

were selected for sequencing with M13 primers by a commercial laboratory (MyTACG, Kuala Lumpur, Malaysia).

2.5. Sequence editing and alignment

The DNA sequences obtained were analysed using Geneious 9.1.6 software (http://www.geneious.com). Sequence analysis and comparison at the nucleotide level were performed against the reference sequence for *P. knowlesi* H-strain (GenBank accession no. AM910985), *P. inui* clone 109 (GenBank accession no. AB287277) and *P. cynomolgi* Berok strain (PlasmoDB Gene ID PCYB_021237). Results were exported to Mega 7.0 software for further alignment and analysis (Kumar et al., 2016). Similarity searches using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed.

2.6. Haplotype network

Haplotype networks for the *P. knowlesi, P. inui* and *P. cynomolgi* A-type 18S SSU rRNA genes were constructed by using the median-joining method in NETWORK version 5.0.0.1 software (Fluxus Technology LTD Suffolk, UK). The genealogical haplotype network for *P. knowlesi, P. inui* and *P. cynomolgi* was inferred using the sequences of human and macaque isolates from other geographical locations. Given the possibility that sequence variations could arise from artefacts of replication during PCR amplification, a stringent selection of the sequences to be included in generating the haplotype networks was applied. An alignment of all the available sequences was examined visually, and only those residues where variation was observed in sequences obtained from independent PCR amplifications from the same sample or from another independently amplified sample were considered reliable, thus, included in the analysis.

3. Results/discussion

3.1. Simian Plasmodium parasites transmitted by An. balabacensis

Mosquitoes were collected from the three villages in order to investigate the seasonal and spatial dynamics of the vector in Kudat, an area of northern Sabah with a high prevalence of *P. knowlesi* zoonotic malaria. In a first study, the dominant species obtained by human landing collection, *An. balabacensis*, was found to represent 95% of all the collected mosquitoes, with detectable macaque *Plasmodium* parasites in 45 of the 1791 specimens (Wong et al., 2015), with *P. coatneyi*, *P. cynomolgi*, *P. inui* and *P. knowlesi* detected but not *P. fieldi*. The predominance of *An. balabacensis* (90% of 1599 specimens) was confirmed in a second study conducted in Kampung Paradason (Chua et al., 2017) in similarly caught mosquitoes, of which 23 were positive for *Plasmodium* species of macaques, including *P. fieldi*.

Partial sequences of the parasites' A-type SSU rRNA genes present in genomic DNA purified from whole mosquitoes collected in this second study were used to establish that the *P. knowlesi* populations from the three hosts in Sabah were highly related phylogenetically (Chua et al., 2017). We wished to extend the molecular analysis of the samples collected in the first study to include sequences of the A-type SSU rRNA genes specifically from sporozoites found in the salivary glands. Indeed, it has been shown in numerous previous studies that oocyst formation in the vector does not necessarily proceeds to sporogony due to incompatibility between the parasites/anopheline species or strain.

Of the 45 positive mosquitoes identified previously, 38 were available for the molecular investigations conducted in this study (Table 1). Infections were restricted to the midgut in 13 of these, to the salivary gland in another 13, while the remaining 12 mosquitoes harboured parasites in both locations. It is noteworthy that the distribution of the parasite species was not biased with respect to midgut or salivary gland, and two or more *Plasmodium* species were present in the majority of the mosquitoes (22/38), with mixed infection being equally found in midguts and in salivary glands (12/25 and 14/25, respectively). It does, therefore, seem likely that *An. balabacensis* can transmit the various *Plasmodium* species between macaques, and to humans.

Amplification of a large fragment of the A-type SSU rRNA and its subsequent cloning and sequencing was attempted for all salivary gland positive samples and obtained good sequencing data from 10 of these. It is possible that in some samples one species of sporozoites may have been predominant. Ultimately, sequences were obtained for *P. knowlesi* from three samples, for *P. cynomolgi* from three samples, and for *P. inui* from six samples (Table 1).

3.2. Haplotype network analysis of macaque Plasmodium parasite species

In this study we wished to focus on the relationship between the zoonotic parasites, *P. knowlesi*, *P. cynomolgi* and *P. inui*, in their natural and human hosts. To this end, we opted to generate a haplotype network encompassing all known suitable SSU rRNA gene sequences. Haplotype networks provide insights into the phylogeography of the parasite populations and help infer their recent evolutionary history.

The number of A-type SSU rRNA sequences from macaque *Plasmodium* species suitable for haplotype analysis that are derived from mosquitoes remains low. When the sequences obtained in this study from 10 infected mosquitoes are combined with those obtained in a previous study (Chua et al., 2017) from 10 positive mosquitoes, a total of five sequences became available for *P. knowlesi*, seven for *P. cynomolgi* and twelve for *P. inui* (three sequences for *P. fieldi* and three for *P. coatneyi* were not considered in the current study). We obtained an additional SSU RNA sequences for each of the three species from samples collected from infected monkeys in Selangor State, Peninsular Malaysia, and retrieved other sequences of suitable length from GenBank, derived from samples collected from macaques for *P. inui* and *P. cynomolgi*, and from both macaques and humans for *P. knowlesi* (Table 2). For each of these species, some of the sequences were derived from parasite isolates first collected many decades ago across broad geographical locations. Thus, a total of 69 A-type SSU rRNA sequences were available for *P. knowlesi*, and of these 52 were deemed suitable for inclusion in the haplotype network analysis (Table 2), while for *P. inui* 43 of 94 were selected (Table 2) and for *P. cynomolgi* 23 of 35 were selected (Table 2). The resulting haplotype networks illustrated the potential of this approach to reveal geographical differentiation within the population of each species, but also highlighted the limitations of sub-optimal sampling.

Sequences from *P. inui* were obtained from macaques sampled from Taiwan, southern Thailand, and for Malaysia (principally Malaysian Borneo). The haplotype network obtained (Fig. 1A) reflects this geographical origin as well as the breadth of sampling. Indeed, the *P. inui* populations from Thailand and Taiwan that were collected from a few animals sampled during single surveys in sites situated close to each other yielded relatively tight clusters. This contrasts with the broader cluster from parasites sampled many years apart from macaques and mosquitoes in Sarawak and Sabah (Malaysian Borneo). However, the extent of sequence diversity was relatively low for most of the sequences (except for H_6, H_14 and H_11), such that only a few sites were sufficient to yield distinct clusters. It can be noted haplotypes found in distinct Thai clusters were derived from the parasites found in the same macaque: three haplotypes from monkey WPN4, H_11 in one cluster and H_12 and H_13 in the other; three haplotypes from monkey WPN6, H_14 in one cluster and H_9 and H_13 in the other (Fig. A1). The full genome sequences available for different *Plasmodium* species revealed the presence of two to three A-type SSU rRNA genes per genome (often located on different chromosomes, and similarly for the S-type genes). This could explain the observation above. However, the extent of the sequence diversity observed for these homologous genes is usually quite low (less than 15 sites for the ca. 2 kb gene), whereas the diversity observed for the *P. inui* parasites from the same monkey exceeds 25 sites for the ca. 1 kb fragments analysed. Thus, our

Table 1
Plasmodium species detected in *An. balabacensis* from Kudat.

ID[a]		Pkn[b]	Pin[b]	Pcy[b]	Pct[b]
LL233	MG		✓	✓	
LL312	MG		✓	✓	
LL369	MG			✓	
LL224	MG		✓	✓	
LL52	SG		✓		
LL184	SG		✓	✓	
LL237	**SG**		✓	✓	✓
LL297	SG	✓			
LL477	**SG**	✓	✓	✓	✓
LL99	MG	✓	✓		
	SG		✓		
LL175	MG			✓	
	SG		✓	✓	
LL179	MG		✓	✓	
	SG	✓		✓	
LL226	MG			✓	
	SG			✓	
LL234	MG	✓		✓	
	SG			✓	
LL248	MG			✓	
	SG	✓	✓	✓	
LL442	MG			✓	
	SG			✓	

ID[a]		Pkn[b]	Pin[b]	Pcy[b]	Pct[b]
KP115	MG		✓		
KP480	MG			✓	
KP570	MG		✓		
KP612	MG		✓		
KP82	**SG**	✓	✓	✓	
KP94	SG	✓	✓		
KP646	**SG**		✓		
KP730	**SG**	✓	✓		
KP206	MG		✓	✓	
	SG			✓	✓
KP267	MG		✓		✓
	SG	✓	✓	✓	

ID[a]		Pkn[b]	Pin[b]	Pcy[b]	Pct[b]
TD247	MG	✓	✓		
TD317	MG		✓	✓	
TD395	MG		✓		
TD434	MG			✓	
TD457	MG		✓	✓	
TD20	SG		✓		
TD397	**SG**	✓	✓	✓	
TD470	**SG**		✓	✓	
TD497	SG		✓		
TD92	MG		✓		
	SG		✓		
TD309	MG			✓	
	SG	✓		✓	
TD456	MG		✓	✓	
	SG			✓	

[a]Each mosquito has a unique ID consisting of the village where it was collected (LL = Limbuak Laut, KP = Kampung Paradason, TD = Timbang Dayang) and a number.
[b]Mosquito midguts (MG) or salivary glands (SG) positive for *Plasmodium* species (Pkn = *P. knowlesi*, Pin = *P. inui*, Pcy = *P. cynomolgi*, Pco = *P. coatneyi*) are indicated by a tick mark. Samples that gave a positive amplification for the SSU rRNA gene fragment are in bold, and the species from which a sequence was obtained subsequent to cloning of the amplicon is denoted by a green tick. Sequencing was not carried out for midgut samples.

observations suggest the presence of two genetically distinct *P. inui* populations in Thailand. Similar observation of two distinct sub-populations of *P. inui* has been recently reported from Sarawak, Malaysian Borneo based on the analysis of their mitochondrial genome and a fragment of an apicoplast gene (Nada Raja et al., 2018). Furthermore, *P. inui* parasites were also found in *M. radiata* monkeys sampled in India, and a limited number of sequences from the *Cyt-b*, *MSP1-42* and the *SSU rRNA* genes indicated a population distinct from the others (Dixit et al., 2018). However, the smaller SSU rRNA sequence (ca. 382 bp) only partially overlapped the fragment we analysed, precluding its inclusion in the analyses.

Although the number of available sequences for *P. cynomolgi* is limited, the extent of sequence diversity surpasses that observed for *P. inui* sequences, and a clear pattern of geographical differentiation is also evident for the parasites obtained from Sabah (Malaysian Borneo) and those from Peninsular Malaysia (Fig. 1B). The one exception concerns the two haplotypes H_3 and H_4 that are both derived from the M strain (Fig. A2) a line derived from an isolate collected in the early 1930's (Mulligan, 1935) and that has been used for experimental studies thereafter. The sequence variation between these two haplotypes, though less extensive than that observed for the distinct *P. inui* haplotypes found in the same sample, could denote that two genetically distinct populations occur in the M strain. The monkey from which Sinton and Mulligan isolated the *P. cynomolgi* line (later known as the M strain) was obtained from Singapore, however, it is not clear whether this animal acquired the infection in or close to Singapore or whether it was imported from further afield.

The *P. knowlesi* haplotype network had the highest number of available sequences, and although nearly all were derived from samples collected in Malaysia (Fig. 1C), the extent of sequence diversity is relatively high. The one sample collected from Thailand was from an infection acquired in southern Thailand, relatively close to the Malaysian border (Jongwutiwes et al., 2004) and its sequence (H_39, Fig. A3) clustered with those collected from parasites present in Peninsular Malaysia. Indications of geographic differentiation is suggested by the network analysis and reflects the recent findings that genetically distinct populations can be discerned between Peninsular Malaysia and Malaysian Borneo (Divis et al., 2017; Yusof et al., 2016) as well as divergent sympatric populations in Malaysian Borneo (Assefa et al., 2015; Divis et al., 2015; Pinheiro et al., 2015). For this parasite species, sequences were available from samples collected from mosquitoes as well as the macaque and the human hosts in Malaysian Borneo, and the resulting haplotype network indicated an even distribution of the parasite populations in these three hosts a result concordant with previous analysis using other genetic markers (Divis et al., 2015, 2017; Fong et al., 2015b; Lee et al., 2011; Yusof et al., 2016).

Although informative and consistent with previous observations, the conclusions drawn from the haplotype analyses remain tentative as

Table 2
Accession numbers of all GenBank sequences included in the analyses and construction of haplotype networks.

Species	Origin[a] (Isolate ID)	Accession no.		
		An. balabacensis	Macaque	Human
P. knowlesi	M (Nuri strain)		AY327557	AM910985
	M (H strain)			L07560
	M-Selangor (B21)		**MF370091-MF370100** (MF370091, MF370093-95, MF370097-99)	
	M-Sarawak		DQ350264-DQ350269 (DQ350264-66, DQ350267-69)	AY327549-AY327556
			DQ641518-DQ641525 (DQ641518-20, DQ641524)	EU807923
			FJ619069	FJ804768
			FJ619087-FJ619090 (FJ619087-88) FJ619097-FJ619098	
	M-Sabah	MF582564-MF582565	MF582566	MF582562-MF582563
	M-Sabah (LL477sg)	**MF370081-MF370090** (MF370081-82, MF370086-87)		
	M-Sabah (KP730sg)	**MF370101-MF370107** (MF370101-05)		
	M-Sabah (TD397sg)	**MF370108-MF370109** (MF370108-09)		
	Thailand (A1)			AY580317
P. inui	M-Selangor (R1)		**MF370156-MF370160** (MF370158)	
	M-Sarawak		FJ619065	
			FJ619067	
			FJ619073-FJ69074 (FJ619073)	
			FJ619076	
			FJ619078-FJ619079 (FJ619078)	
			FJ619081-FJ619082	
			FJ619085	
			FJ619091	
			FJ619093	
			FJ619095-FJ619096 (FJ619095)	
			FJ619104	
	M-Sabah	MF582556-MF582560	MF582561	
	M-Sabah (KP82sg)	**MF370110-MF370119** (MF370110)		
	M-Sabah (KP646sg)	**MF370120-MF370124** (MF370120)		
	M-Sabah (TD92sg)	**MF370125-MF370130** (MF370125)		
	M-Sabah (TD470sg)	**MF370131-MF370140** (MF370132)		
	M-Sabah (LL237sg)	**MF370141-MF370148** (MF370141, MF370145)		
	M-Sabah (LL477sg)	**MF370149-MF370155** (MF370149)		
	Thailand		EU400384-EU400392 (EU400384-90, EU400392)	
			EU400395-EU400397 (EU400396-97)	
	Celebes		AB287277	
	Taiwan		FN256224-FN256230 FN430724-FN430725	
P. cynomolgi	M (Berok strain)		PCYB_021237	
			PCYB_032923	
	M (M strain)		AB287289-AB287290	
	M-Selangor (M28)		**MF370174-MF370183** (MF370174-77, MF370183)	
	M-Sabah	MF582549-MF582552		
	M-Sabah (LL442sg)	**MF370161-MF370163** (MF370161, MF370163)		
	M-Sabah (LL477sg)	**MF370164-MF370166** (MF370164)		
	M-Sabah (TD397sg)	**MF370167-MF370170** (MF370167-68)		
	M-Sabah (TD456sg)	**MF370171-MF370173** (MF370171)		
	M-Sarawak		FJ619084	
	Ceylon		L07559	
	Vietnam		L08241	
	Kalimantan		DQ660816	

[a]M-x = Malaysia-the state in which the sample originated.
*Accession numbers in black bold represent sequences generated in this study and deposited in GenBank; Accession numbers in green represent sequences used in this study to construct haplotype network.

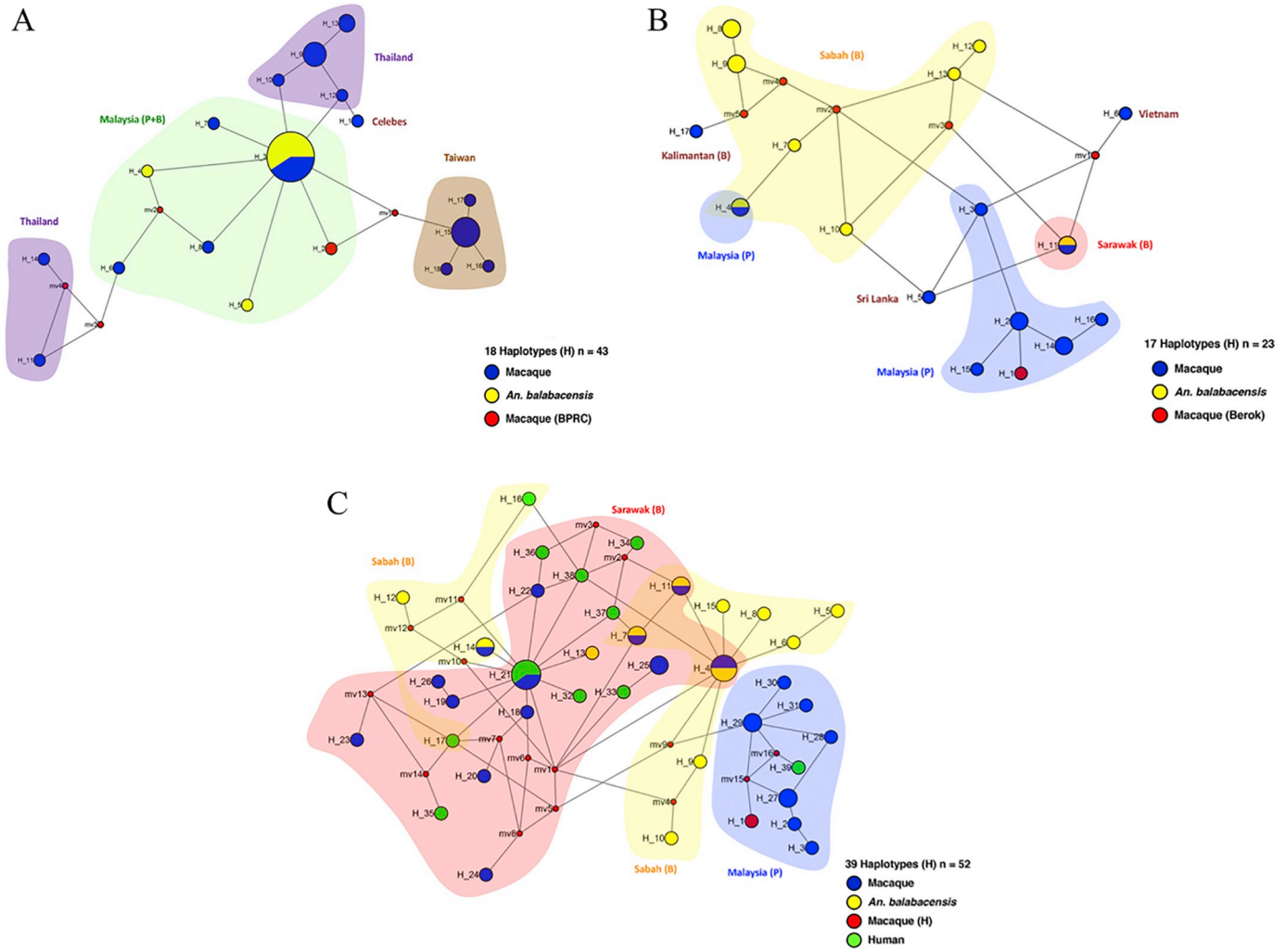

Fig. 1. A-Type SSU rRNA Haplotype Network for: A. *P. inui*, B. *P. cynomolgi*, C. *P. knowlesi.* The size of the circles representing each haplotype is proportional to the number of sequences that correspond to this haplotype. The red circle represents the sequence used as a reference in the alignment (Fig. 1A–C). Small red dots are hypothetical median vectors created by the program to connect sampled haplotypes into a parsimonious network. Distances between nodes are arbitrary. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

they are based on limited data. This will be a stepping stone to future studies. The numbers of sequences available for each species are not only low, but they are derived from a limited number of parasite isolates. Moreover, these isolates do not adequately encompass or reflect the geographical range of the parasite, or the different species/subspecies of macaques or anophelines that naturally harbour them. Whereas the total number of isolates that have been collected over the years by far exceeds those included in this analysis, the genes targeted for sequencing vary between investigations, as does their number and length, making it difficult to collate data from different studies in order to conduct meaningful population-wide analyses. It is also not easy to collect the parasites from the mosquitoes.

4. Conclusions

Given that elimination is the overall objective of malaria control programs in Southeast Asian countries, the zoonotic nature of some of the macaque-infecting parasites, clearly of concern for *P. knowlesi* and as yet potential for *P. cynomolgi* and *P. inui*, makes it highly desirable to undertake systematic investigations on the biology of these parasites. These should further encompass a survey of the parasites across their geographic range, as well as that of the anopheline species that ensures their transmission in the different regions. To this end, we suggest that a systematic approach should be adopted with respect to the collection of adequate number of samples and to the genetic analyses that will be subsequently undertaken. For example, defined sequences from a limited set of phylogenetically relevant genes (mitochondrial, SSU rRNA, etc.) would be obtained for each parasite-positive sample and made available to the community for further analyses. This will provide an expanding database that would enhance control measures in areas where zoonotic malaria occurs.

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Conflict of interest statement

We declare there is no conflict of interest.

Author contributions statement

Conceived and designed the experiments: IV, CD, THC; Funding acquisition: CD, IV; Performed the experiments: IV, MLW, CSL, WYWS, BOM; Analysed the data: MLW, AA, GS, FSQ; Wrote the paper: GS, AA, MLW, IV. All authors read and agreed to final version of manuscript.

Competing interest statement

The authors declare no competing interests.

Data availability statement

All data are included in the manuscript and in the additional files.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.04.010.

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