

New vectors in northern Sarawak, Malaysian Borneo, for the zoonotic malaria parasite, *Plasmodium knowlesi*

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Research

Keywords: Zoonosis, malaria, Plasmodium knowlesi, vector, Anopheles balabacensis, Anopheles barbirostris

Posted Date: January 28th, 2020

DOI: <https://doi.org/10.21203/rs.2.22077/v1>

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Version of Record: A version of this preprint was published at Parasites & Vectors on September 15th, 2020. See the published version at <https://doi.org/10.1186/s13071-020-04345-2>.

Abstract

Background

The vectors for *Plasmodium knowlesi*, a significant cause of human malaria in Southeast Asia, identified previously in nature all belong to the *Anopheles Leucosphyrus* Group. Only one study has been previously undertaken in Sarawak, Malaysian Borneo, to identify vectors of *P. knowlesi*, where *Anopheles latens* was incriminated as the vector in Kapit, central Sarawak. A study was therefore undertaken to identify malaria vectors in a different location in Sarawak.

Methods

Mosquitoes found landing on humans and resting on leaves over a 5-day period at two sites in the Lawas District of northern Sarawak were collected and identified. DNA samples extracted from salivary glands of anophelines were subjected to nested PCR malaria-detection assays. The small sub-unit ribosomal RNA (SSUrRNA) genes of *Plasmodium*, and the internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome c oxidase subunit 1 (CO1) sequences of the mosquitoes were derived from the *Plasmodium*-positive samples for phylogenetic analyses.

Results

A total of 65 anophelines and 127 culicines were collected. By PCR, six *An. balabacensis* and five *An. barbirostris* were found to have single *P. knowlesi* infections while three other *An. balabacensis* had either single, double or triple infections with *P. inui*, *P. fieldi*, *P. cynomolgi* and *P. knowlesi*. Phylogenetic analyses of the *Plasmodium* SSUrRNA genes confirmed 3 *An. barbirostris* and 3 *An. balabacensis* with single *P. knowlesi* infections, while 3 other *An. balabacensis* had two or more *Plasmodium* species of *P. inui*, *P. knowlesi*, *P. cynomolgi* and possibly novel species of *Plasmodium*. Phylogenies inferred from the ITS2 and CO1 sequences of *An. balabacensis* and *An. barbirostris* indicate that the former is genetically indistinguishable from *An. balabacensis* in Borneo while the latter is a novel sibling species belonging to the *Anopheles Barbirostris* Subgroup.

Conclusions

New vectors for *P. knowlesi* in Sarawak were identified, including *An. barbirostris*, which is a species that does not belong to the *Anopheles Leucosphyrus* Group.

Background

Human infections with *P. knowlesi*, a malaria parasite of long- and pig-tailed macaques [1], were thought to be extremely rare until a large number of human cases were described in the Kapit Division of Sarawak State, Malaysian Borneo [2]. Knowlesi malaria cases have been reported from different locations in Malaysia [2–7], in almost all countries in Southeast Asia [8–17] except Timor Leste, and also in the Nicobar and Andaman Islands of India [18]. This simian malaria parasite is now the most common cause

for malaria admissions to hospitals in Malaysia, where in the past 2 years (2017-2018) 7745 cases were reported, with 87% occurring in the states of Sabah and Sarawak in Malaysian Borneo (unpublished data, Ministry of Health Malaysia) [19]. Despite now being recognised as a significant cause of malaria in humans in Southeast Asia [20,21], there remains a paucity of information regarding the vectors of *P. knowlesi* in nature.

Information about vectors of human malaria parasites in Sarawak was first reported by McArthur [22] in his review regarding the importance of the *Anopheles* Leucosphyrus Group in malaria transmission. Dissection of various species of *Anopheles* mosquitoes were carried out in the Kuching General Hospital and oocysts were found in the midguts of *An. latens*. *An. latens*, *An. barbirostris*, and *An. donaldi* were later incriminated as the vectors for human malaria parasites in Upper Baram [23,24]. In Miri, Chang et al. [25] discovered malaria sporozoites in the salivary glands of *An. donaldi* and *An. letifer* in an oil palm plantation. However, the *Plasmodium* species of sporozoites harboured by these vectors could not be determined accurately since molecular tools were not available when these studies were undertaken. Despite this, inoculation of sporozoites into uninfected rhesus monkey by the simiophagic *An. hackeri* demonstrated that it was the natural vector of *P. knowlesi* in Peninsular Malaysia [26]. It was only in 1999, in Belaga, Sarawak that an ELISA was used for detection of *Plasmodium* sporozoites species in mosquitoes but the ELISA could only detect two species of *Plasmodium*; *P. falciparum* and *P. vivax* [27].

The first and only entomological study in Sarawak that identified *Plasmodium* species using molecular methods was conducted from 2005-2006. Using nested PCR assays on DNA extracted from salivary glands of mosquitoes in Kapit district, *An. latens* was incriminated as the vector of *P. knowlesi* in this region [28]. Subsequently, similar studies utilising molecular tools have incriminated *An. cracens* as the vector of *P. knowlesi* in Kuala Lipis, Peninsular Malaysia [29] while *An. balabacensis* was found to harbour sporozoites of *P. coatneyi*, *P. cynomolgi*, *P. inui*, *P. knowlesi*, and unidentified *Plasmodium* species in Sabah, Malaysian Borneo [30]. *An. introlatus* was hypothesised to be a vector in Selangor, Peninsular Malaysia as they discovered one out of 55 *An. introlatus* to be infected with only oocysts, but not sporozoites of *P. knowlesi* [31]. In Southern Vietnam where co-infection of *P. vivax* and *P. knowlesi* was predominant in humans and mosquitoes, *An. dirus* was shown to be the vector of *P. falciparum*, *P. vivax* and *P. knowlesi* using molecular methods [15]. Information derived from these studies was undeniably imperative for vector control but similar knowledge of vectors of *P. knowlesi* is lacking due to restricted sampling only in the Kapit district in Sarawak [28].

Following the increased accessibility of molecular methods in the recent decades, most *Anopheles* malaria vectors have been found to comprise complexes of sibling species which have virtually identical morphological characteristics [32,33]. The second internal transcribed spacer (ITS2) within the ribosomal DNA, mitochondrial cytochrome c oxidase sub-unit one (CO1), and NADH dehydrogenase sub-unit six (ND6) were previously used as target DNA sequences to study the phylogeny of closely-related mosquito species [33–35]. As a result, closely-related species which were thought to be identical were successfully differentiated. This demonstrates that morphological characterisation alone might be inadequate in determining the identity of the vectors of malaria.

The current study was aimed at incriminating the vector(s) of *P. knowlesi* and other malaria parasites in northern Sarawak (Lawas District), as well as providing an accurate identity of the incriminated vector(s). Lawas District was selected as the study site since 173 patients with knowlesi malaria had been admitted to Lawas Hospital in the 3 years prior to the commencement of the study (unpublished data, Lawas Hospital).

Methods

Study site

The study was carried out in the Lawas District of Northern Sarawak, Malaysian Borneo (Figure 1). Lawas District is bordered by Brunei on its west, Sabah on its east, North Kalimantan on its south, and Labuan Federal Territory on its north. The first mosquito collection site was close to Long Tengoa village (DMS: 4 37'5" N 115 20'23" E) which had 3 recent human cases of knowlesi malaria. The collection site was situated in a forested area approximately 500 m eastward from the village and was at an elevation of about 100 m above sea level. The second collection was conducted in an abandoned army camp (4 16'18" N 115 31'49" E) close to Long Luping, approximately 40 km southward from Long Tengoa. This camp was situated at an elevation of approximately 650 m above sea level right beside a stream. Three patients who was admitted to Lawas Hospital with knowlesi malaria within 3 months prior to this study had spent time hunting near this camp site.

Mosquito collection, identification, and dissection

The first mosquito collection was carried out by 6 collectors for a period of 3 days in September 2014 in Long Tengoa village. The second collection at Long Luping was carried out by 5 collectors for 2 days in May 2015. All mosquitoes collected in Long Tengoa (1800 hr to 2300 hr) were collected using the human landing catch method while those collected from Long Luping (Day 1: 1600 hr to 2200 hr; Day 2: 1600 hr to 2100 hr) were collected using both the human landing catch method and the resting catch method. For the human landing catch method, all mosquitoes landing/biting on human bait were caught using a cylindrical specimen tube (18 mm in diameter X 50 mm in height) with moist tissue covering the bottom of the tube. Once a mosquito was caught, the opening of the tube was plugged with cotton wool and labelled according to the time of collection. The procedures for resting catch method were similar to that of the human landing catch method except that mosquitoes found resting under leaves were collected. The mosquitoes were then brought to the field laboratory for morphological identification. Anopheline mosquitoes were identified for their species using taxonomic keys while non-anophelines were identified for their genera [37–39]. Salivary glands of all anophelines were then carefully dissected and preserved in individual 1.5-mL microcentrifuge tubes containing 0.5 mL absolute alcohol. Preserved salivary glands were transported to the Malaria Research Centre, Universiti Malaysia Sarawak, for further molecular analysis.

DNA extraction and detection of *Plasmodium* species

Genomic DNA of the dried salivary glands was extracted using DNeasy® Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. The eluted samples were kept at 4 °C until required. Extracted DNA samples were initially subjected for nested PCR assays for the detection of *Plasmodium* DNA based on the SSU rRNA genes [40]. *Plasmodium*-positive samples were then undergone another rounds of PCR assays for the identification of *P. coatneyi*, *P. cynomolgi*, and *P. knowlesi* using species-specific PCR primers as previously described [1]. Additionally, new PCR primers were developed for *P. fieldi* (PfldF3: 5'-GAT CTT TTT TTG TTT CGG CAT TGA A-3'; PfldR3: 5'-AAG GCA CTG AAG GAA GCA ATC TAA GAG TTT-3') and *P. inui* (PinF5: 5'-GTA TCG ACT TTG TGC GCA TTT TTC TAC-3'; INAR3: 5'-GCA ATC TAA GAG TTT TAA CTC CTC-3') with optimum annealing temperatures at 60 and 62 , respectively.

Generating *Plasmodium* SSUrRNA gene amplicons

The SSUrRNA genes were generated by semi-nested PCR assays in order to increase the DNA yield prior to cloning. Nest 1 PCR amplification was performed using primers rPLU1 and rPLU5 as previously described [40]. Then, PCR product from Nest 1 was used as DNA template for the Nest 2 PCR amplification using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA). PCR primers rPLU3 and rPLU5 [40] were used with an annealing temperature of 68 .

Generating *Anopheles* CO1 and ITS2 region amplicons

Both the CO1 and ITS2 regions of the vectors were amplified by PCR assays using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. To amplify the a 748 bp-long CO1 region, PCR primers AnCOX1F (5'-GGAATGGATGTWGATACWCGAGC-3') and AnCOX1R (5'-CCTAAATTTGCTCATGTTGCC-3') were designed with the annealing temperature of 65. For the ITS2 region, DNA amplification was performed using PCR primers 5.8SF and 28SR as previously described by Paredes Esquivel et al. [41].

Cloning and sequencing

Generated amplicons were ligated with pCR®-Blunt Vector (Invitrogen, USA) and transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, USA), according to the Zero Blunt® PCR Cloning Kit (Invitrogen, USA) protocol. *E. coli* transformed colonies were screened for the presence of target DNA insert and plasmids containing the inserts were purified using the PureLink® Quick Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). DNA sequencing of plasmid DNA was conducted according to the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) protocol, using M13 primers, internal primers rPLU2 and rPLU6 for the *Plasmodium* SSUrRNA inserts, and internal primers JIBF (5'-CTA GTG TGC TTC CCA TGG AGA TAG-3') and JIBR (5'-CAC ACC CAA CCC AAT AAA AAT TG-3') for ITS2 inserts.

Phylogenetic analysis

No modifications were done on the SSUrRNA and CO1 sequences prior to alignment. For the ITS2 region, the central repeat region contained within it was identified as described previously [38] using the Tandem

Repeats Finder software at <https://tandem.bu.edu/trf/trf.html> [42] and trimmed. The trimmed sequences were then aligned for phylogenetic analyses. Reference sequences obtained from GenBank [33,35,43–45] are listed in the additional files [Additional Files 1 & 2]. Multiple sequence alignments were performed using the default parameters of ClustalW within the LaserGene 7.1 programme (DNASTAR). The best nucleotide substitution models were calculated using MEGA 7.0.21 and the models with the lowest Bayesian Information Criterion (BIC) were selected [46,47]. Subsequently, phylogenetic trees were constructed by the Maximum Likelihood (ML) method using MEGA 7.0.21 with bootstrap values calculated from 1000 replicates [48].

Results

Species composition of collected mosquitoes

A total of 192 mosquitoes were collected from Long Tengoa (n=86) and Long Luping (n=106) (Table 1). Based on morphological characteristics, mosquitoes from the genus Anopheles were the most abundant (n=65; 33%), followed by Culex (n=41; 21%), Aedes (n=40; 21%), Armigeres (n=31; 16%), Mansonia (n=7; 4%), Malaya (n=6; 3%), and Uranotaenia (n=2,1%).

A higher proportion of culicines were found in Long Tengoa (n=72; 84%), while anophelines predominated half of the total collection in Long Luping (n=61, 48%). Most *An. balabacensis* (n=31) and *An. barbirostris* (n=18) mosquitoes were caught at Long Luping while members of the *Anopheles Umbrosus* (*An. letifer*, *An. roperi*, and *An. umbrosus*) were collected in Long Tengoa, and these were absent in Long Luping (Table 1).

Table 1. Anopheles mosquitoes collected from two sites in Lawas.

Genera/Species	Number of mosquitoes		
	Long Tengoa	Long Luping	Total
<i>An. balabacensis</i>	0	31	31
<i>An. latens</i>	1	1	2
<i>An. barbirostris</i>	0	18	18
<i>An. donaldi</i>	3	0	3
<i>An. letifer</i>	5	0	5
<i>An. roperi</i>	1	0	1
<i>An. umbrosus</i>	4	0	4
<i>An. tessellatus</i>	0	1	1
<i>Aedes</i>	9	31	40
<i>Armigeres</i>	31	0	31
<i>Culex</i>	31	10	41
<i>Malaya</i>	0	6	6
<i>Mansonia</i>	1	6	7
<i>Uranotaenia</i>	0	2	2
Total	86	106	192

Plasmodium species in Anopheles mosquitoes

DNA extracted from the salivary glands of 65 anopheline mosquitoes were subjected to nested PCR assays and Plasmodium DNA was only detected in samples derived from 10 *An. balabacensis* and 6 *An. barbirostris* collected in Long Luping. Of 31 *An. balabacensis*, 9 (29.0 %) were found to carry *P. knowlesi* and other simian malaria parasites while 5 (27.8 %) *An. barbirostris* were found to be infected with only *P. knowlesi* (Table 2). The identity of the species of Plasmodium could not be determined for 2 of the Plasmodium-positive samples by using the species-specific PCR primers for *P. coatneyi*, *P. cynomolgi*, *P. inui*, *P. fieldi*, and *P. knowlesi*.

Table 2. Summary of results of PCR assays for Plasmodium-positive samples from Long Luping.

Species	Number analysed by PCR assays	Number of positive samples		Sample ID (LW)
		Genus-specific PCR assay	Species-specific PCR assays*	
<i>An. balabacensis</i>	30	10	1: No species identified 6: <i>Pk</i> 1: <i>Pcy</i> + <i>Pk</i> 1: <i>Pcy</i> + <i>Pfld</i> + <i>Pin</i> 1: <i>Pin</i>	101 31, 32, 49, 50, 51, 59 45 67 74
<i>An. barbirostris</i>	18	6	1: No species identified 5: <i>Pk</i>	38 44, 47, 48, 57, 58

**Pct* = *P. coatneyi*; *Pcy* = *P. cynomolgi*; *Pfld* = *P. fieldi*; *Pin* = *P. inui*; *Pk* = *P. knowlesi*.

All 14 samples which were tested positive for at least one simian malaria parasite were subjected to another round of semi-nested PCR to obtain a longer fragment of the SSUrRNA for sequencing. Amplicons could not be generated for 5 (3 *An. balabacensis* and 2 *An. barbirostris*) of the samples infected with only *P. knowlesi*. As a result, a total of 38 SSUrRNA fragments were cloned and sequenced from 9 anophelines (6 *An. balabacensis* and 3 *An. barbirostris*) which were Plasmodium-positive by nested PCR assays. The phylogenetic tree (Figure 2) constructed using the Plasmodium A- and S-type SSUrRNA sequences showed that *An. balabacensis* were harbouring sporozoites of *P. cynomolgi* (LW67), *P. inui* (LW67 and LW74), and *P. knowlesi* (LW45, LW59, LW31, LW49) in their salivary glands while *An. barbirostris* carried only *P. knowlesi* (LW47, LW57, LW58). Three *An. balabacensis* (LW45, LW67, LW74) were shown to carry more than 1 species of Plasmodium while the other 2 *An. balabacensis* (LW31, LW49) were infected with only *P. knowlesi*. Multiple A-type SSUrRNA sequences derived from the salivary glands of mosquitoes LW45, LW67, and LW74 formed several distinct clades instead of grouping with any of the clades formed by the reference sequences (Figure 2). This indicates that *An. balabacensis* is not only harbouring sporozoites of *P. cynomolgi*, *P. inui*, and *P. knowlesi*, but also sporozoites of unknown species of Plasmodium.

Molecular characterisation of Anopheles mosquitoes

The ITS2 region and CO1 gene [see Additional Files 3 & 4 for multiple sequence alignment of CO1] of anophelines carrying simian malaria parasites were further sequenced to confirm their identities. Phylogenetic analyses of the ITS2 region and CO1 gene of *An. barbirostris* s. l. from Thailand, West Sumatra, West Java, and South Kalimantan showed that samples from each localities have formed distinct clades (Figures 3 & 4). The phylogenetic tree constructed using the ML method with the ITS2 sequences showed that *An. barbirostris* collected in this study from the Lawas district clustered together with those from Selangor, Peninsular Malaysia (Accession no.: KJ462243, KJ462248, and KJ462247). In addition, the ITS2 sequences from Selangor are also the only ones with identical length to the ITS2 of *An. barbirostris* characterised in the current study (Table 4). These *An. barbirostris* will be referred to as *An. barbirostris* Clade VI. On the other hand, the ML tree of the CO1 gene of *An. barbirostris* Clade VI collected in Lawas, Malaysian Borneo appeared to be distinct from the other *An. barbirostris* s. l. (Figure 4). *An. barbirostris* Clade VI and *An. barbirostris* s. l. however, are shown to more closely related to each other than to the outgroup, *An. coustani*.

Table 4. Lengths of 5.8S-ITS2-28S region of members of the Barbirostris Subgroup amplified by primers 5.8SF and 28SR.

Species	Reference	Locality	Amplicon size (bp)
<i>An. barbirostris</i> s.s	[33]	South Kalimantan, Thailand	1674-1677
<i>An. vanderwulpi</i>		Sumatra	1858
<i>An. barbirostris</i> Clade III		Thailand	1860-1862
<i>An. barbirostris</i> Clade IV		Sumatra, Thailand	1713-1718
<i>An. campestris</i>		Thailand	1652
<i>An. barbirostris</i> VI	[45]	Selangor, Peninsular Malaysia	1576
<i>An. barbirostris</i> VI	Current study	Sarawak, Malaysian Borneo	

The CO1 fragment of *An. balabacensis* obtained in this study showed close phylogenetic relationship (Figure 5) with the other *An. balabacensis* sequences. Those collected from this study in Sarawak, Malaysian Borneo clustered together with the *An. balabacensis* in Eastern Sabah, Malaysian Borneo (Accession no.: DQ897940), forming a sister clade to the *An. balabacensis* from South Kalimantan, Indonesian Borneo (Accession no.: DQ897941).

Discussion

Variation of mosquito compositions between collection sites

There were variations in the mosquito compositions of Long Tengoa and Long Luping, which are only 40 km apart and with an altitude difference of 500 m, even though the numbers of each species collected were small. Previous studies have consistently shown that mosquito composition differs in collection sites with different ecological settings, even when the sites were only 1,500 m away [24,27]. Peak biting

times of identical mosquito species were also reported to vary between entomological survey sites [30,49]. The fact that both the incriminated vectors of *P. knowlesi* (*An. balabacensis* and *An. barbirostris*) in Long Luping were not found in Long Tengoa implies that a different species of anopheline could potentially be the vector in the latter site. However, one of the limitations of this study is the relatively short duration of collection periods and the small number of mosquitoes collected; a more extensive survey may well have found *An. balabacensis* and *An. barbirostris* in Long Tengoa. Nevertheless, the detection of simian malaria parasites in the salivary glands of wild-caught *An. balabacensis* and *An. barbirostris* calls for more detailed longitudinal studies on seasonal variation in composition and on the bionomics of the vectors in this area, including host preference.

Identification of *Plasmodium*

Sporozoites of *P. knowlesi* and other simian malaria parasites were identified in 9 *An. balabacensis* and 5 *An. barbirostris* by nested PCR assays. The *An. balabacensis* and *An. barbirostris* examined had sporozoite infection rates of 29.0 % and 27.8% respectively, which are exceedingly high compared to other vector studies in Malaysia. In the neighbouring Malaysian Borneo state of Sabah, *An. balabacensis* was identified as the vector for *P. knowlesi* with sporozoite rates ranging from 1.03 to 3.42% at three different sites [30]. Infection rates of <2.00 % were reported among vectors in central Sarawak and among *An. cracens* in Peninsular Malaysia [27,29,49]. The only other study known to have found a comparable sporozoite rate was a study done in Palawan, Philippines where 29.4% of the *An. balabacensis* examined had sporozoites [50]. The high sporozoite rates of the vectors could be attributed to the site of collection as well which is consistent with the finding of the previous study involving *An. latens* in Kapit, Sarawak [49] where it was found that the sporozoite rate was highest in the forest, followed by in the farm at the forest-fringe, and in the long house. Long Luping is an abandoned army camp which is far away from any human settlement. It is potentially a foraging site for the macaques due to the banana trees that grow around its perimeter. No vector control activities have been carried out since the army camp was abandoned and macaques were sighted at the site during the entomological surveys, suggesting that macaques and mosquitoes could forage and breed, respectively, undisturbed in this site. The high infection rates could also be due to the increased sensitivity of the nested PCR assays compared to microscopy which is normally used in detecting sporozoites.

The failure to sequence the SSUrRNA genes of *Plasmodium* from 5 samples that were malaria-positive by nested PCR assays could be caused by the low number of sporozoites present in the salivary glands of the mosquitoes, leading to a low level of *Plasmodium* DNA extracted from the salivary glands.

Phylogenetic analyses of the isolated SSUrRNA gene sequences indicates that at least two species of *Plasmodium* may be novel were found in *An. balabacensis*. However, the low quantity of DNA extracted from the salivary glands prevented the sequencing of another gene which would have been necessary to determine whether these are indeed novel species of *Plasmodium*. It is highly likely that unidentified *Plasmodium* species co-infected the vectors when they fed on macaques, which have been found in Sarawak to harbour at least 7 species of *Plasmodium* [51]. With an increasing number of zoonotic malaria infections in the world [52–54], epidemiological studies of these novel species within human

populations that come into close contact with macaques during activities in the forest and forest-fringe will be required to monitor potential host-switch events.

Molecular characterisation of vectors

While all the *An. balabacensis* identified in this study had formed a monophyletic clade with the other *An. balabacensis* from Sabah and Indonesia, the collected *An. barbirostris* appeared to have formed its own clade with those from Peninsular Malaysia, distinct from the other members of the Barbirostris Subgroup. It is highly likely that the *An. barbirostris* Clade VI reported in this study is a previously uncharacterised, closely related species within the Barbirostris Group. In addition to being morphologically and phylogenetically similar to other members of the Barbirostris Group, the length of its ITS2 region has further supported this hypothesis (Table 4). To the best of our knowledge, members of the *An. barbirostris* are the only *Anopheles* species with an ITS2 sequence length of more than 1.5 kb, mainly due to its multiple internal repeats [33]. Despite the roles of both *An. barbirostris* and *An. balabacensis* in malaria transmission [55–59], the taxonomy of the latter species and its species group was studied more extensively than the former [33,35,60–63]. It is therefore likely that more cryptic species will be discovered in the Barbirostris Group than within the Leucosphyrus Group.

The Leucosphyrus Group was thought to be the only species group capable of transmitting *P. knowlesi* but it was recently shown in the Nicobar and Andaman Islands in India that *An. sundaicus* is potentially a vector of this parasite [64]. Apart of *An. sundaicus*, this is the second time a species (*An. barbirostris*) from an entirely different subgenus than the Leucosphyrus Group, is incriminated as a vector for *P. knowlesi* in a natural setting. This suggests that there are possibly more *Anopheles* species that could transmit the disease and maintain the high level of endemicity in the macaque populations.

Nevertheless, in Sarawak, more detailed studies need to be conducted on the bionomics of both vectors to provide data for the implementation of appropriate vector control. Species-specific molecular assays should also be designed and utilised in future vector incrimination studies for this species range in order to correctly identify malaria infective mosquitoes.

Implications for vector control in Sarawak

The incrimination of *An. balabacensis* and *An. barbirostris* as novel vectors for *P. knowlesi* in Northern Sarawak calls for re-evaluation of current and future vector control methods in the state. Detailed studies first need to be undertaken to determine the feeding behaviour and host preference of these vectors. From the aspect of insecticide-based preventative measures, future insecticide resistance surveys should include both *An. balabacensis* and *An. barbirostris* Clade VI to ensure that the insecticide used would still be efficient in killing these vectors. Residual spraying of insecticide could also be considered for uninhabited buildings like the army camp in Long Luping, where human presence is intermittent. Apart from insecticides, clustered regularly interspaced short palindromic repeat (CRISPR)-based gene drive has been recently suggested as one possible prospect for the control of *P. knowlesi* vector(s). Gene drive is a biotechnology method used to increase the spread of a genetic trait (e.g. mosquito sterility/mosquito

immunity against *Plasmodium* infection) into the wild population. As gene drive spreads a certain genetic trait in a mosquito population by means of mating and inheritance, it is an extremely species-specific vector control method [65]. Southeast Asia however, might not be ready for this technology as the primary vector of *P. knowlesi* in Southeast Asia has yet to be determined.

Conclusion

An. balabacensis has been incriminated as a vector of *P. knowlesi* and other simian malaria parasites, and *An. barbirostris* Clade VI as a vector of *P. knowlesi* in Lawas, Northern Sarawak in Malaysian Borneo. Phylogenetic analyses have shown that the *An. balabacensis* in Lawas were indistinguishable from the *An. balabacensis* in Eastern Sabah, Malaysian Borneo and South Kalimantan, Indonesian Borneo. Phylogenetic analyses also indicate that the *An. barbirostris* Clade VI present in Lawas and Selangor were probably a sibling species to the other five members (*An. barbirostris* s.s., *An. vanderwulpi*, *An. barbirostris* Clade III, *An. barbirostris* Clade IV, and *An. campestris*) of the Barbirostris Group.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Universiti Malaysia Sarawak and by the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-10-1194-7854). All field staff and volunteers who carried out mosquito collections were provided with antimalarial prophylaxis.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by research grants from the Ministry of Higher Education in Malaysia (FRGS/1/2014/SKK01/UNIMAS/03/1), Universiti Malaysia Sarawak (grant no.: F05/SpFRC/1433/16/1, F05/DPP/1508/2016), and by a postgraduate scholarship to JAXD from the Ministry of Higher Education in Malaysia.

Authors' contributions

BS designed the study and supervised JAXD, KAK and DSAM while they carried out the molecular studies. BS, PCSD and JAXD analysed the results. KY organised the fieldwork logistics, AM organised and supervised staff from Sarawak Department of Health involved in the field trips and JAXD undertook the fieldwork. BS and JAXD wrote the paper and all the authors read and approved the final manuscript.

Acknowledgements

We would like to thank staff from the Sarawak State Health Department and Lawas District Health Office for their assistance during the field trips. We also thank the Director General of Health Malaysia for permission to publish this article.

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Figures

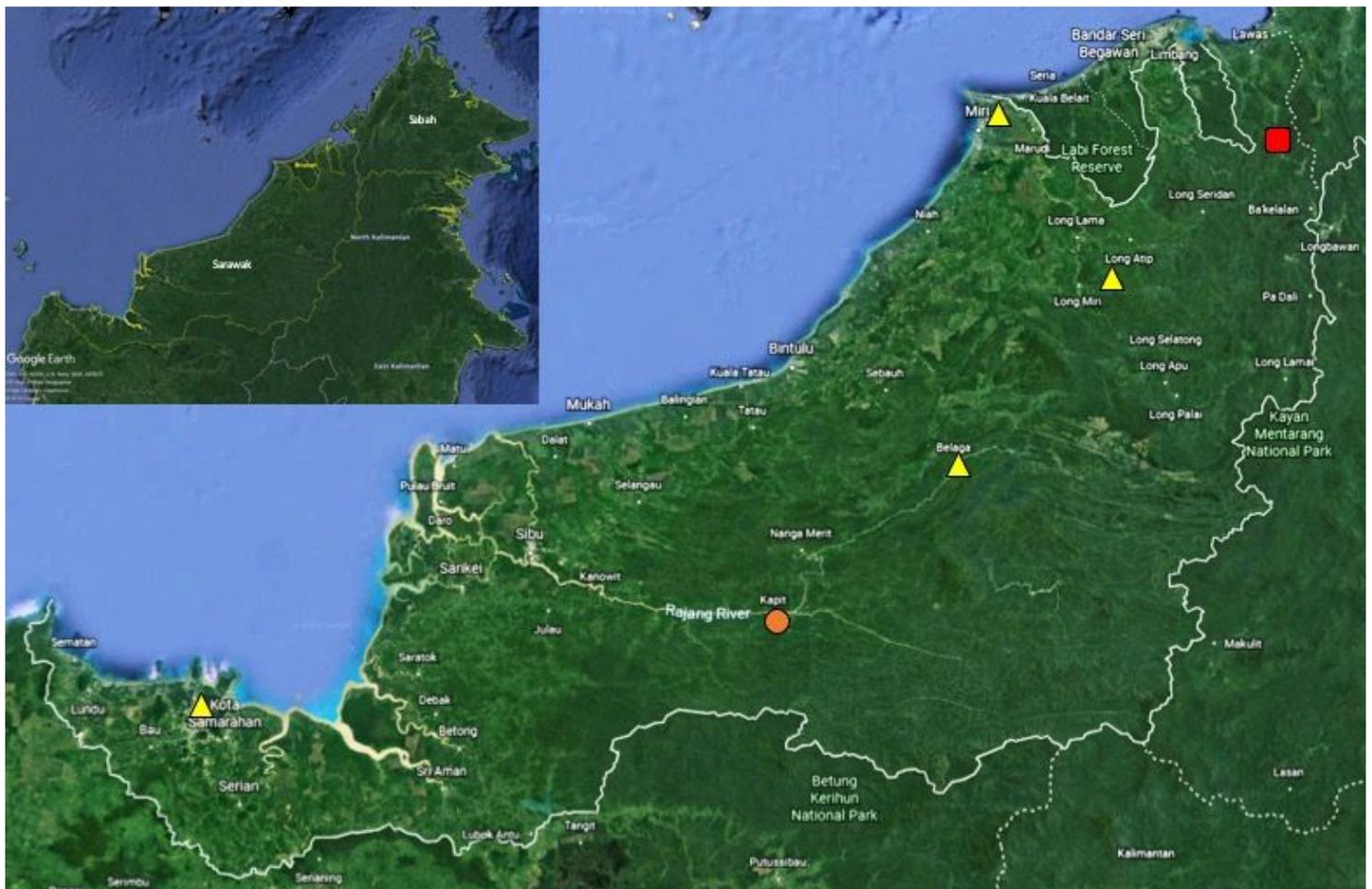


Figure 1

Locations of entomological surveys conducted previously and for the current study [36]. Human malaria parasites were discovered from dissected mosquitoes from these sites [22–25,27] *P. knowlesi* and other simian malaria parasites were discovered from dissected mosquitoes from this site [28] Study site for the current study

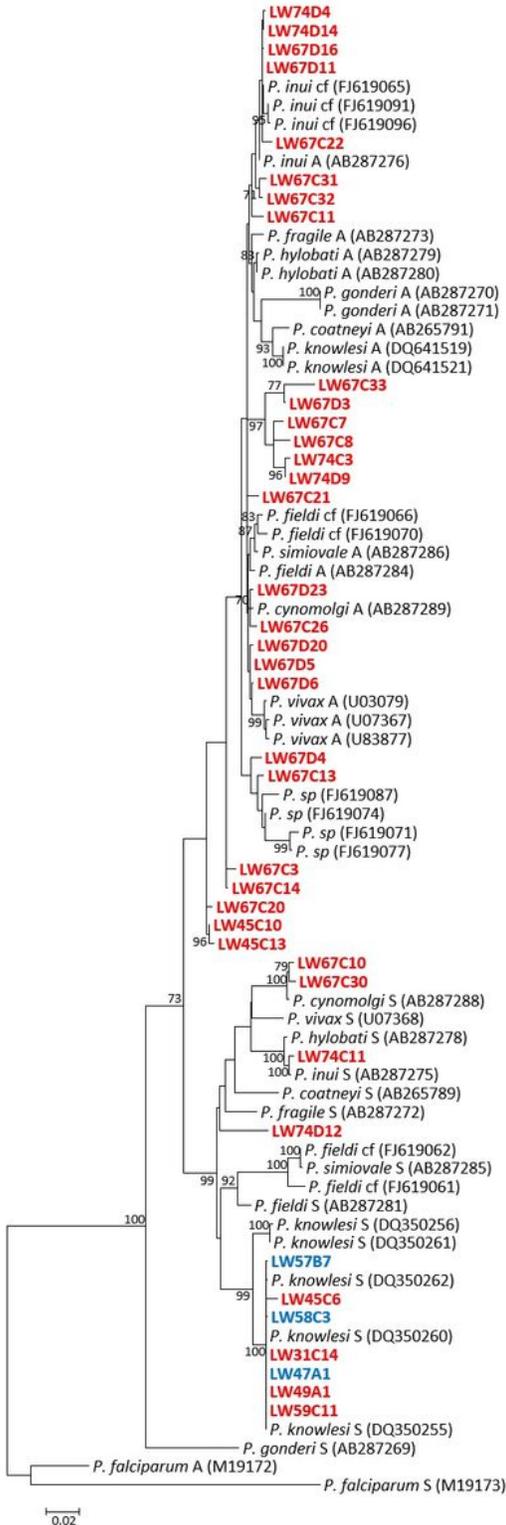


Figure 2

Phylogenetic tree based on the Plasmodium SSUrRNA genes using the ML method. The analysis was based in the Tamura 3-parameter + G + I substitution model. GenBank accession numbers are in brackets while letters 'A' and 'S' represent the different isoforms of the SSUrRNA gene. Only bootstrap values >70% are shown on the nodes. Blue and red colours represent parasites obtained from *An. barbirostris* and *An. balabacensis*, respectively.

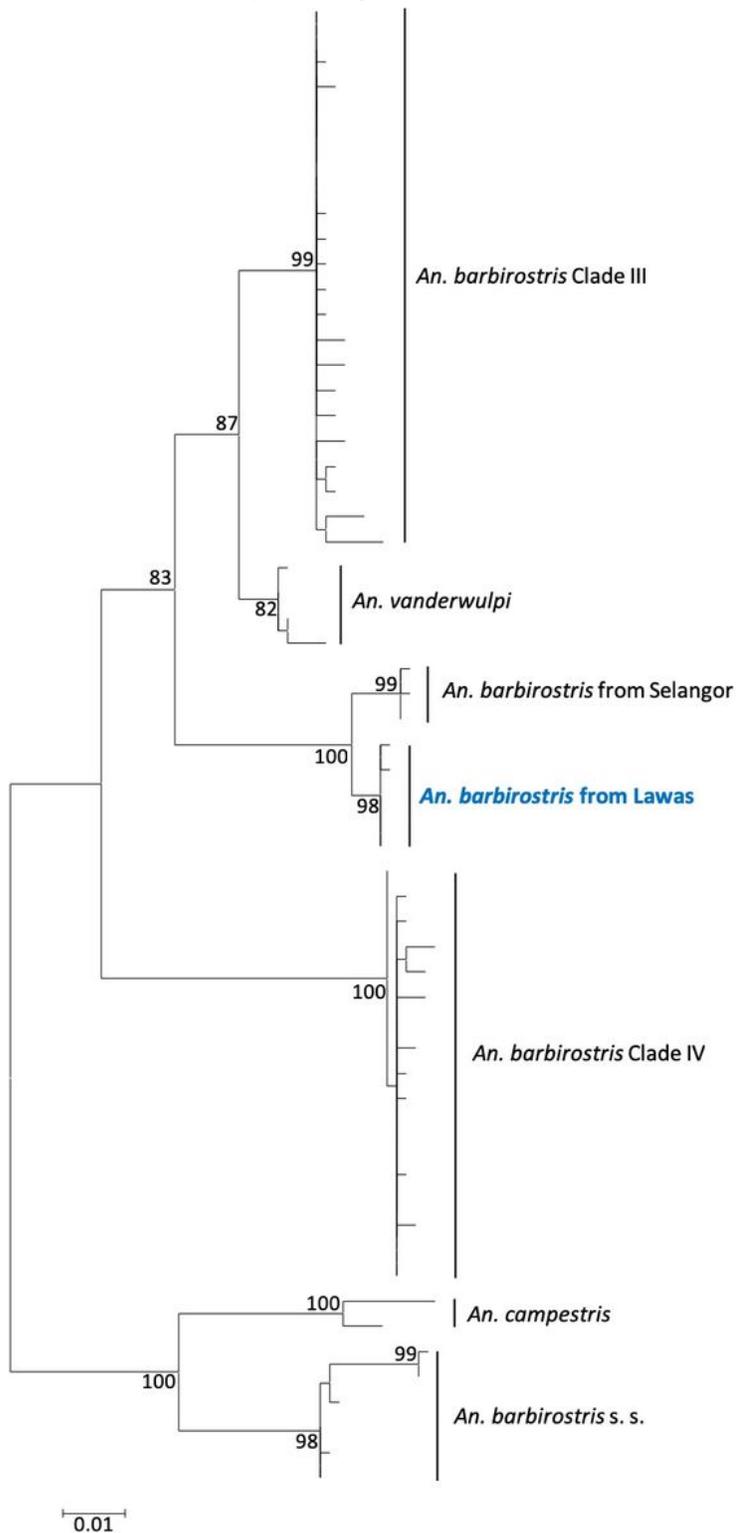


Figure 3

. Phylogenetic tree based on the Anopheles ITS2 region using the ML method. The analysis was based in the Kimura 2-parameter + G substitution model. Only bootstrap values >70% are shown on the nodes.

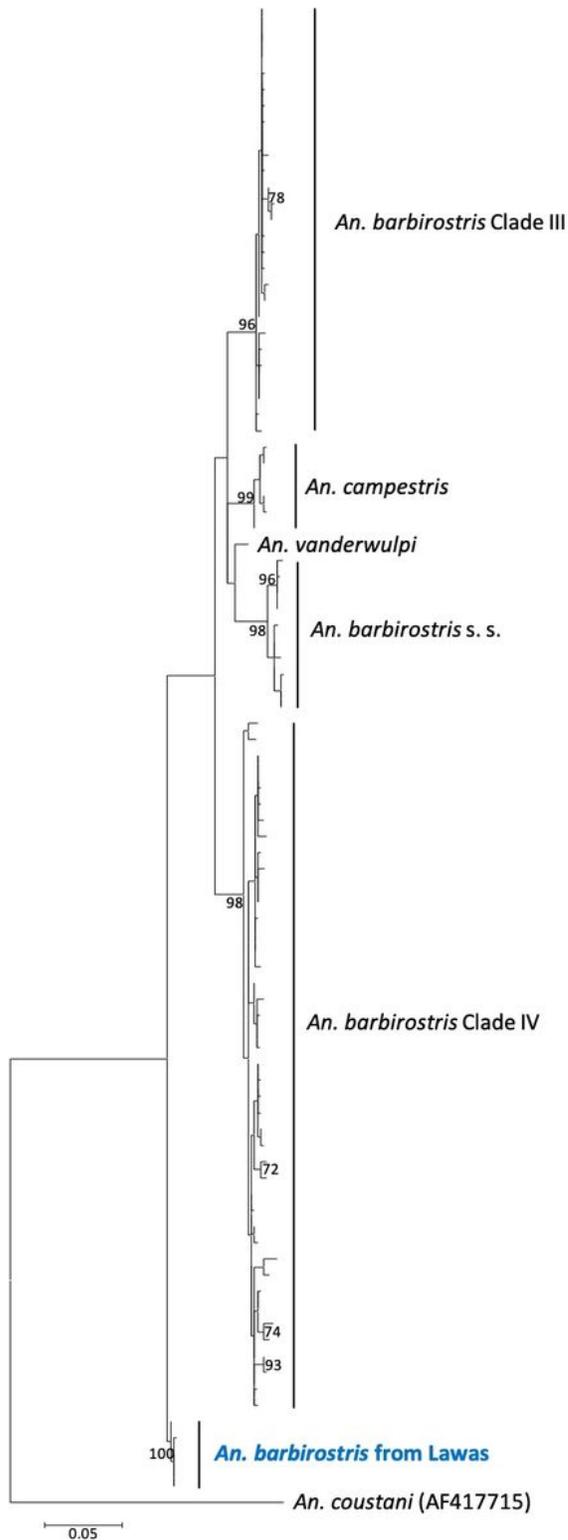


Figure 4

Phylogenetic tree based on the Anopheles CO1 gene using the ML method. The analysis was based in the Tamura 3-parameter + G + I substitution model. Only bootstrap values >70% are shown on the nodes. GenBank accession number is in brackets.

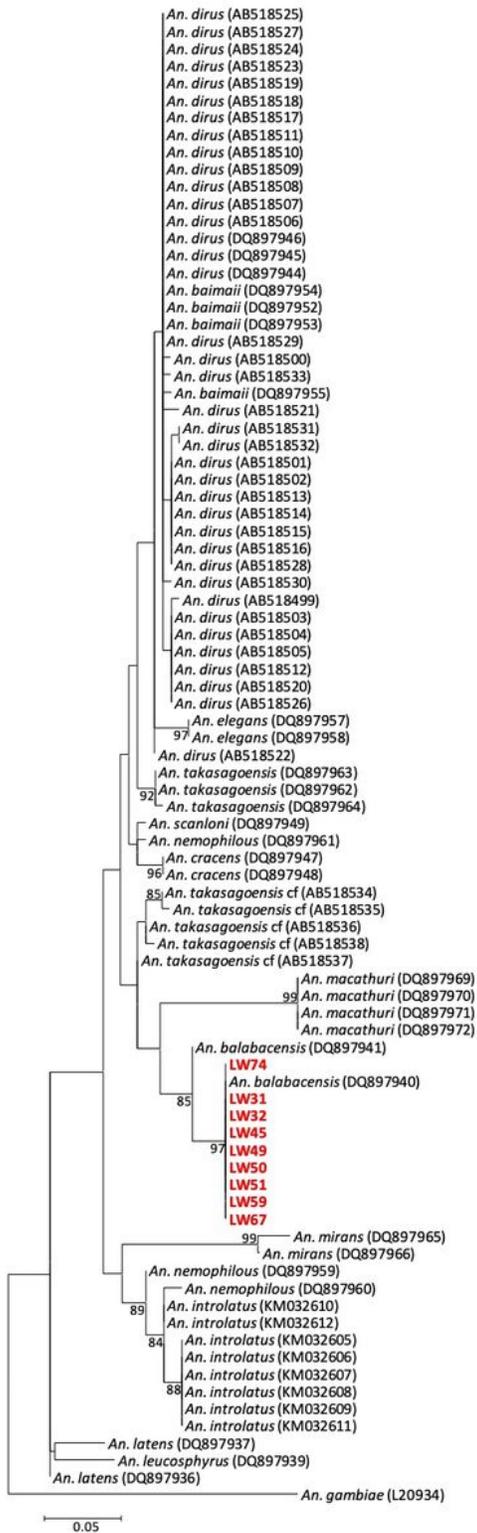


Figure 5

Phylogenetic tree based on the Anopheles CO1 gene using the NJ method. The analysis was based in the Tamura 3-parameter + G substitution model. Only bootstrap values >70% are shown on the nodes. GenBank accession number is in brackets. Red colour represents *An. balabacensis* obtained from Lawas district.

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