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

Joshua Ang Xin De
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences https://orcid.org/0000-0002-4710-8831
Khamisah Abdul Kadir
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences
Dayang Shuaisah Awang Mohamad
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences
Asmad Matusop
Sarawak State Health Department
Paul Cliff Simon Divis
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences
Khatijah Yaman
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences
Balbir Singh (bsingh@unimas.my)
Universiti Malaysia Sarawak Faculty of Medicine and Health Sciences

Research

Keywords: ZOONOSIS, MALARIA, PLASMODIUM KNOWLESI, VECTOR, ANOPHELES BALABACENSIS, ANOPHELES BARBIROSTRIS CLADE VI

Posted Date: April 2nd, 2020

DOI: https://doi.org/10.21203/rs.2.22077/v2

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 Anopheles mosquitoes were subjected to nested PCR malaria-detection assays. The small sub-unit ribosomal RNA (SSUrRNA) genes of Plasmodium , and the internal transcribed spacer 2 (ITSII) and mitochondrial cytochrome c oxidase subunit 1 (COI) 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 Clade VI were found to have single P. knowlesi infecions 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 Clade VI 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 ITSII and COI sequences of An. balabacensis and An. barbirostris Clade VI 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 Clade VI , 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 years 2017 and 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 and an additional challenge to malaria elimination in Southeast Asia [20–23], there remains a paucity of information regarding the vectors of *P. knowlesi* in nature.

In the period between 1951 to 1997, malaria vectors in Sarawak were determined by examining dissected salivary glands for the presence of sporozoites. Information about vectors of human malaria parasites was first reported by McArthur [24] in his 1951 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*. In 1956 and 1995, *An. latens, An. barbirostris* s. l., and *An. donaldi* were later incriminated as the vectors for human malaria parasites in Upper Baram [25,26]. In Miri, Chang et al. [27] discovered malaria sporozoites in the salivary glands of *An. donaldi* and *An. letifer* in an oil palm plantation in Miri in 1997. 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, intravenous inoculation of sporozoites isolated from the simiophilic *An. hackeri* in 1961 into an uninfected rhesus monkey demonstrated that it was the natural vector of *P. knowlesi* in Peninsular Malaysia [28]. The parasite was morphologically identified from the blood of the rhesus monkey inoculated with the sporozoites. 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* [29].

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 [30]. Subsequently, similar studies utilising molecular tools have incriminated *An. cracens* as the vector of *P. knowlesi* in Kuala Lipis, Peninsular Malaysia [31] 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 [32]. *Anopheles 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* [33]. 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].

Molecular studies of malaria vectors have indicated that morphological keys cannot differentiate sibling species within a species complex and have revealed extensive cryptic speciation, with most nominal species in Southeast Asia being found to comprise species complexes [34–36]. For example, within the Dirus Complex, at least eight species have been described [37,38]. For the purpose of accurate identification, DNA barcodes such as the second internal transcribed spacer (ITSII) within the ribosomal DNA, mitochondrial cytochrome c oxidase sub-unit 1 and 2 (COI and COII), and NADH dehydrogenase sub-unit six (ND6) were previously used to study the phylogeny of closely-related mosquito species [35,39–45]. Accurate identification of vectors is especially important to inform malaria vector control programmes in Southeast Asia, where most *Anopheles* malaria vectors are comprised of species complexes [23,36], and where malaria elimination is on the agenda [46]. It is therefore critical to utilise

available molecular methods to precisely identify *Anopheles* mosquitoes found to be vectors for zoonotic malaria parasites.

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 in 2014 (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 prior to mosquito collection in September 2014. 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 were admitted to Lawas Hospital with knowlesi malaria within 3 months prior to mosquito collection in May 2015 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. *Anopheles* mosquitoes were identified for their species/group using taxonomic keys while non-anophelines were identified for their genera [47–49]. Salivary glands of individual *Anopheles* mosquitoes were then

carefully dissected and preserved in 1.5-ml microcentrifuge tubes (1 specimen/tube) containing 0.5 ml absolute alcohol. The dissection pins were wiped with dispensable 70% alcohol swabs after every dissection to prevent cross-contamination. Preserved salivary glands were transported to the Malaria Research Centre, Universiti Malaysia Sarawak, for further molecular analysis.

DNA extraction and detection of *Plasmodium* species

Absolute alcohol preserving the salivary glands was first dried prior to DNA extraction. 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 to nested PCR assays for the detection of *Plasmodium* DNA based on the SSU rRNA genes using the *Plamosium*-specific primers rPLU3 and rPLU4 [50]. Subsequently, *Plasmodium*-positive samples were tested using a species-specific PCR assays to identify *P. coatneyi, P. cynomolgi,* and *P. knowlesi* [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. The new primers were found to have higher specificity (data not shown) compared to the *P. fieldi*- and *P. inui*-specific primers developed previously [1]. The processes for genomic DNA extraction, PCR mastermix preparation, pipetting of template for primary PCR, and pipetting of template for nested PCR were each conducted in a different room, using filtered pipette tips and micropipettes dedicated to each room to prevent cross contamination. These PCR assays were repeated for samples that were positive for the initial nested PCR assays and only samples which were consistently positive on both occasions were subjected to sequencing of *Plasmodium* SSUrRNA genes.

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 [50]. 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 [50] were used with an annealing temperature of 68 .

Generating *Anopheles* COI and ITSII region amplicons

Both the COI and ITSII 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 COI region, PCR primers AnCOX1F (5'-GGAATGGATGTWGATACWCGAGC-3') and AnCOX1R (5'-CCTAAATTTGCTCATGTTGCC-3') were designed with the annealing temperature of 65. For the ITSII region, DNA amplification was performed using PCR primers 5.8SF and 28SR as previously described by Paredes Esquivel et al. [35].

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 with PCR using M13 primers, 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 ITSII inserts.

Phylogenetic analysis

For the ITSII region, the central repeat region contained within it was identified using the Tandem Repeats Finder software (https://tandem.bu.edu/trf/trf.html) as described previously and trimmed [35,51]. The trimmed sequences were then aligned for phylogenetic analyses. No trimming was done on the SSUrDNA and COI sequences prior to alignment. Reference sequences obtained from GenBank [35,40,52–54] 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 [55,56]. Subsequently, phylogenetic trees were constructed by the Maximum Likelihood (ML) method using MEGA 7.0.21 with bootstrap values calculated from 1000 replicates [57].

Analysis of primer binding sites in the ITSII sequence of *An. barbirostris* s. l.

A total of 18 full length ITSII sequences from various *An. barbirostris* s. l., including those generated in this study, were also aligned without trimming using the parameters stated above. Primer binding sites for the multiplex PCR assay developed by Brosseau et al. [58] to identify the members of the Barbirostris Complex were then identified from the alignment. Amplicon size(s) resulting from the multiplex PCR assay was also determined for the ITSII sequences generated from this study.

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; 34%), 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 *Anopheles* mosquitoes predominated in Long Luping (n=51, 48%). Most *An. balabacensis* (n=31) and *An. barbirostris* s. l. (n=18) mosquitoes were caught at Long Luping while all specimens of the Umbrosus Group (*An. letifer*, *An. roperi*, and *An. umbrosus*) were collected in Long Tengoa, and these were not collected in Long Luping (Table 1).

Plasmodium species in *Anopheles* mosquitoes

DNA extracted from the salivary glands of 65 *Anopheles* mosquitoes were subjected to nested PCR assays and *Plasmodium* DNA was only detected in samples derived from 10 *An. balabacensis* and 6 *An. barbirostris* s. l. 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* s. l. 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*.

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* s. l.) of the samples infected with only *P. knowlesi.* For the 9 samples where amplicons were successfully obtained, cloned and transformed into chemically competent *E. coli*, colony PCR was conducted on at least 10 colonies. Colonies originating from the same transformation which produced amplicons of slightly different sizes (1,500 bp – 2,000 bp) were subjected to sequencing. As a result, a total of 38 SSUrRNA fragments were cloned and sequenced from these *Anopheles* mosquitoes (6 *An. balabacensis* and 3 *An. barbirostris* s. l.) 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* s. l. 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 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 probably sporozoites of unknown species of *Plasmodium*.

Despite detecting *P. cynomolgi* and *P. fieldi* by PCR in LW45 and LW67, respectively, we were unable to obtain SSUrRNA sequences of these two species of *Plasmodium* from the clones that were sequenced (Figure 2). Binding sites for *P. cynomolgi*- and *P. fieldi*-specific primers were subsequently searched for in sequences isolated from LW45 and LW67 to confirm their specificities in detecting the species they were developed to identify. Clones LW45C10 and LW45C13 were found to have fully complementary binding sites for the *P. cynomolgi*-specific primers (CY2F + CY4R) although they did not form a clade with *P. cynomolgi* in the phylogenetic tree (Figure 2). CY2F binding site was also discovered to be fully conserved in several other clones isolated from LW67 (C3, C10, C14, C20, and C30) while one and/or two SNPs were present for primer CY4R (Figure 3). As the SNPs were not near to the 3' end of the primer binding site, CY2F + CY4R could potentially amplify a 137-bp fragment from these aligned clones in a PCR. On the other hand, *P. fieldi*-specific primer sequences were not conserved in any of the clones isolated from LW67. This suggests that the *P. fieldi* detected by PCR was not recovered among the 23 clones sequenced.

Molecular characterisation of *Anopheles* mosquitoes

The ITSII region and COI gene [see Additional Files 3 & 4 for multiple sequence alignment of COI] of *Anopheles* mosquitoes carrying simian malaria parasites were further sequenced to confirm their identities. Phylogenetic analyses of the ITSII region and COI 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 4 & 5). The phylogenetic tree constructed using the ML method with the ITSII 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). The ITSII sequences from Selangor are also the only ones with identical length to the ITSII of *An. barbirostris* s. l. characterised in the current study (Table 3). In addition, it is found that only primers fBDSVW and rBAR&Van in the multiplex PCR assay for *An. barbirostris* s.l. [59] could bind to the ITSII sequences generated in our study, and they amplified a 404-bp fragment (Additional File 5). When the PCR assay was used to identify the other five members of the Barbirostris Complex (*An. barbirostris, An. dissidens, An. saeungae, An. wejchoochotei,* and *An. campestris*), they either produced multiple amplicons or amplicons of different sizes to 404 bp. The *An. barbirostris* s. l. that were collected and sequenced in our study will henceforth be referred to as *An. barbirostris* Clade VI. On the other hand, the phylogenetic tree by the Maximum Likelihood method of the COI gene, indicated that the *An. barbirostris* Clade VI collected in Lawas, Malaysian Borneo appeared to be distinct from the other *An. barbirostris* s. l. (Figure 5).

The COI fragment of *An. balabacensis* obtained in this study showed close phylogenetic relationship (Figure 6) 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

Mosquito composition in 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 [26,29]. Peak biting times of identical mosquito species were also reported to vary between entomological survey sites [32,60]. The fact that both vectors in Long Luping (*An. balabacensis* and *An. barbirostris* Clade VI) were not found in Long Tengoa implies that a different species of *Anopheles* mosquito 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. Similarly, only one *An. latens* was collected at each of the two sites in Lawas and it is possible that if more *An. latens* had been collected and analysed, one or more may have tested *Plasmodium*-positive*. Anopheles latens* would then have been incriminated as a vector for *P. knowlesi* in Lawas, just like it has been for the Kapit District in Central Sarawak[30]. Nevertheless, the detection of simian malaria parasites in the salivary glands of wild-caught *An. balabacensis* and *An. barbirostris* Clade VI 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* Clade VI by nested PCR assays. The *An. balabacensis* and *An. barbirostris* Clade VI 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 [32]. Infection rates of <2.00 % were reported among vectors in central Sarawak and among *An. cracens* in Peninsular Malaysia [29,31,60]. 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 sprorozoites [61]. 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 [60] 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 way sampling was conducted compared to other reported studies [4,30–32,62,63]. Firstly, the current method recovers both sides of the salivary glands without rupturing any to check for sporozoites, increasing the yield of extracted *Plasmodium* DNA. Secondly, the current method does not retain the head of the specimens which might contain inhibitors which would disrupt PCR assays. PCR inhibitors were previously found to be present in the heads of *Culex pipiens* and *An. punctipennis* which caused false negative results in detection of *Wolbachia pipientis* in samples tested [64]. The efficiency of the PCR assay was later restored when specimens were decapitated prior to DNA extraction. Although we had high sporozoite infection rates by nested PCR assays, we failed to generate the longer (1,500 bp – 2,000 bp) *Plasmodium* SSUrDNA amplicons from 5 samples that were malaria-positive by nested PCR assays. This could be caused by the very low number of sporozoites present in the salivary glands of the mosquitoes and the low sensitivity of the PCR assay to amplify long fragments from a sample with low template concentration.

Diversity and density of *Plasmodium* infection in vectors

In line with the discovery of at least 7 *Plasmodium* species infecting long-tailed macaques in Sarawak [65], it is unsurprising that multiple unidentified *Plasmodium* species were recovered from *An. balabacensis* in this study (Figure 2). However, the low quantity of DNA extracted from the salivary glands prevented the sequencing of another gene, such as the mitochondrial genome, 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 are known to host a diverse range of *Plasmodium* [1,4,65,66].

Accurate identification of *Plasmodium* by PCR might also be impeded when uncharacterised *Plasmodium* such as those found in sample LW45 (C10 and C13) could be detected by *P. cynomolgi*-specific primers (Figure 3). This demonstrates the need for the sequencing of a considerable genomic locus length for proper identification of the species of *Plasmodium*. On the other hand, the unsuccessful attempts to sequence *P. fieldi* from sample LW67, and *P. cynomolgi* from LW45, could be due to the low density of *P. fieldi* and *P. cynomolgi* respectively among the other *Plasmodium* co-infecting each of these mosquitoes. As the PCR amplification prior to cloning amplifies the SSUrRNA genes of all *Plasmodium* species indiscriminately, the scarcity of any species of *Plasmodium* DNA in the sample reduces the chance of its amplicon being produced during PCR amplification. The difficulty in obtaining sequencing data of genes of *Plasmodium* derived from vectors is probably the main reason why previous studies on

vectors of knowlesi malaria have only used nested PCR assays [15,30,32,33,63] and for the lack of studies describing the diversity and density of *Plasmodium* infection in vectors. With an increasing number of zoonotic malaria infections in the world [67–69], epidemiological studies of these inadequately studied species within human populations that come into close contact with macaques during activities in the forest and forest-fringe will also 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* Clade VI 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 ITSII region has further supported this hypothesis (Table 3). To the best of our knowledge, members of the Barbirostris Complex are the only *Anopheles* species with an ITSII sequence length of more than 1.5 kb, mainly due to its multiple internal repeats [35]. Despite the roles of both *An. barbirostris* s. l. and *An. balabacensis* in malaria transmission [70–74], the taxonomy of the latter species and its species group was studied more extensively than the former [35,38,40,75–77]. It is therefore likely that more cryptic species will be discovered in the Barbirostris Group than within the Leucosphyrus Group.

The Leucosphyrus Group has been long thought to be the only species group capable of transmitting *P. knowlesi*. *Anopheles kochi* from the Kochi Group was suspected as a vector due to its high susceptibility to *P. knowlesi* infection under experimental conditions and its simiophilic biting behaviour but the parasite was never recovered from any *An. kochi* collected in the natural environment [4,78,79]. DNA of *P. knowlesi* was recently detected from the carcasses of *An. donaldi* and *An. sundaicus* collected from Sabah (Malaysia) and the Nicobar and Andaman Islands (India), respectively [63,80]. Apart from these two, our study now reports *An. barbirostris* Clade VI, a species from an entirely different subgenus than the Leucosphyrus Group, to be a vector for *P. knowlesi* in a natural setting. This suggests that there are possibly more *Anopheles* species that could transmit the pathogen 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* Clade VI 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. As the main vector control method currently adopted by the Sarawak State Health Department is the regular spraying of residual insecticide on houses in malarious area, spraying 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) [81]. 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 [82]. Southeast Asia however, might not be ready for this technology as primary vectors of *P. knowlesi* in many parts of Southeast Asia have yet to be determined.

Conclusion

Anopheles 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. dissidens*, *An. saeungae*, and *An. wejchoochotei*) 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.

References

1. Lee KS, Divis PCS, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: Reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog. 2011;7.
2. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SSG, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:1017–24.

3. Cox-singh J, Davis TME, Lee K, Shamsul S, Matusop A, Ratnam S, et al. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life-threatening. 2008;46:165–71.
4. Vythilingam I, Noorazian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, et al. *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. Parasites and Vectors. 2008;1:1–10.
5. Barber BE, William T, Jikal M, Jilip J, Dhararaj P, Menon J, et al. *Plasmodium knowlesi* Malaria in Children. 2011;17.
6. William T, Menon J, Rajahram G, Chan L, Ma G, Donaldson S, et al. Malaria in a Tertiary Care Hospital, Sabah, Malaysia. 2011;17.
7. Cooper DJ, Rajahram GS, William T, Jelip J, Mohammad R, Benedict J, et al. *Plasmodium knowlesi* malaria in Sabah, Malaysia, 2015-2017: ongoing increase in incidence despite near-elimination of the human-only *Plasmodium* species . Clin Infect Dis. 2019;1–7.
8. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Study T. Naturally Acquired knowlesi Malaria. 2004;10:2211–3.
9. Putaporntip C, Hongsrimuang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, et al. Differential Prevalence of *Plasmodium* Infections and Cryptic *Plasmodium knowlesi* Malaria in Humans in Thailand. 2009;199:1143–50.
10. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, et al. Human Infections with *Plasmodium* the Philippines. 2008;14:811–4.
11. Ng O, Ooi E, Lee C, Lee P, Ng L, Wong P, et al. Naturally Acquired Human *Plasmodium knowlesi* Infection, Singapore. 2008;14:814–7.
12. Jiang N, Chang Q, Sun X, Lu H, Yin J, Zhang Z, et al. Co-infections with *Plasmodium knowlesi* and other malaria parasites, Myanmar. Emerg Infect Dis. 2010;16:1476–8.
13. Lubis IN, Wijaya H, Lubis M, Lubis CP, Divis PC, Beshir KB, et al. Contribution of *Plasmodium knowlesi* to multi-species human malaria infections in North Sumatera, Indonesia. J Infect Dis. 2017;215:1148–55.
14. Figtree M, Lee R, Bain L, Kennedy T, Mackertich S, Urban M, et al. Plasmodium knowlesi in human, Indonesian Borneo. Emerg Infect Dis. 2010;16:672–4.
15. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S. Co-infections of *Plasmodium knowlesi*, *P. falciparum*, and *P. vivax* among Humans and *Anopheles dirus* Mosquitoes, Southern Vietnam. Emerg Infect Dis. 2011;17:1232–9.
16. Iwagami M, Nakatsu M, Khattignavong P, Soundala P, Lorphachan L, Keomalaphet S, et al. First case of human infection with *Plasmodium knowlesi* in Laos. PLoS Negl Trop Dis. 2018;12:e0006244.
17. Khim N, Siv S, Kim S, Mueller T, Fleischmann E, Singh B, et al. *Plasmodium knowlesi* Infection in Humans, Cambodia, 2007-2010. 2011;17:1900–3.
18. Tyagi RK, Das MK, Singh SS, Sharma YD. Discordance in drug resistance-associated mutation patterns in marker genes of *Plasmodium falciparum* and *Plasmodium knowlesi* during coinfections. J Antimicrob Chemother. 2013;68:1081–8.

19. World Malaria Report. Geneva World Heal. Organ. 2018.
20. Cox-Singh J. Zoonotic malaria: *Plasmodium knowlesi*, an emerging pathogen. Curr Opin Infect Dis. 2012;25:530–6.
21. Ahmed M, Cox-Singh J. *Plasmodium knowlesi* – an emerging pathogen. 2015;10:134–40.
22. Zaw MT, Lin Z. Human Plasmodium knowlesi infections in South-East Asian countries. J Microbiol Immunol Infect. Elsevier Taiwan LLC; 2019;52:679–84.
23. Hii J, Vythilingam I, Roca-Feltrer A. Human and Simian Malaria in the Greater Mekong Subregion and Challenges for Elimination. Towar Malar Elimin - A Leap Forw. InTech; 2018.
24. McArthur J. The importance of anopheles leucosphyrus. Trans R Soc Trop Med Hyg. 1951;44:683–94.
25. De Zulueta J, Lachance F. A malaria-control experiment in the interior of Borneo. Bull World Health Organ. 1956;15:673–93.
26. Chang MS, Doraisingam P, Hardin S, Nagum N. Malaria and filariasis transmission in a village/forest setting in Baram District, Sarawak, Malaysia. J Trop Med Hyg. 1995;98:192–8.
27. Chang MS, Hii J, Buttner P, Mansoor F. Changes in abundance and behaviour of vector mosquitoes induced by land use during the development of an oil palm plantation in Sarawak. Trans R Soc Trop Med Hyg. 1997;91:382–6.
28. Wharton RH, Eyles DE. *Anopheles hackeri*, a vector of *Plasmodium knowlesi* in Malaya. Science (80-). 1961;134:279–80.
29. Chang MS, Matusop A, Sen FK. Differences in *Anopheles* composition and malaria transmission in the village settlements and cultivated farming zone in Sarawak, Malaysia. Southeast Asian J Trop Med Public Health. 1999;30:454–9.
30. Vythilingam I, Tan CH, Asmad M, Chan ST, Lee KS, Singh B. Natural transmission of *Plasmodium knowlesi* to humans by *Anopheles latens* in Sarawak, Malaysia. Trans R Soc Trop Med Hyg. 2006;100:1087–8.
31. Jiram AI, Vythilingam I, NoorAzian YM, Yusof YM, Azahari AH, Fong M-Y. Entomologic investigation of *Plasmodium knowles*i vectors in Kuala Lipis, Pahang, Malaysia. Malar J. 2012;11:213.
32. Wong ML, Chua TH, Leong CS, Khaw LT, Fornace K, Wan-Sulaiman WY, et al. Seasonal and Spatial Dynamics of the Primary Vector of *Plasmodium knowlesi* within a Major Transmission Focus in Sabah, Malaysia. PLoS Negl Trop Dis. 2015;9:1–15.
33. Vythilingam I, Lim Y AI, Venugopalan B, Ngui R, Leong CS, Wong ML, et al. Plasmodium knowlesi malaria an emerging public health problem in Hulu Selangor, Selangor, Malaysia (2009-2013): epidemiologic and entomologic analysis. Parasit Vectors. 2014;7:436.
34. Sungvornyothin S, Garros C, Chareonviriyaphap T, Manguin S. How reliable is the humeral pale spot for identification of cryptic species of the Minimus Complex? J Am Mosq Control Assoc. 2006;22:185–91.

35. Paredes-Esquivel C, Donnelly MJ, Harbach RE, Townson H. A molecular phylogeny of mosquitoes in the Anopheles barbirostris Subgroup reveals cryptic species: Implications for identification of disease vectors. Mol Phylogenet Evol. Elsevier Inc.; 2009;50:141–51.
36. Manguin S, Garros C, Dusfour I, Harbach RE, Coosemans M. Bionomics, taxonomy, and distribution of the major malaria vector taxa of *Anopheles* subgenus Cellia in Southeast Asia: An updated review. Infect Genet Evol. 2008;8:489–503.
37. Takano KT, Nguyen NTH, Nguyen BTH, Sunahara T, Yasunami M, Nguyen MD, et al. Partial mitochondrial DNA sequences suggest the existence of a cryptic species within the Leucosphyrus group of the genus *Anopheles* (Diptera: Culicidae), forest malaria vectors, in northern Vietnam. Parasites and Vectors. 2010;3.
38. Sallum MAM, Peyton EL, Harrison BA, Wilkerson RC. Revision of the Leucosphyrus group of *Anopheles* (Cellia). Rev Bras Entomol. 2005;49:1–152.
39. Walton C, Handley JM, Kuvangkadilok C, Collins FH, Harbach RE, Baimai V, et al. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. Med Vet Entomol. 1999;13:24–32.
40. Sallum MAM, Foster PG, Li C, Sithiprasasna R, Wilkerson RC. Phylogeny of the Leucosphyrus Group of Anopheles (Cellia) (Diptera: Culcidae) based on mitochondrial gene sequences. Ann Entomol Soc Am. 2007;100:27–35.
41. Phuc HK, Ball AJ, Son L, Hanh N V., Tu ND, Lien NG, et al. Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. Med Vet Entomol. 2003;17:423–8.
42. Surendran SN, Sarma DK, Jude PJ, Kemppainen P, Kanthakumaran N, Gajapathy K, et al. Molecular characterization and identification of members of the *Anopheles subpictus* complex in Sri Lanka. Malar J. Malaria Journal; 2013;12:1.
43. Goswami G, Singh OP, Nanda N, Raghavendra K, Gakhar SK, Subbarao SK. Identification of all members of the Anopheles culicifacies complex using allele-specific polymerase chain reaction assays. Am J Trop Med Hyg. 2006;75:454–60.
44. Dusfour I, Blondeau J, Harbach RE, Vythilingham I, Baimai V, Trung HD, et al. Polymerase Chain Reaction Identification of Three Members of the *Anopheles sundaicus* (Diptera: Culicidae) Complex, Malaria Vectors in Southeast Asia. J Med Entomol. 2007;44:723–31.
45. Ma Y, Li S, Xu J. Molecular identification and phylogeny of the Maculatus group of *Anopheles* mosquitoes (Diptera: Culicidae) based on nuclear and mitochondrial DNA sequences. Acta Trop. 2006;99:272–80.
46. World Health Organization. WHO Malaria Terminology. World Heal Organ. 2018;31.
47. Rattanarithikul R, Harrison BA, Harbach RE, Panthusiri P, Coleman RE. Illustrated Keys to the Mosquitoes of Thailand V. Genera Ortho. Southeast Asian J Trop Med Public Health. 2006;37:9–36.
48. Reid JA. Anophelines mosquitoes of Malaya and Borneo. 1968.

49. Rattanarithikul R, Harrison BA, Panthusiri P, Coleman RE. Illustrated Keys to the Mosquitoes of Thailand I. Background; Geographic Distribution; Lists of Genera, Subgenera, and Species; and a Key to the Genera. Southeast Asian J Trop Med Public Health. 2005;36:59–69.
50. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus-and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687.
51. Benson G. Tandem repeats finder: A program to analyze DNA sequences. Nucleic Acids Res. 1999;27:573–80.
52. Sallum MAM, Schultz TR, Foster PG, Aronstein K, Wirtz RA, Wilkerson RC. Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. Syst Entomol. 2002;27:361–82.
53. Beard CB, Hamm DM, Collins FH. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Mol Biol. 1993;2:103–24.
54. Sum J-S, Lee W-C, Amir A, Braima K a, Jeffery J, Abdul-Aziz NM, et al. Phylogenetic study of six species of *Anopheles* mosquitoes in Peninsular Malaysia based on inter-transcribed spacer region 2 (ITS2) of ribosomal DNA. Parasit Vectors. 2014;7:309.
55. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772.
56. Guindon S, Gascuel O. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst Biol. 2003;52:696–704.
57. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. Molecular Biology and Evolution. 2015;7.
58. Brosseau L, Udom C, Sukkanon C, Chareonviriyaphap T, Bangs MJ, Saeung A, et al. A multiplex PCR assay for the identification of five species of the *Anopheles barbirostris* complex in Thailand. Parasites and Vectors. BioMed Central; 2019;12:1–10.
59. Brosseau L, Udom C, Sukkanon C, Chareonviriyaphap T, Bangs MJ, Saeung A, et al. A multiplex PCR assay for the identification of five species of the *Anopheles barbirostris* complex in Thailand. Parasites and Vectors. BioMed Central; 2019;12:1–10.
60. Tan CH, Vythilingam I, Matusop A, Chan ST, Singh B. Bionomics of *Anopheles latens* in Kapit, Sarawak, Malaysian Borneo in relation to the transmission of zoonotic simian malaria parasite *Plasmodium knowlesi*. Malar J. 2008;7:52.
61. Schultz GW. Biting activity of mosquitos (Diptera: Culicidae) at a malarious site in Palawan, Republic of The Philippines. Southeast Asian J Trop Med Public Health. 1992;23:464–9.
62. Chua TH, Manin BO, Daim S, Vythilingam I, Drakeley C. Phylogenetic analysis of simian *Plasmodium* spp. infecting Anopheles balabacensis Baisas in Sabah, Malaysia. PLoS Negl Trop Dis. 2017;11:1–13.

63. Hawkes FM, Manin BO, Cooper A, Daim S, Homathevi R, Jelip J, et al. Vector compositions change across forested to deforested ecotones in emerging areas of zoonotic malaria transmission in Malaysia. Sci Rep. 2019;9:1–12.
64. Beckmann JF, Fallon AM. Decapitation improves detection of *Wolbachia pipientis* (Rickettsiales: Anaplasmataceae) in *Culex pipiens* (Diptera: Culicidae) mosquitoes by the polymerase chain reaction. J Med Entomol. 2012;49:1103–8.
65. Nada Raja T, Hu TH, Zainudin R, Lee KS, Perkins SL, Singh B. Malaria parasites of long-tailed macaques in Sarawak, Malaysian Borneo: A novel species and demographic and evolutionary histories. BMC Evol Biol. BMC Evolutionary Biology; 2018;18:1–12.
66. Gamalo LE, Dimalibot J, Kadir KA, Singh B, Paller VG. *Plasmodium knowlesi* and other malaria parasites in long-tailed macaques from the Philippines. Malar J. BioMed Central; 2019;18:1–7.
67. Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. Malar J. 2014;13:68.
68. Lalremruata A, Magris M, Vivas-Martinez S, Koehler M, Esen M, Kempaiah P, et al. Natural infection of *Plasmodium brasilianum* in humans: Man and monkey share quartan malaria parasites in the Venezuelan Amazon. EBioMedicine. Elsevier B.V.; 2015;2:1186–92.
69. Imwong M, Madmanee W, Suwannasin K, Kunasol C, Peto TJ, Tripura R, et al. Asymptomatic natural human infections with the simian malaria parasites *Plasmodium cynomolgi* and *Plasmodium knowlesi*. J Infect Dis. 2019;219:695–702.
70. Hii J, Kan S, Pereira M, Parmar SS, Campos RL, Chan MKC. Bancroftian filariasis and malaria in island and hinterland populations in Sabah, Malaysia. Trop Geogr Med. 1985;37:93–101.
71. Hii JLK, S VY. A study of dispersal, survival and adult population estimates of the malaria vector, *Anopheles balabacensis* Baisas (Diptera: Culicidae) in Sabah, Malaysia Jeffrey. Trop Biomed. 1985;2:121–31.
72. Cooper RD, Edstein MD, Frances SP, Beebe NW. Malaria vectors of Timor-Leste. Malar J. 2010;9:40.
73. Alam MS, Khan MGM, Chaudhury N, Deloer S, Nazib F, Bangali AM, et al. Prevalence of anopheline species and their *Plasmodium* infection status in epidemic-prone border areas of Bangladesh. Malar J. 2010;9:1–8.
74. Amerasinghe PH, Amerasinghe FP, Konradsen F, Fonseka KT, Wirtz RA. Malaria vectors in a traditional dry zone village in Sri Lanka. Am J Trop Med Hyg. 1999;60:421–9.
75. Sallum MAM, Peyton EL, Wilkerson RC. Six new species of the *Anopheles leucosphyrus* group, reinterpretation of *An . elegans* and vector implications. 2005;19:158–99.
76. Taai K, Harbach RE. Systematics of the *Anopheles barbirostris* species complex (Diptera: Culicidae: Anophelinae) in Thailand. Zool J Linn Soc. 2015;174:244–64.
77. Townson H, Dyer N, Mcalister E, Satoto TBT, Bangs MJ, Harbach RE. Systematics of *Anopheles barbirostris* van der Wulp and a sibling species of the Barbirostris Complex (Diptera: Culicidae) in eastern Java, Indonesia. Syst Entomol. 2013;38:180–91.

78. Jeslyn WPS, Huat TC, Vernon L, Irene LMZ, Sung LK, Jarrod LP, et al. Molecular Epidemiological Investigation of *Plasmodium knowlesi* in Humans and Macaques in Singapore. Vector-Borne Zoonotic Dis. 2011;11:131–5.
79. Coatney GR, Collins WE, Warren M, Contacos PG. The Primate Malarias. Natl. Inst. Allergy Infect. Dis. Bethesda. 1971.
80. Vidhya PT, Pulikkottil I, Maile A, Zahid AK. *Anopheles sundaicus* mosquitoes as vector for *Plasmodium knowlesi*, Andaman and Nicobar Islands, India. Emerg Infect Dis. 2019;25:817–20.
81. Feachem RGA, Chen I, Akbari O, Bertozzi-Villa A, Bhatt S, Binka F, et al. Malaria eradication within a generation: ambitious, achievable, and necessary. Lancet. 2019;394:1056–112.
82. Esvelt KM, Smidler AL, Catteruccia F, Church GM. Concerning RNA-guided gene drives for the alteration of wild populations. Elife. 2014;3:1–21.
83. Google Earth. Map of Lawas, Sarawak. 2018.

Tables

Table 1. Genera/species of mosquitoes collected and identified morphologically in Lawas.

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

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*	
An. balabacensis	30	10	1: No species identified 6: *Pk* 1: *Pcy* + *Pk* 1: *Pcy* + *Pfld* + *Pin* 1: *Pin*	101 31, 32, 49, 50, 51, 59 45 67 74
An. barbirostris s. l.	18	6	1: No species identified 5: *Pk*	38 44, 47, 48, 57, 58

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

Table 3. Lengths of 5.8S-ITSII-28S region of members of the Barbirostris Subgroup amplified by primers 5.8SF and 28SR.

Species	Reference	Locality	Amplicon size (bp)
An. barbirostris s.s	[35]	South Kalimantan, Thailand	1674-1677
An. vanderwulpi		Sumatra	1858
An. dissidens		Thailand	1860-1862
An. saeungae		Sumatra, Thailand	1713-1718
An. wejchoochotei		Thailand	1652
An. barbirostris s. l.	[54]	Selangor, Peninsular Malaysia	1576
An. barbirostris VI	Current study	Sarawak, Malaysian Borneo	

Figures

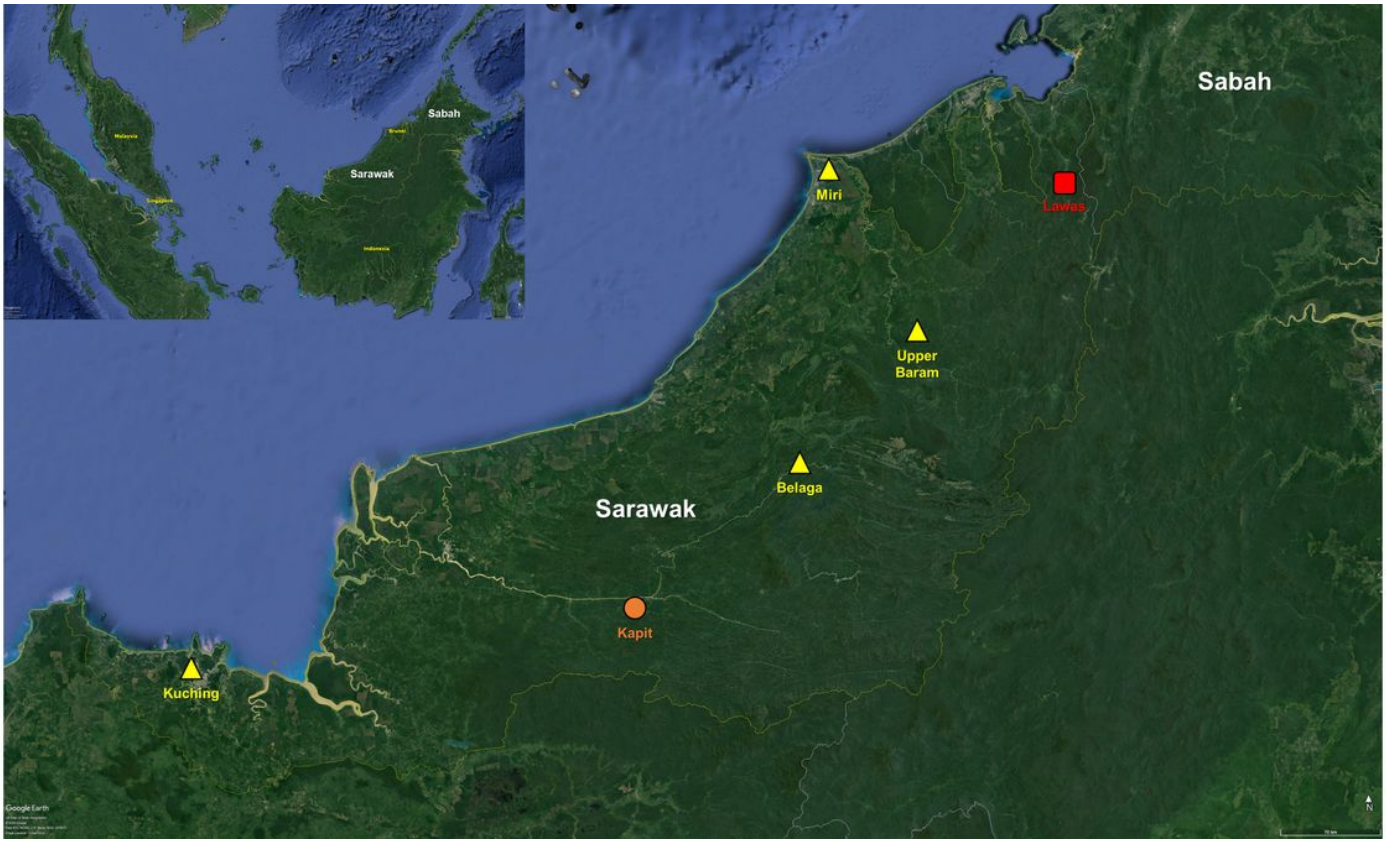

Figure 1

Locations of entomological surveys conducted previously and for the current study [83]. Human malaria sporozoites were discovered from dissected mosquitoes from these sites between 1951-1999 [24–27,29] P. knowlesi and other simian malaria sporozoites were discovered from dissected mosquitoes and by nested PCR assays from this site between 2005-2006 [30] Study site for the current study

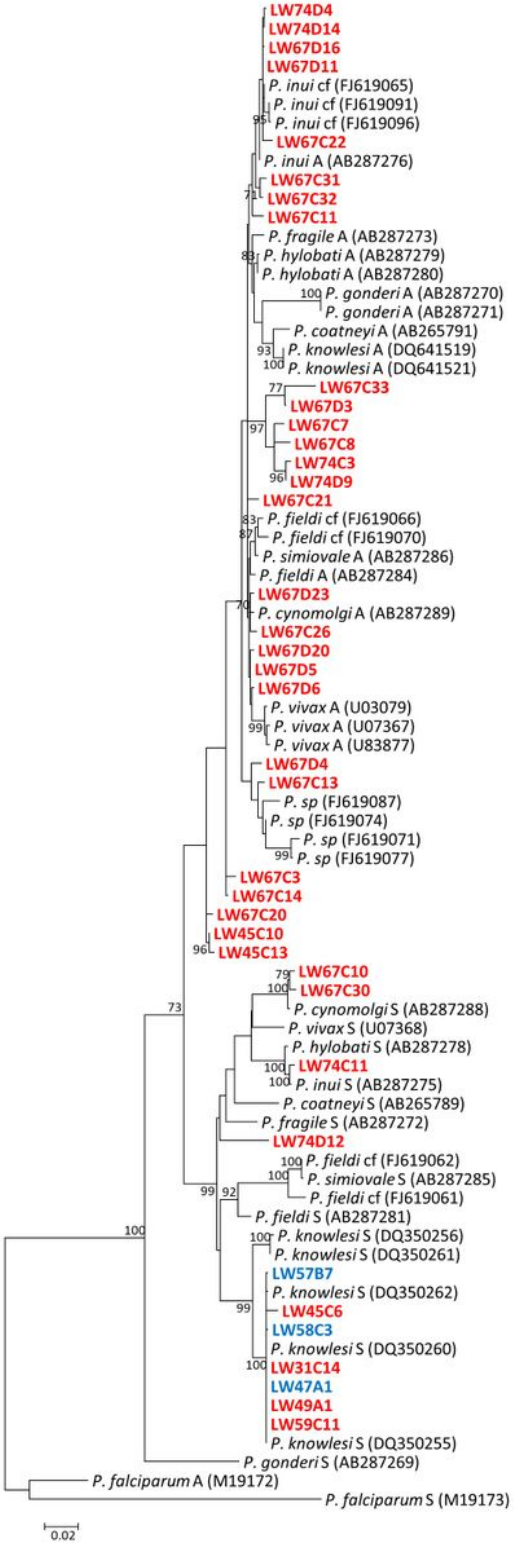

Figure 2

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.

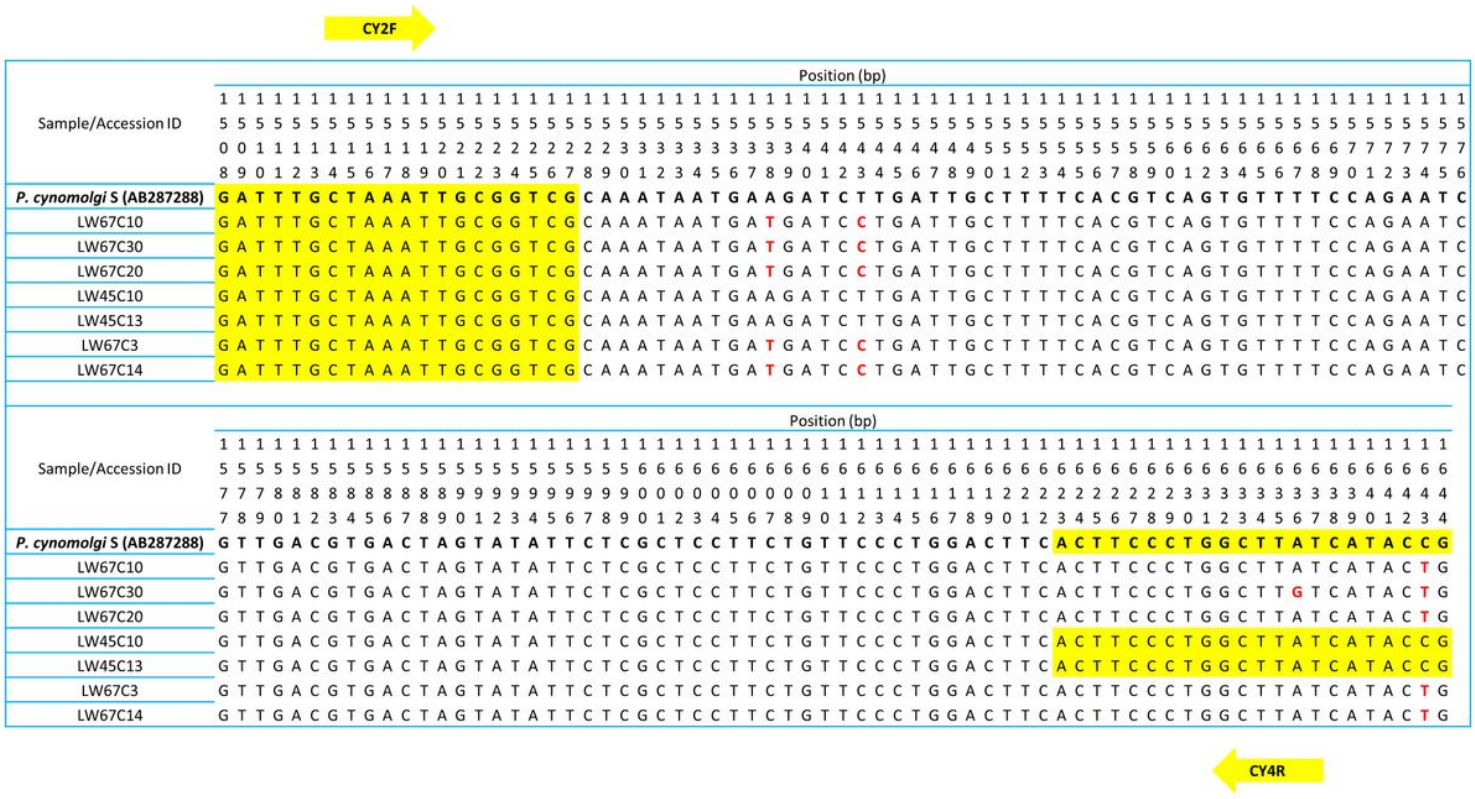

Figure 3

Alignment of clones which could potentially be detected by PCR with primers CY2F and CY4R. Primer binding sites are highlighted in yellow and SNPs are represented in red.

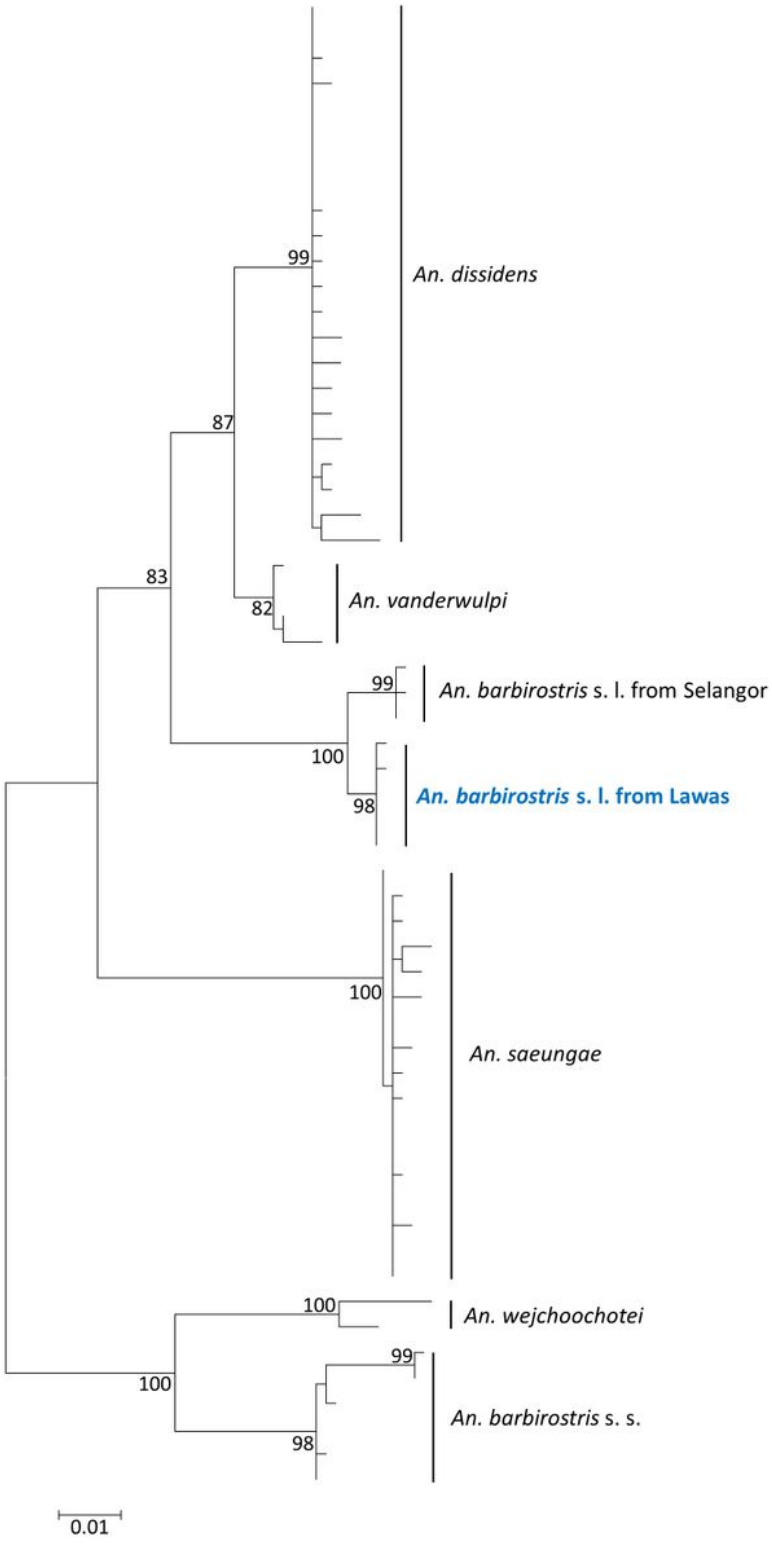

Figure 4

Phylogenetic tree based on the Anopheles ITSII 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.

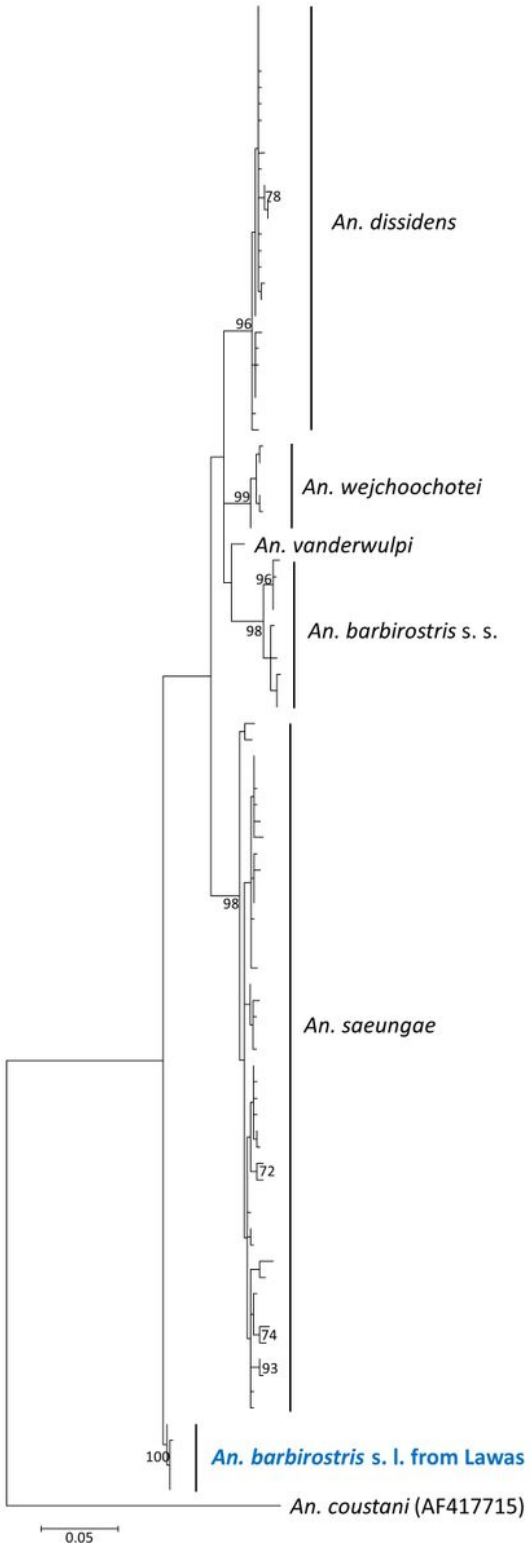

Figure 5

Phylogenetic tree based on the Anopheles COI 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.

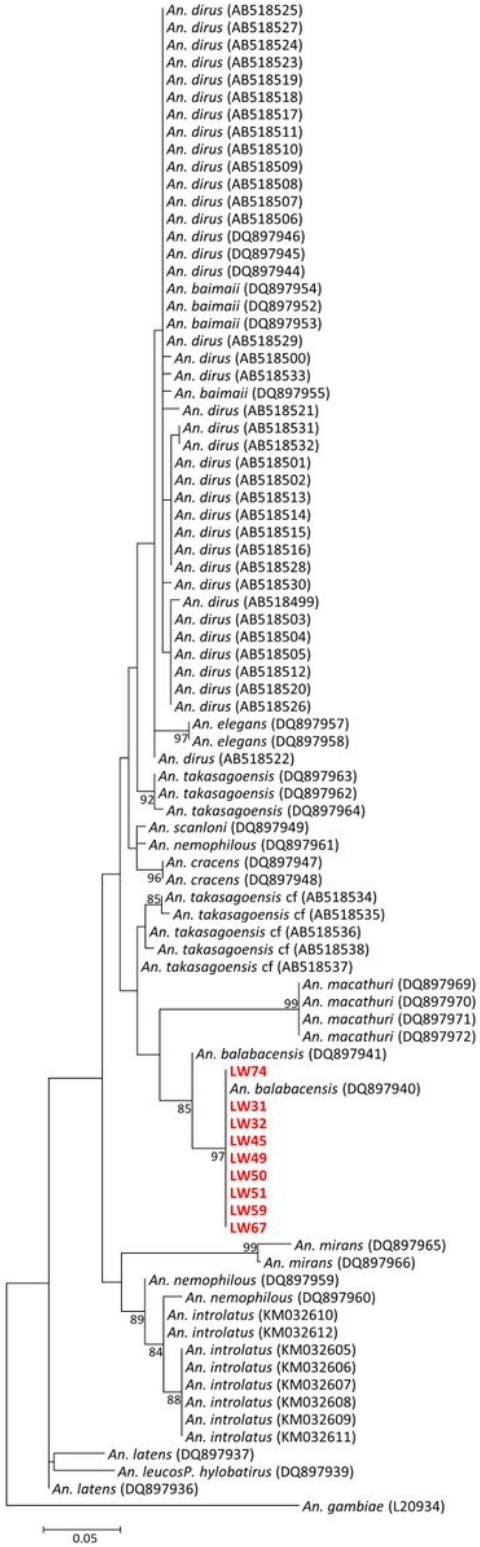

Figure 6

Phylogenetic tree based on the Anopheles COI gene using the ML 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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.docx
- AdditionalFile5.docx
- AdditionalFile3.docx
- Graphicalabstract.jpg
- AdditionalFile4.docx
- AdditionalFile2.docx