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Myzorhynchus series of *Anopheles* mosquitoes as potential vectors of *Plasmodium bubalis* in Thailand

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Ungulate malaria parasites and their vectors are among the least studied when compared to other medically important species. As a result, a thorough understanding of ungulate malaria parasites, hosts, and mosquito vectors has been lacking, necessitating additional research efforts. This study aimed to identify the vector(s) of *Plasmodium bubalis*. A total of 187 female mosquitoes (133 *Anopheles* spp., 24 *Culex* spp., 24 *Aedes* spp., and 6 *Mansonia* spp. collected from a buffalo farm in Thailand where concurrently collected water buffalo samples were examined and we found only *Anopheles* spp. samples were *P. bubalis* positive. Molecular identification of anopheline mosquito species was conducted by sequencing of the PCR products targeting *cytochrome c oxidase subunit 1* (*cox1*), *cytochrome c oxidase subunit 2* (*cox2*), and internal transcribed spacer 2 (*ITS2*) markers. We observed 5 distinct groups of anopheline mosquitoes: Barbirostris, Hyrcanus, Ludlowae, Funestus, and Jamesii groups. The Barbirostris group (*Anopheles wejchoochotei* or *Anopheles campestris*) and the Hyrcanus group (*Anopheles peditaeniatus*) were positive for *P. bubalis*. Thus, for the first time, our study implicated these anopheline mosquito species as probable vectors of *P. bubalis* in Thailand.

Malaria parasites of the genus *Plasmodium*, particularly in most of the medically important species, have undergone intensive studies, and they are well manageable as a result. Historically, descriptions of *Plasmodium* species infecting even-toed ungulates (order Artiodactyla), on the other hand, have appeared intermittently in literature (see review in Templeton et al.¹). Among these, *Plasmodium bubalis* was discovered in Murrah buffalo (Bovidae: *Bubalus bubalis*) in India² and was later reported in water buffaloes in several other countries (see for example Templeton et al.³; Kandel et al.⁴). *Plasmodium traguli* was found in mousedeer (Tragulidae: *Tragulus javanicus*) in Malaysia decades ago and has not appeared in literature since^{5,6}. *Plasmodium caprae* was first recorded in African goats (Bovidae: *Capra aegagrus hircus*)⁷ and more recently in several countries outside Africa, including Thailand^{3,8}. Among the various ungulate malaria parasites described thus far, at least three are endemic in Southeast Asia, suggesting the presence of mosquito vectors in this region. Most of the first discoveries of ungulate malaria occurred prior to the implementation of PCR in 1980, and thus vector identification efforts relied solely on morphological investigations. As a result, a comprehensive picture of these taxa's transmission cycle could not be drawn.

According to Rattanarithikul et al.⁹ and the Walter Reed Biosystematics Unit¹⁰, at least 464 mosquito species have been recorded in Thailand, with 83 of these belonging to the Anophelinae subfamily. It is not surprising that vector studies on malaria of medical importance have gained greater attention and achieved greater accomplishments than others^{11,13}. The majority of anopheline species in Southeast Asian countries are cryptic species complexes^{14–16}. The Barbirostris complex, for example, includes at least six species, five of which are found in Thailand^{17,18}. Misidentification is a common pitfall in vector studies, particularly when dealing with species

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complexes¹⁵. Such deceptive results could have an impact on vector control programs or mislead subsequent studies^{19–21}.

Despite several limitations and difficulties, a study of mosquitoes feeding on infected mousedeer in Malaysia resulted in the successful incrimination of *Anopheles umbrosus* and *Anopheles letifer* as probable vectors of *P. traguli*²². After decades of inactivity, sporozoites of unknown malaria parasites were isolated from the salivary glands of *Anopheles gabonensis* and *Anopheles obscurus* in Gabon²³. The cytochrome b sequences isolated from these sporozoites share the same clade with *Plasmodium* DNA detected from African ungulates. *Plasmodium* sporozoites were observed in the salivary glands of *Anopheles punctipennis* in a separate study conducted in the United States of America. Phylogenetic analysis revealed that their sequences were related to *Plasmodium* from white-tailed deer (Cervidae: *Odocoileus virginianus*)²⁴. However, little is known about the vectors of *P. bubalis* and *P. caprae*, both of which are endemic in Southeast Asia. *Anopheles minimus* has been suspected of transmitting *P. bubalis*²⁵. However, incrimination of this mosquito species remains controversial without clear evidence to support it. In Thailand and other countries, very limited research works on *P. bubalis* and its vector have been published^{3,4}. Recently, a high prevalence of *P. bubalis* infection has been reported in Thailand; however, no information on the transmission or the probable vector has been provided²⁶. We hypothesized that the mosquito vectors of *P. bubalis* are likely endemic species in the country. Therefore, we conducted this study aiming to identify the anopheline mosquito species transmitting *P. bubalis* in Thailand.

Results

***P. bubalis* detected in buffalo blood samples on a farm.** Previous investigation in Thailand revealed that 35% of buffaloes on a farm located in Chachoengsao Province were infected with *P. bubalis*²⁶. Thus, this study selected the same farm to identify the vector mosquitoes of *P. bubalis*. A total of 90 buffalo blood samples were collected in June 2020 (n = 45) and November 2021 (n = 45) from the farm. Mosquitoes were captured then underwent PCR screening for *P. bubalis* infection using primers targeting the *cytb* gene. Two buffalo blood samples (IDs THBuff20_37 and THBuff20_39) from the 2020 collection were positive (4.4%), indicating that *P. bubalis* infection occurred on this farm when mosquito samples were collected. In the 2021 collection, no blood or anopheline mosquito samples were positive.

Species composition of mosquitoes collected from buffalo farm by morphology. A total of 1,571 female mosquitoes were collected from a farm in Chachoengsao. Morphological examination indicated that anopheline mosquitoes accounted for 8.53% (n = 134), while *Culex* spp. accounted for 74.6% (n = 1172), *Aedes* spp. accounted for 13.05% (n = 205), *Mansonia* spp. accounted for 0.38% (n = 6), and unidentifiable due to body part destruction accounted for 3.44% (n = 54) (Fig. 1A). Among 134 anopheline mosquitoes, 5 different *Anopheles* groups were identified including Barbirostris, Hyrcanus, Funestus, Ludlowae, and Jamesii groups; 1 mosquito was unable to be identified in any group due to missing wings and legs (Fig. 1B).

Identification of *P. bubalis* DNA from mosquito salivary gland samples. For a total of 133 identified anopheline mosquitoes, salivary glands with the head and thorax were carefully separated from the rest of the mosquitoes' bodies, then the salivary glands and midguts were stained with 0.1% mercurochrome dye and examined under a microscope. However, no oocysts and sporozoites were found. Then, one to three samples consisting of the salivary glands, head, and thorax were combined based on the group and, finally, 51 pooled samples were prepared (Table 1). DNA was extracted from the samples and PCR screening was performed for *Plasmodium cytb*, *18S rRNA*, and *cox1* genes. The number of each pool was as follows: Barbirostris group (n = 35, 23 pools), Hyrcanus group (n = 81, 19 pools), Ludlowae group (n = 14, 7 pools), Funestus group (n = 1, 1 pool), and Jamesii group (n = 2, 1 pool). Out of 51 pools of anopheline mosquitoes, 3 pools were PCR positive for *Plasmodium*. These samples were the Barbirostris group (IDs THMosqBuff20_P6_3, THMosqBuff20_P8_2) and Hyrcanus group (ID THMosqBuff20_P20_3) (Table 1). The minimum infection rates (MIR) were 5.7% (0.015–0.186) in the Barbirostris group mosquito and 2.5% (0.004–0.128) in the Hyrcanus group mosquito (Table 2). Additionally, for those of non-anopheline mosquitoes, a total of 22 pools (*Culex* spp. n = 24, 8 pools; *Aedes* spp. n = 24, 8 pools; and *Mansonia* spp. n = 6, 6 pools) were tested. *Plasmodium bubalis* was not detected in any *Culex* spp., *Aedes* spp., or *Mansonia* spp. pools.

Analysis by the BLASTN program using *cytb* and *cox1* sequences obtained from 3 pools against non-redundant nucleotide collection revealed that they were 100% identical to *P. bubalis* type I (accession no. LC090213). Analysis by the BLASTN program using putative *P. bubalis*'s *18S rRNA* sequences did not identify any sequences in the database with 100% identity. The maximum identity was 92% with *18S rRNA* sequences of *Plasmodium falciparum* (accession no. LR131366) as well as those of other *Plasmodium* species. Because no *18S rRNA* sequences derived from any ungulate malaria parasites were available in the GenBank™ database, we used two buffalo-derived samples (IDs THBuff20_37 and THBuff20_39) for PCR-amplification with the same universal primers for *Plasmodium* *18S rRNA* and sequences were determined. Sequences derived from 3 mosquito samples showed 100% identity with the sequences from 2 buffalo samples, further supporting the presence of *P. bubalis* in the mosquitoes.

Phylogenetic analyses using the *cytb* (789 bp), *cox1* (254 bp), and *18S rRNA* (351 bp) genes revealed that *Plasmodium* sequences from this study belong to the same cluster as *P. bubalis* type I isolates previously reported from Thailand (Fig. 2, Suppl. Figure 2, Suppl. Figure 3). The current findings indicated that all *Plasmodium* sequences obtained from mosquitoes in this study were *P. bubalis* type I.

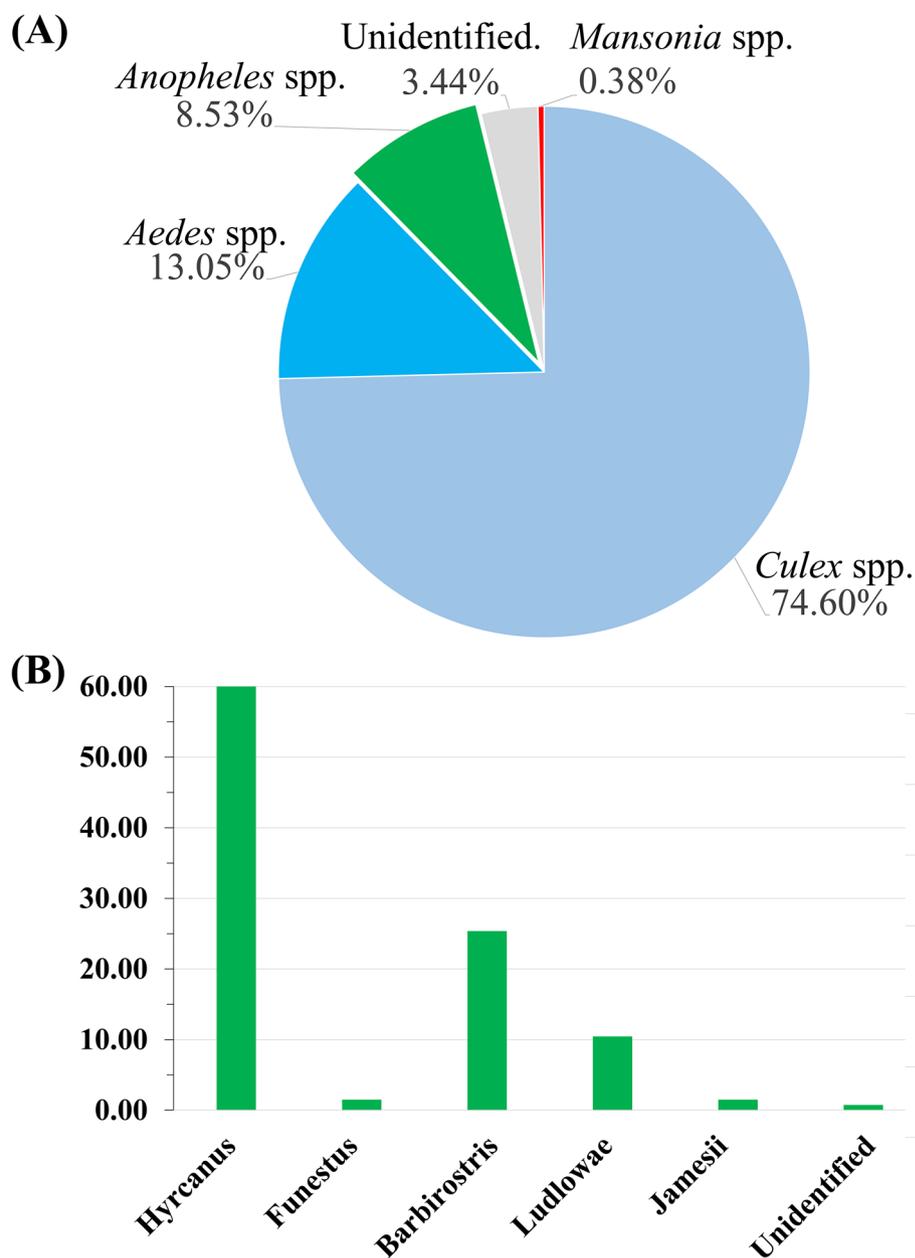


Figure 1. Chart illustrating the percentage of mosquitoes, according to morphological identification. (A) Percentages of each genus of mosquitoes collected in this study. (B) *Anopheles* mosquito groups.

Sampling sites	Group	No. collected	No. of pools	No. of positive pools			No. of pools sequenced for mosquito genes and determined species name
				<i>cytb</i>	<i>18S rRNA</i>	<i>cox1</i>	
Chachoengsao	Barbirostris	35	23	2	2	2	17 (<i>An. campestris</i> or <i>An. wejchoochotei</i>)
	Hyrcanus	81	19	1	1	1	15 (<i>An. peditaeniatus</i>), 1 (<i>An. sinensis</i>)
	Funestus	1	1	0	0	0	1 (<i>An. varuna</i>)
	Ludlowae	14	7	0	0	0	6 (<i>An. vagus</i>)
	Jamesii	2	1	0	0	0	1 (<i>An. pseudojamesi</i>)
Total		133	51	3	3	3	41

Table 1. Summary of *P. bubalis*'s PCR screening results of anopheline mosquitoes collected from the buffalo farm.

Species	Total no. mosquitoes	Pool size (range)	No. tested	No. positive pools	MIR (%) (95% CI)
<i>An. campestris</i> or <i>wejchoochotei</i>	35	1–3	35	2	5.7 (0.015–0.186)
<i>An. peditaeniatus</i>	81	1–3	52	1	2.5 (0.004–0.128)

Table 2. Minimum infection rates of *Plasmodium* in collected mosquitoes.

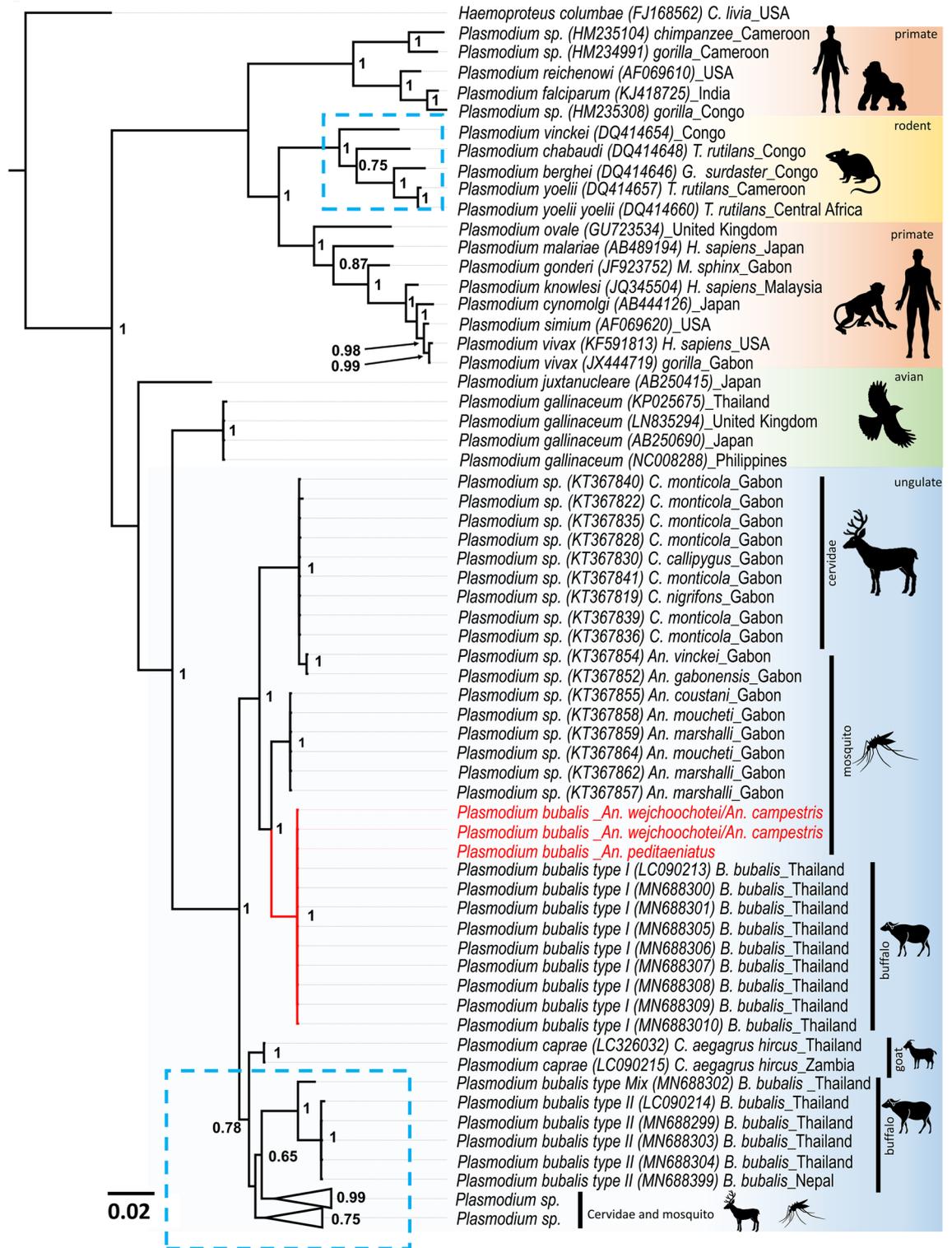


Figure 2. Phylogenetic positions of *Plasmodium* detected from *Anopheles* mosquitoes in this study. The phylogenetic tree was inferred by Bayesian inference method using partial *cytb* sequences (789 bp). *Haemoproteus columbae* was used to root all sequences. At the nodes, Bayesian posterior probabilities (PP \geq 0.65) are indicated. *Plasmodium* sequences obtained in this study are highlighted in red. The length for the substitutions/site (0.02) is indicated.

Molecular identification of anopheline mosquitoes collected from a buffalo farm. To identify the species of anopheline mosquitoes collected from buffalo farms by molecular analysis, *cox1*, *cox2*, and *ITS2* gene sequences were determined for three *Plasmodium*-positive *Anopheles* mosquito pools, as well as 15 additional *Plasmodium*-negative pools in this study. The obtained sequences were initially assessed by the BLASTN program against a non-redundant nucleotide collection for species identification. Based on the sequence of DNA barcoding region for mosquito identification, several studies have suggested an evolutionary divergence of 2–3% as a threshold for intraspecific variation^{27–29}. Thus, sequences with the highest identity (minimum $\geq 97\%$) are listed in Supplementary Table 2. BLASTN analysis of some *cox1* sequences obtained in this study was unable to reach this threshold, indicating the limitation of this approach due to the insufficient collection of mosquito sequences in the database. Nonetheless, analysis of all 3 genes of 1 Funestus group pool was matched to *An. varuna*. All 3 gene sequences of one Ludlowae group mosquito hit *An. vagus*. *An. peditaeniatus* was hit by two Hyrcanus group pools with all 3 gene sequences including a *P. bubalis* sequence-positive pool (THMosqBuff20_P20_3), and by one Hyrcanus group pool with *cox2* and *ITS2* sequences. *An. pseudojamesi* was hit by one Jamesii group pool.

The *ITS2* sequences of 9 pools of Barbirostris group mosquitoes showed 98.8–100% identity to *An. campestris* or *An. wejchoochotei*. The *Cox2* sequences of 9 pools of Barbirostris group mosquitoes showed 99–100% identity to *An. campestris*. However, there were no *An. wejchoochotei cox2* sequences available in the database, which limited the assessment of the *cox2* sequence with *An. wejchoochotei*. BLASTN search using 8 *cox1* sequences (1,416 bp) hit *An. donaldi* with $\sim 97\%$ identity and one *cox1* sequence (333 bp) showed 99.1% identity to *An. campestris*. Because all *An. wejchoochotei cox1* sequences deposited in the database are much shorter than the 8 sequences in this study, we aligned our *cox1* sequences with *An. wejchoochotei cox1* sequences (AB971335, AB971336, AB971337, AB971338, AB971339, and AB971340) from the morphologically well-described samples¹⁸ and found they were matched with $> 99\%$ identity (Supplementary Fig. 1). Because *An. wejchoochotei* sequences were reported in 2015 from morphologically defined samples¹⁸, and the identities of "*An. campestris*" from which DNA sequences were deposited to the database before this report were not clear, it was impossible to distinguish *An. campestris* and *An. wejchoochotei* molecularly at the time. Thus, we concluded that *P. bubalis*-positive anopheline mosquitoes from the Barbirostris group (THMosqBuff20_P6_3 and THMosqBuff20_P8_2) were either *An. campestris* or *An. wejchoochotei*; one from the Hyrcanus group (THMosqBuff20_P20_3) was *An. peditaeniatus*.

Discussion

The current study aimed to identify potential *P. bubalis* vectors in Thailand. *An. wejchoochotei* or *An. campestris*, *An. peditaeniatus*, *An. varuna*, *An. vagus*, and *An. pseudojamesi* were molecularly confirmed on a farm where *P. bubalis* was detected from water buffaloes, and *P. bubalis* DNA sequences were detected from *An. wejchoochotei* or *An. campestris*, and *An. peditaeniatus*. According to Rattanarithikul et al.⁹ and the Walter Reed Biosystematics Unit¹⁰, all of these anopheline mosquitoes have previously been recorded in districts throughout Thailand as well as across Southeast Asian countries. *An. wejchoochotei* is found in Thailand and Cambodia, whereas *An. peditaeniatus* can be found in Thailand, Cambodia, Indonesia, Malaysia, Myanmar, the Philippines, and Vietnam^{9,17,18,30–33}. *An. wejchoochotei* and *An. peditaeniatus* were recently found to harbor human *Plasmodium* species in Cambodia¹³.

In this study, we detected *P. bubalis*'s DNA in salivary gland samples, but oocysts and sporozoites were not observed under a microscope. This was most likely due to the low infection rate of the parasite in the water buffaloes, which resulted in a low parasite burden in the mosquitoes³. *P. traguli* oocysts and sporozoites have been discovered in *An. umbrosus* and *An. letifer* by microscopic examination in a historic mousedeer study in Malaysia²². The successful observation of *P. traguli* in mosquitoes may be due to a relatively higher infection rate in mousedeers than *P. bubalis* in water buffaloes because the *P. traguli* detection rate in the mousedeer blood samples was high ($\geq 37\%$)²².

Furthermore, nucleotide sequence analysis using Bayesian Inference (BI) confirmed that *Plasmodium* parasites isolated from *An. wejchoochotei* or *An. campestris* and *An. peditaeniatus* in this study were genetically identical and were grouped to previously described *P. bubalis* type I isolated from buffaloes²⁶, suggesting that these mosquito species were plausible vectors for *P. bubalis*.

Taai and Harbach¹⁸ described *An. wejchoochotei* for the first time, while Reid³⁴ recorded *An. campestris* in 1962. It should be noted that mosquitoes from Thailand that have since been identified as *An. wejchoochotei* were initially referred to as *An. campestris*-like by Harrison and Scanlon³⁵ due to their resemblance to *An. campestris*. Both are members of the Barbirostris complex group and cannot be distinguished solely by the morphology of the adult mosquitoes; morphological information of the larva is required. Previous research suggested that *cox1*, *cox2*, and *ITS2* are reliable genetic markers for distinguishing cryptic species within the complex group of anopheline mosquitoes^{36,37}. A recent study in Sulawesi, Indonesia, used approximately 700 bp of the *cox1* gene to distinguish members of mosquito species complexes³⁸. Furthermore, *cox1* and *ITS2* sequences have been used to identify cryptic mosquito species³⁹. Based on the *cox1* barcode region, an evolutionary divergence of 0.5% (range 0.0–3.9%) was proposed as a threshold for intraspecific variation²⁸. Consequently, we carried out an investigation into the *cox1*, *cox2*, and *ITS2* markers of anopheline mosquitoes in this study. We found that sequences from *Plasmodium*-positive mosquitoes (THMosqBuff20_P6_3 and THMosqBuff20_P8_2) showed high similarity with either *An. campestris* or *An. wejchoochotei* sequences in the GenBank™ database. The conflicting species discrimination of the previously deposited sequences between *An. campestris* and *An. wejchoochotei* (formerly, *An. campestris*-like) will be solved by molecular analysis of the morphologically confirmed *An. campestris* samples in the future.

The Barbirostris and Hyrcanus groups belong to the Myzorhynchus series of *Anopheles* mosquitoes, which contains most vectors of human malaria except for *An. punctipennis*, which belongs to the *Anopheles* series. *An.*

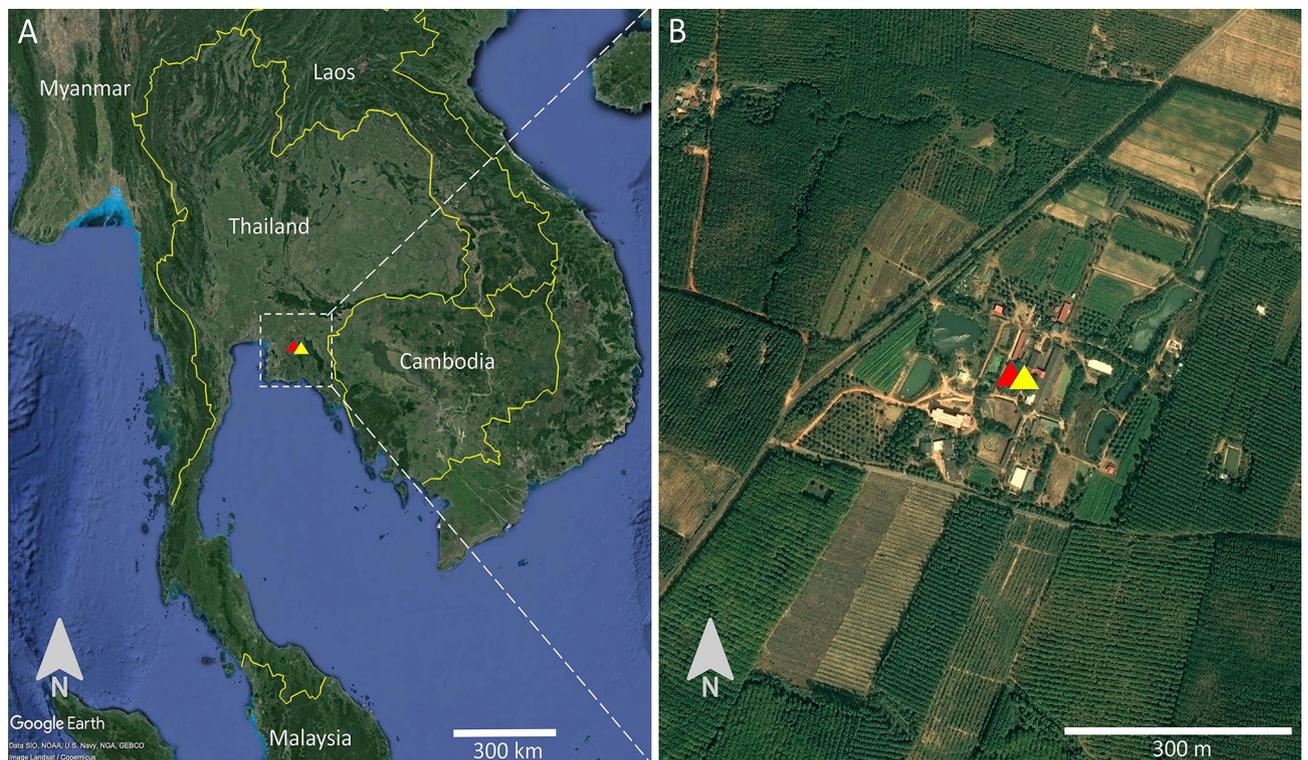


Figure 3. (A) Map depicting a buffalo farm in Chachoengsao for sample collection in Thailand. (B) The landscape of mosquito sampling sites in a buffalo farm in Chachoengsao. The images were obtained and modified from Google Earth Pro version 7.3.4.8248. The red triangle indicates blood sample collection sites, while the yellow triangle indicates mosquito sampling sites.

umbrosus and *An. letifer*, suspected vectors of *P. traguli*, and *An. gabonensis* and *An. obscurus*, the vectors of African ungulate malaria parasites, also belong to the *Myzorrhynchus* series. Thus, *Myzorrhynchus* series mosquitoes appear to have a dominant role in the transmission of ungulate malaria parasites.

Conclusions

An. wejchoochotei or *An. campestris* and *An. peditaeniatus* were identified as vectors of *P. bubalis* type I.

Methods

Study site, mosquito collection, dissection, and DNA extraction. This study was conducted on a buffalo farm in Chachoengsao province of Thailand (Fig. 3A). To investigate mosquito composition and identify the probable vector of *P. bubalis*, we carried out a survey of Murrah dairy buffaloes in Chachoengsao Province (13°28'53.98"N 101°27'35.23"E) for 14 consecutive nights in June 2020 and 2 nights in November 2021. The Murrah dairy buffalo farm is located 1 km away from the Nong Mai Kaen community. The area is surrounded by rubber trees with small ponds to wallow the water buffaloes (Fig. 3B).

CDC light traps with dry ice were set overnight at less than 1.5 m above ground level. Peripheral nets were placed surrounding the buffalo stable. Mosquitoes on the peripheral net were captured from 7.30 PM to 11.30 PM using tube aspirators (10 mm in diameter × 200 mm in length). The mosquitoes were then brought to the laboratory for morphological and molecular analysis. All anopheline mosquitoes were identified into group/species levels using taxonomic keys^{9,40}, while non-anopheline mosquitoes were identified up to only genus level according to the pictorial identification key of important disease vectors in the WHO Southeast Asia⁴¹. Anopheline mosquitoes were carefully dissected within three days after collection to obtain the salivary glands of each mosquito. A 26G and ½ inch-long sterile needle was used to dissect individual mosquitoes, which was changed after each dissection to prevent cross-contamination. In addition, 0.1% mercurochrome dye was used to stain oocysts on the midgut wall and sporozoites in the salivary glands, and samples were examined under a microscope at 1,000-times magnification. Salivary glands, which were still attached to the head and thorax, were kept in 0.2 mL of 1 × PBS at 4 °C for further DNA extraction for mosquito species identification and malaria parasite detection.

DNA samples from mosquitoes were extracted using NucleoSpin® Tissue (Macherey–Nagel, Düren, Germany) according to the manufacturer's guidelines with a minor modification in the elution step (elution volume reduced to 30 µL). Previous studies suggested that it is possible to detect higher infectivity in mosquito pool samples^{42,43}. Thus, adult female mosquitoes were grouped based on their morphology. Mosquito pools were made following morphological identification and were subsequently confirmed by molecular identification. Each pool was made up of one to three mosquitoes from the same groups depending on sample availability.

Blood collection from buffaloes, DNA extraction, and microscopic examination. To evaluate the malaria infection status in buffaloes, we carried out a survey of Murrah dairy buffaloes on a farm in Chachoengsao in June 2020 and November 2021, during which mosquitoes were captured ($n = 45$ and $n = 45$, respectively). These blood samples were drawn from the jugular vein using 21 G needles and BD vacutainers containing acid citrate dextrose (ACD). It should be noted that *P. bubalis* have been detected from buffaloes on this farm in our previous surveys^{3,26}. DNA was extracted as described above.

Anopheline mosquito's *cox1*, *cox2*, and *ITS2* gene amplification. Three genes of anopheline mosquito comprising *cox1*, *cox2*, and *ITS2* were amplified by PCRs using KOD FX Neo Polymerase (Toyobo, Japan) according to the manufacturer's protocol. The AnplCOXIF (5'-GGATCCCTTCAGCCATTAAATCGCG-3') and AnplCOXIR primers (5'-TCGAGCTTAAATTCATTGCACTAATCTGCC-3') were designed to amplify the *cox1* region with 1,584 bp-long products. The *Cox2* region was amplified by Anplcox2F-Anplcox2R primers (5'-GGATCCAGATTAGTGCAATGAATTTAAGC-3') and (5'-CTGCAGGATTTAAGAGATCATTACTTGC-3') to generate a total of 792 bp-long products. For the *ITS2* region, PCR amplification was carried out using ITS2A and ITS2B primers, as previously described⁴⁴. The PCR product size of the *ITS2* region varied depending on the mosquito group (~ 1,500 bp for Barbirostris complex, ~ 562 bp for Hyrcanus, ~ 697 bp for Ludlowae, ~ 518 bp for Funestus, and ~ 555 bp for Jamesii).

PCR detection of *Plasmodium's cytb*, *18S rRNA*, and *cox1* genes. DNA samples from buffalo blood underwent nested PCR screening for *Plasmodium* using primers targeting *cytb* gene DW2 (5'-TAATGCCTA GACGTATTCCTGATTATCCAG-3') and DW4 (5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3') as the outer primers and NCYBINF (5'-TAAGAGAATTATGGAGTGGATGGTG-3') NCYBINR (5'-CTTG TG GTAATTGACATCCA-ATCC-3') for the inner primers, as previously described⁴⁵. Subsequently, *Plasmodium*-positive samples were further confirmed using primer sets targeting the *18S rRNA* and *cox1* genes. The first amplification of the *18S rRNA* gene was carried out using *Plasmodium* universal primers, rPLU5 (5'-CCTGTT GTTGCTTAAACTTC-3') and rPLU6 (5'-TTAAAATTGTTGCAGTTAAAACG-3'), as previously described by Snounou et al.⁴⁶. New inner primers were designed based on the conserved region of the *18S rRNA* gene among the genus *Plasmodium*, PlSSUF1 (5'-CTTAGTTACGATTAATAGGAGTAG-3') and PlSSUR1 (5'-TCCTACT-CTT GTCTTAAACTAG-3') for forward and reverse directions, respectively, for the second amplification. In addition, PCR targeting the *Plasmodium's cox1* gene was conducted using the following primers: Cox1-F3-2 (5'-ATT ATGTAATTGCACATTTCCATTTTG-3') and Pbucox1-4B3 (5'-CCAAATAAAGTCATTGTWGAACC-3'). Each PCR amplification was carried out in a reaction volume of 12.5 μ L, consisting of 2 \times PCR buffer KOD FX Neo, 2.0 mM of dNTP, 0.4 μ M of each primer, 1.0 Unit of KOD FX Neo DNA Polymerase (Toyobo, Japan), 1 μ L genomic DNA as a template, and additional sterile distilled water up to 12.5 μ L. The cycling conditions and product size of each PCR assay are described in Supplementary Table 1. Subsequently, 5 μ L of PCR products were run on 1.5% agarose gel electrophoresis before being stained by Red Safe (Intron Biotechnology, Korea) and visualized under a UV transilluminator. The PCR products of positive samples were scaled up to 50 μ L for purification and sequencing. Gel purification was carried out using NucleoSpin[®] Gel and PCR clean up (Macherey–Nagel, Düren, Germany) according to the manufacturers' protocols. Purified PCR products were sequenced in both directions. DNA samples extracted from mosquitoes were subjected to PCR screening for *P. bubalis* in the same way as mentioned in blood samples. Additionally, *Plasmodium's cytb*-positive samples underwent PCR confirmation using primers targeting the *18S rRNA* and *cox1* genes, which were subsequently subjected to sequencing.

Sequence analyses. The chromatogram files of all target genes were edited manually using BioEdit software version 7⁴⁷. Low-quality sequences were excluded, resulting in a total of 41 mosquito pools being used for molecular analysis of each gene. Once the alignment was completed, sequences were compared to published sequence data in the GenBank[™] database using the BLASTN program. The alignment of multiple sequences obtained from this study and additional sequences from the GenBank[™] were made using the ClustalW via BioEdit version 7.

The ClustalW implemented in BioEdit version 7 was used to align sequences obtained in this study and additional sequences from GenBank[™] database. MrBayes v3.2.750 was used to create phylogenetic trees using the Bayesian Inference (BI) method and the Markov chain Monte Carlo method. BI phylogenetic analysis was performed using two independent runs of four chains, each for 10 million generations. As a result of burn-in, the first 25% of trees were discarded. Tracer v1.751 was used to assess the mixing and convergence of runs, as well as effective sample sizes (EES > 200). FigTree v1.4.4 was used to visualize the trees (available at <http://tree.bio.ed.ac.uk/software/figtree/>).

Statistical analysis. To evaluate the infection rate of positive mosquitoes, the minimum infection rate (MIR) was calculated for each species in which *Plasmodium* DNA was detected. If *Plasmodium* was detected from a mosquito pool, it was assumed that the pools contained at least one infected mosquito. Therefore, MIR was calculated as (number of positive pools/total number of analyzed mosquitoes) \times 100, as previously described^{48,49}. The MIR was calculated using the Wilson confidence interval method for binomial proportions, and the results were expressed as a percentage with a 95% confidence interval (CI).

Ethics statement and biosafety. This study has been reviewed and approved by Chulalongkorn University Animal Care and Use Committee (Approval No. 1931027). All protocol in this study was performed according to the Institutional Biosafety Committee of Chulalongkorn University (No. 2031033).

Data availability

All data in this article are available. Nucleotide sequences obtained in the present study were deposited in the GenBank™ database under the following accession numbers: OK338063, OL627356-57, OL672204-05 (*P. bubalis's cox1*), OL624705-09 (*P. bubalis's 18S rRNA*), and OL672206-09 (*P. bubalis's cyt b*).

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References

1. Templeton, T. J., Martinsen, E., Kaewthamasorn, M. & Kaneko, O. The rediscovery of malaria parasites of ungulates. *Parasitology* **143**, 1501–1508. <https://doi.org/10.1017/S0031182016001141> (2016).
2. Sheather, A. A malaria parasite in the blood of a buffalo. *J. Comp. Pathol. Ther.* **32**, 80026–80027 (1919).
3. Templeton, T. J. *et al.* Ungulate malaria parasites. *Sci. Rep.* **6**, 23230. <https://doi.org/10.1038/srep23230> (2016).
4. Kandel, R. C. *et al.* First report of malaria parasites in water buffalo in Nepal. *Vet. Parasitol. Reg. Stud. Rep.* **18**, 100348. <https://doi.org/10.1016/j.vprsr.2019.100348> (2019).
5. Garnham, P. & Edeson, J. Two new malaria parasites of the Malayan mousedeer. *Riv. Malariol.* **41**, 1–8 (1962).
6. Hoo, C. & Sandosham, A. The early forms of *Hepaticystis fieldi* and *Plasmodium traguli* in the Malayan mouse-deer *Tragulus javanicus*. *Med. J. Malays.* **22**, 299–301 (1968).
7. de Mello, F.d., Paes, S. Sur une plasmodie du sang des chèvres. *Cr. Séanc. Soc. Biol.* **88**, 829–830 (1923).
8. Kaewthamasorn, M. *et al.* Genetic homogeneity of goat malaria parasites in Asia and Africa suggests their expansion with domestic goat host. *Sci. Rep.* **8**, 5827. <https://doi.org/10.1038/s41598-018-24048-0> (2018).
9. Rattananarithikul, R. H., Bruce, A., Harbach, R. E., Panthusiri, P. & Coleman, R. E. Illustrated keys to the mosquitoes of Thailand IV. Anopheles. *Southeast Asian J Trop Med Public Health.* **37** (2006).
10. Walter Reed Biosystematics Unit. 2021. Systematic catalogue of Culicidae. <http://mosquitocatalog.org>. Last accessed on 20/09/2021.
11. Sinka, M. E. *et al.* The dominant Anopheles vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. *Parasit. Vectors.* **4**, 89. <https://doi.org/10.1186/1756-3305-4-89> (2011).
12. Syafruddin, D. *et al.* Malaria prevalence in Nias District, North Sumatra Province, Indonesia. *Malar. J.* **6**, 116. <https://doi.org/10.1186/1475-2875-6-116> (2007).
13. Vantaux, A. *et al.* Anopheles ecology, genetics and malaria transmission in northern Cambodia. *Sci. Rep.* **11**, 6458. <https://doi.org/10.1038/s41598-021-85628-1> (2021).
14. Manguin, S., Garros, C., Dusfour, I., Harbach, R. & 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.* **8**, 489–503. <https://doi.org/10.1016/j.meegid.2007.11.004> (2008).
15. Paredes-Esquivel, C., Donnelly, M. J., Harbach, R. E. & Townson, H. A molecular phylogeny of mosquitoes in the Anopheles barbirostris Subgroup reveals cryptic species: implications for identification of disease vectors. *Mol. Phylogenet. Evol.* **50**, 141–151. <https://doi.org/10.1016/j.ympev.2008.10.011> (2009).
16. 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.* **22**, 185–191. [https://doi.org/10.2987/8756-971X\(2006\)22\[185:HRITHP\]2.0.CO;2](https://doi.org/10.2987/8756-971X(2006)22[185:HRITHP]2.0.CO;2) (2006).
17. Brosseau, L. *et al.* A multiplex PCR assay for the identification of five species of the Anopheles barbirostris complex in Thailand. *Parasite. Vectors.* **12**, 223. <https://doi.org/10.1186/s13071-019-3494-8> (2019).
18. Taai, K. & Harbach, R. E. Systematics of the Anopheles barbirostris species complex (Diptera: Culicidae: Anophelinae) in Thailand. *Zool. J. Linn. Soc.* **174**, 244–264. <https://doi.org/10.1111/zoj.12236> (2015).
19. Dahan-Moss, Y. *et al.* Member species of the Anopheles gambiae complex can be misidentified as *Anopheles leesoni*. *Malar. J.* **19**, 1–9. <https://doi.org/10.1186/s12936-020-03168-x> (2020).
20. De Ang, J. X., Yaman, K., Kadir, K. A., Matusop, A. & Singh, B. New vectors that are early feeders for *Plasmodium knowlesi* and other simian malaria parasites in Sarawak, Malaysian Borneo. *Sci. Rep.* <https://doi.org/10.1038/s41598-021-86107-3> (2021).
21. Van Bortel, W. *et al.* Confirmation of *Anopheles varuna* in Vietnam, previously misidentified and mistargeted as the malaria vector *Anopheles minimus*. *Am. J. Trop. Med. Hyg.* **65**, 729–732. <https://doi.org/10.4269/ajtmh.2001.65.729> (2001).
22. Wharton, R., Eyles, D. E., Warren, M., Moorhouse, D. & Sandosham, A. Investigations leading to the identification of members of the Anopheles umbrosus group as the probable vectors of mouse deer malaria. *Bull. World. Health. Organ.* **29**, 357 (1963).
23. Boundenga, L. *et al.* Haemosporidian parasites of antelopes and other vertebrates from Gabon, Central Africa. *PLoS ONE* **11**, e0148958. <https://doi.org/10.1371/journal.pone.0148958> (2016).
24. Martinsen, E. S. *et al.* Hidden in plain sight: Cryptic and endemic malaria parasites in North American white-tailed deer (*Odocoileus virginianus*). *Sci. Adv.* **2**, e1501486. <https://doi.org/10.1126/sciadv.1501486> (2016).
25. Garnham, P. C. C. *Malaria Parasites and Other Haemosporidia* (Blackwell Sci. Pub, 1966).
26. Nguyen, A. H. L., Tiawsirirup, S. & Kaewthamasorn, M. Low level of genetic diversity and high occurrence of vector-borne protozoa in water buffaloes in Thailand based on 18S ribosomal RNA and mitochondrial cytochrome b genes. *Infect. Genet. Evol.* **82**, 104304. <https://doi.org/10.1016/j.meegid.2020.104304> (2020).
27. Hebert, P. D., Cywinska, A. & Ball, S. L. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 313–321. <https://doi.org/10.1098/rspb.2002.2218> (2003).
28. Cywinska, A., Hunter, F. & Hebert, P. D. Identifying Canadian mosquito species through DNA barcodes. *Med. Vet. Entomol.* **20**, 413–424. <https://doi.org/10.1111/j.1365-2915.2006.00653.x> (2006).
29. Ogola, E. O., Chepkorir, E., Sang, R. & Tchouassi, D. P. A previously unreported potential malaria vector in a dry ecology of Kenya. *Parasites Vectors* **12**, 80. <https://doi.org/10.1186/s13071-019-3332-z> (2019).
30. Maquart, P.-O., Fontenille, D., Rahola, N., Yean, S. & Boyer, S. Checklist of the mosquito fauna (Diptera, Culicidae) of Cambodia. *Parasite* **28**, 60. <https://doi.org/10.1051/parasite/2021056> (2021).
31. Saeung, A. *et al.* Geographic distribution and genetic compatibility among six karyotypic forms of *Anopheles peditaeniatus* (Diptera: Culicidae) in Thailand. *Trop. Biomed.* **29**, 613–625 (2012).
32. Tainchum, K., Kongmee, M., Manguin, S., Bangs, M. J. & Chareonviriyaphap, T. Anopheles species diversity and distribution of the malaria vectors of Thailand. *Trends Parasitol.* **31**, 109–119. <https://doi.org/10.1016/j.pt.2015.01.004> (2015).
33. Chookaew, S. *et al.* Anopheles species composition in malaria high-risk areas in Ranong Province. *Dis. Cont. J.* **46**, 483–493. <https://doi.org/10.14456/dcj.2020.45> (2020).
34. Reid, J. A. The *Anopheles barbirostris* group (Diptera, Culicidae). *Bull. Entomol. Res.* **53**, 1–57 (1962).
35. Harrison, B. A. & Scanlon, J. E. Medical entomology studies—II. The subgenus *Anopheles* in Thailand (Diptera: Culicidae). Contributions of the American Entomological Institute (Ann Arbor) **12** (1): iv + 1–iv 307 (1975).
36. Wang, Y., Xu, J. & Ma, Y. Molecular characterization of cryptic species of *Anopheles barbirostris* van der Wulp in China. *Parasite Vectors* **7**, 592. <https://doi.org/10.1186/s13071-014-0592-5> (2014).

37. Wang, G. *et al.* An evaluation of the suitability of COI and COII gene variation for reconstructing the phylogeny of, and identifying cryptic species in, anopheline mosquitoes (Diptera Culicidae). *Mitochondrial DNA Part A*, **28**, 769–777. <https://doi.org/10.1080/24701394.2016.1186665> (2017).
38. Davidson, J. R. *et al.* Molecular analysis reveals a high diversity of Anopheles species in Karama, West Sulawesi, Indonesia. *Parasite Vectors* <https://doi.org/10.1186/s13071-020-04252-6> (2020).
39. Beebe, N. W. DNA barcoding mosquitoes: advice for potential prospectors. *Parasitology* **145**(5), 622–633. <https://doi.org/10.1017/S0031182018000343> (2018).
40. Gunathilaka, N. Illustrated key to the adult female Anopheles (Diptera: Culicidae) mosquitoes of Sri Lanka. *Appl. Entomol. Zool.* **52**, 69–77. <https://doi.org/10.1007/s13355-016-0455-y> (2017).
41. WHO. Pictorial identification key of important disease vectors in the WHO South-East Asia Region. <https://apps.who.int/iris/handle/10665/332202> (accessed 20 August 2021).
42. Rigg, C. A., Hurtado, L. A., Calzada, J. E. & Chaves, L. F. Malaria infection rates in Anopheles albimanus (Diptera: Culicidae) at Ipeti-Guna, a village within a region targeted for malaria elimination in Panamá. *Infect. Genet. Evol.* **69**, 216–223. <https://doi.org/10.1016/j.meegid.2019.02.003> (2019).
43. Torres-Cosme, R. *et al.* Natural malaria infection in anophelines vectors and their incrimination in local malaria transmission in Darién, Panama. *PLoS ONE* **16**, e0250059. <https://doi.org/10.1371/journal.pone.0250059> (2021).
44. Beebe, N. W. & Saul, A. Discrimination of all Members of the *Anopheles punctulatus* complex by polymerase chain reaction-restriction fragment length polymorphism analysis. *Am. J. Trop. Med. Hyg.* **53**, 478–481. <https://doi.org/10.4269/ajtmh.1995.53.478> (1995).
45. Perkins, S. L. & Schall, J. J. A molecular phylogeny of malaria parasites recovered from cytochrome b gene sequences. *J. Parasitol.* **88**, 972–978. [https://doi.org/10.1645/0022-3395\(2002\)088\[0972:AMPOMP\]2.0.CO;2](https://doi.org/10.1645/0022-3395(2002)088[0972:AMPOMP]2.0.CO;2) (2002).
46. Snounou, G. *et al.* High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol.* **61**, 315–320. [https://doi.org/10.1016/0166-6851\(93\)90077-b](https://doi.org/10.1016/0166-6851(93)90077-b) (1993).
47. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids. Symp. Ser.* **41**, 95–98 (1999).
48. Schoener, E. *et al.* Avian Plasmodium in Eastern Austrian mosquitoes. *Malar. J.* **16**, 389. <https://doi.org/10.1186/s12936-017-2035-1> (2017).
49. Ventim, R. *et al.* Avian malaria infections in western European mosquitoes. *Parasitol. Res.* **111**, 637–645. <https://doi.org/10.1007/s00436-012-2880-3> (2012).
50. Huelsenbeck, J. P. & Ronquist, F. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755 (2001).
51. Rambaut, A., Drummond, A. J., Xie, D., Baele, G. & Suchard, M. A. Posterior summarization in Bayesian phylogenetics using tracer 1.7. *Syst. Biol.* **67**, 901–904 (2018).

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Author contributions

Y.R.N. contributed to the investigation, methodology, data analysis; writing—original draft. A.A. contributed to sample collection and morphological identification. T.T.N., D.N., H.L.A.N., and J.P. contributed to methodology and resources. M.A. and O.K. contributed to conceptualization; funding acquisition; writing—reviewing & editing. M.K. contributed to conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; validation; writing—reviewing & editing. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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