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.
complexes. Such deceptive results could have an impact on vector control programs or mislead subsequent studies.

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. tragului. After decades of inactivity, sporozoites of unknown malaria parasites were isolated from the salivary glands of Anopheles gabonensis and Anopheles obscurus in Gabon. 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). 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. 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. 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 provided. 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. 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 and 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 = 1,172), Aedes spp. accounted for 12.05% (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 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% mercuricchrome 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 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; 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 sequences (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.

Table 1. Summary of P. bbaru's PCR screening results of anopheline mosquitoes collected from the buffalo farm.
Table 2. Minimum infection rates of *Plasmodium* in collected mosquitoes.

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

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 ≥ 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. Thus, sequences with the highest identity (minimum ≥ 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 Ludoilow group mosquito hit $An. vagus$. $An. petidaeniatus$ was hit by two Hycarus group pools with all 3 gene sequences including a $P. bubalis$ sequence-positive pool (THM Mosq Buff20-P20_3), and by one Hycarus group pool with $cox2$ and $ITS2$ sequences. $An. pseudojamesi$ was hit by one Jamesi 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 ~ 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 samples and found they were matched with > 99% identity (Supplementary Fig. 1). Because $An. wejchoochotei$ sequences were reported in 2015 from morphologically defined samples, 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 (THM Mosq Buff20-P6_3 and THM Mosq Buff20-P8_2) were either $An. campestris$ or $An. wejchoochotei$; one from the Hycarus group (THM Mosq Buff20-P20_3) was $An. petidaeniatus$.

Discussion

The current study aimed to identify potential $P. bubalis$ vectors in Thailand. $An. wejchoochotei$ or $An. campestris$, $An. petidaeniatus$, $An. varuna$, $An. vagus$, 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 $An. petidaeniatus$. According to Rattanarithikul et al. and the Walter Reed Biosystematics Unit, 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 $An. petidaeniatus$ can be found in Thailand, Cambodia, Indonesia, Malaysia, Myanmar, the Philippines, and Vietnam. $An. wejchoochotei$ and $An. petidaeniatus$ were recently found to harbor human $Plasmodium$ species in Cambodia.

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 mosquitoes. $P. traguli$ oocysts and sporozoites have been discovered in $An. umbrosus$ and $An. letifer$ by microscopic examination in a historic mousedeer study in Malaysia. 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 (≥ 37%).

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

Tai and Harbach described $An. wejchoochotei$ for the first time, while Reid 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 Scanlon 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. A recent study in Sulawesi, Indonesia, used approximately 700 bp of the $cox1$ gene to distinguish members of mosquito species complexes. Furthermore, $cox1$ and $ITS2$ sequences have been used to identify cryptic mosquito species. Based on the $cox1$ barcode region, an evolutionary divergence of 0.5% (range 0.0–3.9%) was proposed as a threshold for intraspecific variation. 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 (THM Mosq Buff20-P6_3 and THM Mosq Buff20-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 Hycarus 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 species. An.
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 Myzorhynchus series. Thus, Myzorhynchus 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, 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 Asia. 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. 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 21G 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.

Anopheles 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 AmpCoxI (5’-GGATCCCTTGCACATTTAATGGC-3’) and AmpCOXIR primers (5’-TGGAGCTAATTCATTGGAATCTGAGGCC-3’) were designed to amplify the *cox1* region with 1,584 bp-long products. The Cox2 region was amplified by Anplcox2F-Anplcox2R primers (5’-GGATCCGATTAGTGCAATGAATTTAGG-3’) and (5’-CTCGAGGATTTAGGACCATCTTGGC-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 described\(^{44}\). The PCR product size of the *ITS2* region varied depending on the mosquito group (~ 1,500 bp for Barbariostris 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’-TAATGGCTTAACATTTTGGCAGTTT-3’) and DW4 (5’-GGATTGGTACGCTTTCTTACGTAATGCT-3’) as the outer primers and NCBINF (5’-TAAGGAATTTATGGGATGTTG-3’) NCBINR (5’-CTTGTTGTAAATTGCAATCCCAATCC-3’) for the inner primers, as previously described\(^{45}\). 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, rPLUS (5’-CCTGTTGTTGCGATTTAAGTACC-3’) and rPLU6 (5’-TTAAATTTTCCAATGTTACAG-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* PlasSUFI (5’-CTTAGTTACGTTAATAGAGATG-3’) and PlasSUF2 (5’-TCCACTCTGTCTTAACACT-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’-ATTATGTAATTTGCAATTTACATTG-3’) and *cox1*-R3 (5’-CCAAATAAAGCTATTGAAGACC-3’). Each PCR amplification was carried out in a reaction volume of 12.5 μL, consisting of 2× PCR buffer KOD FX Neo, 2.0 μM of dNTP, 0.4 μM of each primer, 1.0 Unit of KOD FX Neo DNA Polymerase (Toyobo, Japan), 1 μL genomic DNA as a template, and additional sterile distilled water up to 12.5 μL. The cycling conditions and product size of each PCR assay are described in Supplementary Table 1. Subsequently, 5 μL 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 μL 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 7.0. 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 7.0.

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/](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) \* 100, as previously described\(^{44,45}\). 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. bubali's cox1), OL624705-09 (P. bubali's 18S rRNA), and OL672206-09 (P. bubali's cyt b).

Received: 2 December 2021; Accepted: 28 March 2022
Published online: 06 April 2022

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**Acknowledgements**

We would like to thank Dr. Winai Kaewlamun and all staff for their support and advice. We also appreciate the logistic support during our sample collection by farm’s owners.

**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; methodology; project administration; resources; supervision; validation; writing—reviewing & editing. All authors reviewed the manuscript.

**Funding**

This work was supported by the National Research Council of Thailand (NRCT) [NRCT5-RSA63001-10] and the 90th Anniversary of Chulalongkorn University Scholarship under the Ratchadapisek Somphot Endowment Fund [GCUGR1125643038D] to M. K. and Y. R. N., respectively. A. A. was supported by the Royal Golden Jubilee (RGI) Ph.D. Program (grant number PHD/0028/2561). D.N. was supported by the C2F Ph.D. scholarship of Chulalongkorn University. J. P. was supported by the C2F postdoctoral research fellowship of Chulalongkorn University. This project was also supported by the National Research Center for Protozoan Diseases—Obihiro University of Agriculture and Veterinary Medicine (NRCPD-OUAVM) joint research in FY2020-2021 and FY2021-2022 to M. A. and M. K. M. K. was funded by Chulalongkorn University under the Ratchadapisek Somphot Fund for the Veterinary Parasitology Research Unit and Thailand Science Research and Innovation Fund Chulalongkorn University (CU_FRB65_food (24) 188_31_07).

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-09686-9.

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