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Molecular survey and genetic diversity of *Plasmodium* sp. infesting domestic poultry in northeastern Thailand

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Abstract

Introduction: Haemosporidian parasites are prevalent worldwide and can cause economic losses in poultry production. These parasites are arousing interest in Thailand and are found in many avian species. There is insufficient information on the genetic diversity of these alveolates from the largest families – Plasmodidae, Haemoprotidae and Leucocytozoidae – specifically parasitising ducks, turkeys, and geese. **Material and Methods:** Blood samples from 116 backyard poultry (60 ducks, 36 turkeys and 20 geese) in northeastern Thailand were investigated for *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. infections using microscopic examination and molecular approaches. **Results:** A total of 37/116 birds (31.9%) had confirmed *Plasmodium* infections. The prevalence was 69.4% (25/36) in turkeys, 18.3% (11/60) in ducks, and 5.0% (1/20) in geese. Of these 37 positives, 86.5% were *Plasmodium* sp., 10.8% were *P. gallinaceum* and 2.7% were *P. juxtanucleare*. Sequence analysis based on the cytochrome *b* gene identified seven lineages, of which two were new lineages in backyard poultry. **Conclusion:** This is the first report on the prevalence of haemosporidian parasites in backyard poultry in northeastern Thailand. The results provide important data for better understanding the molecular epidemiology of haemosporidian parasites infection in poultry in this region, which will be helpful in controlling these blood parasites.

Keywords: avian, haemosporidian pathogens, *Haemoproteus* spp., *Leucocytozoon* spp., *Plasmodium* spp.

Introduction

Infections with intracellular parasites in the Haemosporida order and *Plasmodium*, *Haemoproteus* and *Leucocytozoon* genera occur in many avian species and are public health problems. Infections with *Leucocytozoon* spp. and *Plasmodium* spp. are probably more virulent than those with *Haemoproteus* spp. (5, 7). These parasites are endemic globally and are especially frequent in tropical and subtropical areas where their insect vectors are present (6). Infection with these blood-borne parasites affects various organs within a bird's body, causing loss of function and affecting poultry production. Clinical symptoms in birds infected with haemosporidian parasites regularly include lethargy, loss of appetite, dizziness, diarrhoea, cyanosis, anaemia and thrombocytopaenia (4, 38). After postmortem examination, hepatomegaly, splenomegaly, and haemorrhagic areas in internal organs are frequently noted in infected animals (19, 32, 36).

The blood-sucking insects that transmit these parasites are mosquitoes for *Plasmodium* (15, 30), biting midges (*Culicoides*) and louse flies (Hippoboscidae) for *Haemoproteus* spp. (3), and black flies (*Simulium*) and biting midges for *Leucocytozoon* spp. (22). Birds are susceptible to at least 55, 86 and 200 species of *Plasmodium* (37), *Leucocytozoon* (29) and *Haemoproteus* (5, 7), respectively. Currently, the diagnosis of blood parasites in birds relies on laboratory testing and clinical observation. The laboratory routine for detection of haemosporidian infection is the thin blood-smear technique, which identifies blood parasites by examining their morphology under a microscope. However, the morphological characteristics of the parasite species may not be distinct with this technique and its sensitivity is low. Polymerase chain reaction (PCR) can detect parasites rapidly and with higher specificity and sensitivity. Primers for the cytochrome *b* gene (cyt *b*) are sound choices for PCR detection because this gene has been widely used to identify

haemosporidian parasite infections in several host species including chicken, waterfowl and other birds (1, 9, 33, 35).

Thailand is an agricultural country in Southeast Asia where ducks, chickens, geese and other birds are raised for consumption or sale. These poultry species have been reported to serve as reservoirs for haemosporidian parasites, but detailed information was lacking about the frequency of infection in domestic ducks (*Anas platyrhynchos domesticus*), domestic turkeys (*Meleagris gallopavo*) and swan geese (*Anser cygnoides*) in Thailand. Therefore, the aim of this study was to investigate the prevalence of haemosporidian parasites of the *Plasmodium*, *Leucocytozoon* and *Haemoproteus* families in ducks, geese, and turkeys in northeastern Thailand by blood smear examination and the nested-PCR method. Partial sequences obtained from the cyt *b* gene were used to confirm the identity of the pathogens and to construct a phylogenetic tree to gain further understanding of their molecular epidemiology in Thailand compared to other regions of the world.

Material and Methods

Blood sample collection. A total of 116 blood samples were collected from backyard birds in two provinces (Maha Sarakham and Nongkhai) in the northeastern part of Thailand (Fig. 1). Fifty-one blood samples were from Nongkhai (30 from ducks, 10 from turkeys and 11 from geese) and sixty-five were from Maha Sarakham (30 from ducks, 26 from turkeys and 9 from geese). Samples were taken from March to September 2022 of approximately 0.1–0.5 mL of blood drawn from the brachial vein into anticoagulant ethylenediaminetetraacetic acid tubes. These were stored on ice during transport to the laboratory at the Faculty of Veterinary Sciences of Mahasarakham University. The blood samples were screened for haemosporidian parasite infection by a thin-blood-smear technique and measurement of pack cell volume (PCV). The remaining blood was stored at −20°C until DNA extraction. All animal handling and blood collection steps were performed by veterinarians and access to all backyard birds was approved by their owners. All blood samples were randomly collected from symptomatic birds that their owners selected for blood testing. In addition, samples were collected from asymptomatic (healthy) animals.

Examination of haemosporidian parasites by blood smear. Blood samples were examined for haemosporidian parasite infection under a light microscope by thin-blood-smear technique. The blood smear was prepared by dropping blood onto a slide, smearing it with a spreader, air-drying it for approximately 10 s and fixing it with 100% methanol for 5 min. Subsequently, the blood films were stained with 10% Giemsa solution for 15 min and the parasites were detected in monolayer fields under a light microscope (Nikon, Tokyo, Japan).

Packed cell volume (PCV) examination. The percentage of total blood volume which was red blood cells, or the PCV value, was assessed from the height of the erythrocyte column in a microhaematocrit tube after centrifugation. Blood was directly placed into the heparinised microhaematocrit tube and centrifuged at 10,000 rpm for 3 min (Hettich Zentrifugen, Tuttlingen, Germany). The ratio of the volume of packed red blood cells to the total blood volume was measured and expressed as a percentage. The PCVs (%) of the infected and uninfected groups were compared using the independent samples *t*-test. A P-value less than 0.05 was considered statistically significant.

DNA extraction and haemosporidian parasite examination. Approximately 20 µL of each whole blood sample was mixed homogenously with 180 µL of 1× phosphate-buffered saline. DNA was extracted from 200 µL of the mixed solutions following the protocol with the GF-1 Blood DNA Extraction Kit (Vivatis, Shah Alam, Malaysia) and stored at −20°C.

Fig. 1. Map showing the area of sample collection in two provinces in the northeastern region of Thailand, consisting of Nongkhai (NK) and Maha Sarakham (MK)

A nested-PCR method using primers targeting a partial mitochondrial cyt *b* gene was performed. For the external nested PCR, the primer pair of HaemNFI (5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) which can amplify the DNA of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was used (10). For the internal nested PCR, the primers HaemF (5′-ATGGTGCTTTCG ATATATGCATG-3′) and HaemR2 (5′-GCATTATCT GGATGTGATAATGGT-3′) were used to amplify the DNA from *Plasmodium* and *Haemoproteus* (2), and HaemFL (5′-ATGGTGTTTTAGATACTTACATT-3′) and HaemR2L (5′-CATTATCTGGATGAGATAATGGIGC-3′) were used to amplify the *Leucocytozoon* DNA (10).

Both amplification steps of the nested-PCR reaction were performed in a 25 µL reaction volume consisting of 1 U of *Taq* polymerase (Thermo Scientific, Waltham, MA, USA), 1.5 mM of $MgSO_4$, 0.2 mM of dNTPs, 1× PCR buffer, 0.4 µM of each primer and template DNA (5 µL of extracted DNA for the first PCR and 2 µL of PCR product from the first amplification for the second PCR). The PCR conditions comprised 35 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 50°C for the first step and at 50°C (*Leucocytozoon*) and 53°C (*Plasmodium* and *Haemoproteus*) for the second steps, extension for 90 s at 72°C and a final extension at 72°C for 5 min. DNA from fighting cocks previously tested for *Leucocytozoon* and *Plasmodium*/ *Haemoproteus* infections served as positive controls. The PCR master mixes containing only the primers with no DNA template served as negative controls. The nested PCR generated approximately 480 base pairs (bp), which were subsequently identified by 1% agarose gels stained with ViSafe Red Gel Stain (Vivantis) and visualised under ultraviolet light on a gel documentation system.

Nucleotide sequencing and phylogenetic analysis. All positive samples containing the partial cyt *b* gene were purified and sequenced directly at a commercial sequencing company (ATGC Co., Pathum Thani, Thailand). The sequences obtained were multiply aligned using ClustalW in the BioEdit program with final adjustment performed manually, and compared for similarity with sequences deposited in the GenBank database using the BLAST program hosted by NCBI (https://www.ncbi.nlm.nih.gov/). The haplotype diversity of *Plasmodium* sp. was calculated in the DnaSP6 program (25).

The sequences of the partial cyt *b* gene of each *Plasmodium* haplotype in this study were deposited in GenBank (accession Nos OR341178–OR341184). A total of 40 *Plasmodium* sequences (7 haplotypes from this study and 33 related sequences from the MalAvi and GenBank databases) were analysed using the maximum-likelihood method in MEGA X (13) for construction of a phylogenetic tree, with sequences of two *Haemoproteus* sp. used as an outgroup. Bootstrap analysis with 1,000 replications was used to estimate the confidence of the branching patterns of the trees.

Results

Prevalence of *Plasmodium* in backyard poultry. In total, 116 blood smears from backyard poultry were examined under the microscope for haemosporidian parasite infections. *Plasmodium* sp. was observed in ducks and turkeys in Nongkhai and Maha Sarakham provinces (Fig. 2). However, *Leucocytozoon* sp. and *Haemoproteus* sp. were not found in all backyard poultry blood smears. In Nongkhai, the prevalence of *Plasmodium* spp. was 3.3% in ducks and 10% in turkeys. In Maha Sarakham, the prevalence of *Plasmodium* spp. was 6.7% in ducks and 26.9% in turkeys (Table 1). All positive samples identified by light microscopy were successfully amplified by nested PCR.

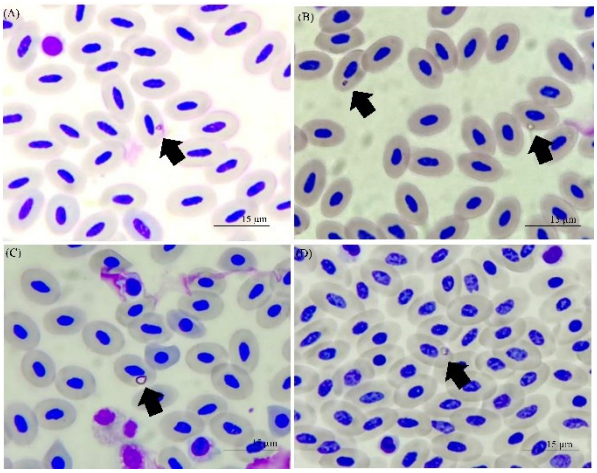

Fig. 2. *Plasmodium* spp. infections in erythrocytes of backyard ducks, turkeys and geese (indicated by arrows). (A) *Plasmodium* sp.; (B) *Plasmodium gallinaceum*; (C) *Plasmodium gallinaceum*; (D) *Plasmodium juxtanucleare*. Parasite species were identified by DNA sequencing

The molecular approach using the nested PCR method detected an additional 26 positive samples from microscopically negative samples. Microscopic examination indicated that no DNA fragments of *Leucocytozoon* sp. or *Haemoproteus* sp. were amplified. In Nongkhai and Maha Sarakham provinces, the molecular prevalence of *Plasmodium* spp. in ducks was 13.3% and 23.3%, and in turkeys was 50% and 76.9%, respectively. The prevalence of *Plasmodium* spp. in geese was 9.1% in Nongkhai. In summary, the overall prevalence of *Plasmodium* spp. was 31.9% (95% confidence interval from 23.4 to 40.4), with the highest prevalence observed in turkeys at 25/36 (69.4%), lower prevalence in ducks at 11/60 (18.3%) and the lowest in geese at 1/20 (5%).

The obtained sequences of the partial mitochondrial cyt *b* gene of *Plasmodium* parasites in this study were approximately 446-bp-long fragments (416–470 bp). All 37 positive PCR products were sequenced and seven haplotypes were identified. Among 37 sequences, 32, 4 and 1 sequences were identified as *Plasmodium* sp., *P. gallinaceum* and *P. juxtanucleare*, respectively.

There were 32 sequences of *Plasmodium* sp. which represented four lineages (accession Nos OR341178–OR341180 and OR341184), 4 sequences of *P. gallinaceum* which represented two lineages (accession Nos OR341181 and OR341183), and 1 sequence of *P. juxtanucleare* which represented one lineage (accession No. OR341182). In addition, two lineages of *Plasmodium* sp. found in this study had not been previously described (Table 2).

The ACCBAD01 lineage (accession No. OR341180) identified in ducks and turkeys was found to be identical (100% similarity) to *Plasmodium* sp. in a *Tyto alba* (barn owl) (accession No. MK390829.1) and an *Accipiter badius* (shikra) (accession No. JN639001.1), both from Thailand. The ORW1 lineage (accession No. OR341179) identified in ducks and turkeys had 100% similarity to *Plasmodium* sp. in a *Gyps bengalensis* (white-rumped vulture) from India (accession No. EF552403.1) and a *Pycnonotus sinensis* (light-vented bulbul) from China (accession No. KJ145050.1). The GALLUS01 lineage (accession No. OR341181) identified in turkeys was closely related (96.63–100% similarity) to *P. gallinaceum* in a *Gallus gallus* (chicken) from Thailand (accession No. LC506179.1). Under accession No. OR341182, the GALLUS02 lineage identified in turkeys was 99.56% identical to *P. juxtanucleare* in a *Gallus gallus spadiceus* (Burmese red junglefowl) from Thailand (accession No. KU248845.1) and another from Malaysia (accession No. KT290918.1).

Table 1. Prevalence of *Plasmodium* spp. infections in backyard poultry

Locations (Provinces)	Host		Prevalence of *Plasmodium* spp.		*Plasmodium* species (n)
	Common name	Scientific name	Microscopic examination (%)	PCR examination (%)	
Nongkhai	Domestic duck	*Anas platyrhynchos domesticus*	1/30 (3.3)	4/30 (13.3)	*Plasmodium* sp. (4)
	Domestic turkey	*Meleagris gallopavo*	1/10 (10)	5/10 (50)	*Plasmodium* sp. (2) *P. juxtanucleare* (1) *P. gallinaceum* (2)
	Swan goose	*Anser cygnoides*	0/11 (0)	1/11 (9.1)	*Plasmodium* sp. (1)
Maha Sarakham	Domestic duck	*Anas platyrhynchos domesticus*	2/30 (6.7)	7/30 (23.3)	*Plasmodium* sp. (7)
	Domestic turkey	*Meleagris gallopavo*	7/26 (26.9)	20/26 (76.9)	*Plasmodium* sp. (18) *P. gallinaceum* (2)
	Swan goose	*Anser cygnoides*	0/9 (0)	0/9 (0)	-
Total			11/116 (9.5)	37/116 (31.9)	

Table 2. Lineages of 37 cytochrome *b* gene sequences from backyard poultry in Thailand with matches to MalAvi database and National Center for Biotechnology Information (NCBI) GenBank database sequences

Lineage names	Parasites	Sample IDs	NCBI GenBank accession No.	Closest sequences in NCBI GenBank (% similarity)
ACCBAD01	*Plasmodium* sp.	D3, D5, D11, D34, D38, D42, D43, D51, D53, T4, T6, T13, T15, T26, T34, T17	OR341180	MK390829.1 (100%), JN639001.1 (100%)
ORW1	*Plasmodium* sp.	D36, T11, T12, T14, T16, T18, T21, T22, T25, T27, T29, T31, T30, T32	OR341179	EF552403.1 (100%), KJ145050.1 (100%), KJ396632.1 (100%)
FANTAIL01	*Plasmodium* sp.	G5	OR341184	AY714196.1 (100%), HF543648.1 (100%)
ANAPLA	*Plasmodium* sp.	D1	OR341178	MK390829.1 (99.78%), JN639001.1 (99.78%)
GALLUS01	*P. gallinaceum*	T2, T7	OR341181	LC506179.1 (96.63-100%), LN835294.1 (96.63-100%)
MELGAL	*P. gallinaceum*	T20, T28	OR341183	LC506179.1 (99.77%), LN835294.1 (99.77%)
GALLUS02	*P. juxtanucleare*	T9	OR341182	KU248845.1 (99.56%), KT290918.1 (99.56%)

New lineages in the present study are shown in bold

Table 3. Association among *Plasmodium* spp. infections with PCV levels

Host		Parasites	Levels of PCV (%) (mean ± SD)	95% CI of average PCV value (%)	P-value	Normal range (24)
Common name	Scientific name	*Plasmodium* spp. infections				
Duck	*Anas platyrhynchos domesticus*	infected (n = 11)	34.91 ± 4.87	31.64–38.18	0.62	36–58%
		uninfected (n = 49)	33.83 ± 6.68	31.89–35.77		
Turkey	*Meleagris gallopavo*	infected (n = 25)	38.92 ± 5.50	36.59–41.24	0.07	36–41%
		uninfected (n = 11)	35.09 ± 5.82	31.18–39.00		
Geese	*Anser cygnoides*	infected (n = 1)	47.00	N/A	N/A	38–58%
		uninfected (n = 19)	41.10 ± 3.03	38.93–43.27		

PCV – packed cell volume; SD – standard deviation; CI – confidence interval

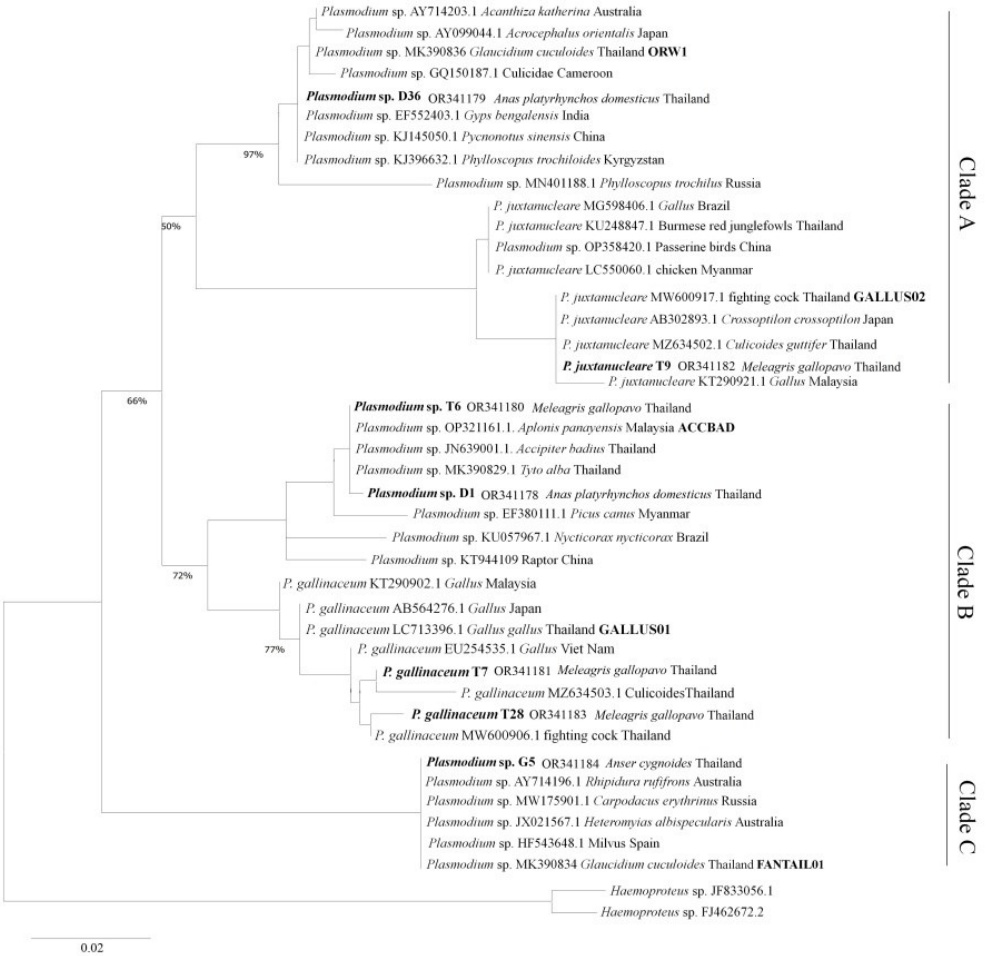

Fig. 3. Phylogenetic tree based on partial cytochrome *b* gene sequences of *Plasmodium* in this study (indicated in bold typeface) together with 33 related sequences from different distributions in Thailand and neighbouring countries in the GenBank and MalAvi databases. Sequences were compared with the maximum likelihood method. The cytochrome *b* gene of two *Haemoproteus* sp. was used as the outgroup. The percentage of trees in which associated taxa clustered together is shown next to the branch

A duck was the source of the ANAPLA lineage (accession No. OR341178), which was highly (99.78%) similar to *Plasmodium* sp. in a *Tyto alba* (accession No. MK390829.1) and an *Accipiter badius* (accession No. JN639001.1), both from Thailand. The MELGAL lineage (accession No. OR341183) identified in a turkey was a very close match (99.77% similarity) to *P. gallinaceum* in a *Gallus gallus* from Thailand (accession No. LC506179.1). Finally, the lineage designated FANTAIL01 (accession No. OR341184) identified in geese was identical (100% similarity) to *Plasmodium* sp. in a *Rhipidura rufifrons* (rufous fantail) from Australia (accession No. AY714196.1) and *Milvus* spp. (kites) from Spain (accession No. HF543648.1).

Phylogenetic analysis showed that the *Plasmodium* spp. could be divided into three main clades – Clade A, Clade B and Clade C. Most of the *Plasmodium* examples isolated from this backyard bird population belonged to Clades A and B, while Clade C contained a sequence of *Plasmodium* sp. from geese. Clade A consisted of the ORW1 lineage detected in ducks and turkeys and the GALLUS02 lineage detected in turkeys. Clade B contained the ACCBAD01 lineage detected in ducks and turkeys, the GALLUS01 lineage detected in turkeys, the ANAPLA lineage detected in ducks and the MELGAL lineage detected in turkeys (Fig. 3). The results showed that *P. gallinaceum* and *P. juxtanucleare* were found only in turkeys, while unidentified *Plasmodium* was found in turkeys, ducks and geese. However, the unidentified *Plasmodium* in geese was genetically different from the unidentified *Plasmodium* in ducks and turkeys.

Concerning PCV values, the mean averages in each animal species were in the normal range. Although the infected groups showed a lower trend of PCV, the results revealed no statistical difference between the infected and uninfected groups (Table 3). Moreover, there were no clinical signs in any poultry infected with *Plasmodium* spp.

Discussion

Plasmodium sp., *Haemoproteus* sp. and *Leucocytozoon* sp. infections are a global poultry health problem, especially in Thailand and nearby countries. In Thailand, these pathogens have been reported in several avian hosts such as chickens (17, 18, 23), Burmese red junglefowls (34),

Thai native fowls (20) and wild birds including owls (Strigiformes) and shikra (*Accipiter badius*) (21, 28). However, insufficient studies have been performed to determine the prevalence of these pathogens, especially in backyard birds such as ducks, turkeys and geese, even though these birds can play roles as reservoir hosts and have chances to come into contact with other domestic and wild birds. Therefore, we examined *Plasmodium* sp., *Haemoproteus* sp. and *Leucocytozoon* sp. infections in ducks, turkeys and geese in Nongkhai and Maha Sarakham provinces from northeastern Thailand, doing so for the first time using PCR.

Based on microscopy and PCR techniques, haemosporidian parasites in the genus *Plasmodium* were the only parasites detected in blood samples of backyard poultry birds, and their prevalence was 31.9%. We found that the prevalence of *Plasmodium* infection in backyard birds was higher in Maha Sarakham than in Nongkhai province. The variance of prevalence between different regions can be described as a result of habitat affecting vector dispersal, avian health management programmes and vector prevention and control. The results of DNA analysis showed that 0.9% of *Plasmodium* infections were identified as *P. juxtanucleare*, 3.4% as *P. gallinaceum* and 27.6% as unidentified *Plasmodium.* In our previous report, *P. juxtanucleare* was the most common avian malaria-inducing parasite identified in fighting cocks in Maha Sarakham (35). Nevertheless, this finding showed that an unidentified *Plasmodium* species was the most common malaria-inducing parasite found in other poultry in this region. This finding was possibly due to the variety in the types of bird which act as the vertebrate host. We found turkeys showed the highest prevalence of *Plasmodium* infections, followed by ducks and geese. This may be explained by individual species of host expressing susceptibility or resistance to infection, which was related to infection status (14, 31). Although the nested-PCR method used to detect *Plasmodium* infections in this study is considered highly sensitive (27, 35), the lower molecular occurrence found in ducks and geese in the neighbouring areas could have resulted from resistance of the hosts. Previous studies suggested that variation in the prevalence of avian haemosporidians in bird communities is mainly determined by host species' susceptibility to particular parasite lineages (8). Our finding correlated with previous studies in Pakistan which reported that *Plasmodium* was the main species of haemosporidian parasites in birds including turkeys, which overall showed 29.3% *Plasmodium* infection while ducks and geese were uninfected (26). Moreover, a study on avian species in Nigeria found the turkey was the species most infected with malaria-inducing parasites, and chickens, pigeons, ducks, geese and guinea fowls were all infected less frequently (16).

The identical PCV levels in the infected and uninfected groups might indicate that *Plasmodium* had a low pathogenicity in these backyard poultry, which concurs with our observation that no clinical signs were observed in the collected samples. A study in chickens in northeastern Nigeria supported our report, showing that PCV levels of uninfected and infected domestic chickens did not differ significantly (11).

Previous studies reported that the cytochrome *b* fragments are appropriate markers to determine the genetic diversity for haemosporidian parasites (12, 39). This study analysed gene fragments encoding cytochrome *b* and showed that the *Plasmodium* population was highly diverse with the presence of five haplotypes in ducks, six haplotypes in turkeys and one haplotype in geese. Most *Plasmodium* lineages found in backyard poultry in this study were ACCBAD01 and ORW1, which infected both turkeys and ducks. These lineages have been previously reported in barn owls and Asian barred owlets in Thailand (21). *Plasmodium* FANTAIL01 is the only lineage detected in geese which has also been detected in Asian barred owlets in Thailand (21).

In the present study, we identified and demonstrated the prevalence of *Plasmodium* sp., *P. gallinaceum* and *P. juxtanucleare* from backyard poultry in Nongkhai and Maha Sarakham provinces in Thailand. We discovered that *P. gallinaceum* and *P. juxtanucleare* were only found in infected domestic turkeys (*M. gallopavo*), but *Plasmodium* sp. could be found among domestic ducks (*Anas platyrhynchos domesticus*), swan geese (*Anser cygnoides*) and domestic turkeys. *Plasmodium* sp. in infected geese showed genetic differences and was retrieved in a different clade from *Plasmodium* sp. in ducks and turkeys.

Conclusion

This is the first report on the prevalence of haemosporidian parasites in backyard poultry in northeastern Thailand. The results provide important data for better understanding the molecular epidemiology of haemosporidian parasites infection in poultry in this region, which will be helpful in controlling these blood parasites.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: This research project was reviewed and approved by the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU-36/2022).

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