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Prevalence and genetic diversity of *Haemoproteus* and *Plasmodium* in raptors from Thailand: Data from rehabilitation center

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ABSTRACT

The diurnal raptors (Family: Accipitridae and Falconidae) are important as ecosystem bioindicators. Unfortunately, the global number of these birds has fallen, and they are close to extinction. This study reports the molecular prevalence and genetic diversity of *Haemoproteus* and *Plasmodium* in raptors admitted to the Kasetsart University Raptor Rehabilitation Unit over a period of 6 years. A total of 198 raptors, including 22 species from 30 provinces in Thailand, were admitted. The prevalence of parasites in raptors was low: *Haemoproteus* was 4.04% (95% CI: 1.29–6.78), and *Plasmodium* 2.53% (95% CI: 0.34–4.71). Eleven lineages of haemosporidian parasites were identified, and four lineages (ACCBAD02, NISALB01, NISALB02, and AEGMO03) are new globally. Interestingly, six lineages were isolated from birds belonging to the Accipitridae and Falconidae families (TYTAL4, TYTAL6, GLACUC08, MILANS06, OTUSCO02, and ORW1), indicating host shift of these parasites. Furthermore, the low prevalence of *Haemoproteus* and *Plasmodium* in raptors compared with that in previous reports suggests a relationship between the activity of avian hosts and vectors. This information is valuable for application in raptor rehabilitation and further research.

1. Introduction

The raptorial birds, raptors, or birds of prey (Family: Accipitridae and Falconidae) are important as ecosystem bioindicators. Furthermore, these birds are recognized as umbrella or flagship species for conservation purposes. Recently, raptors have been threatened, and their global number has fallen close to extinction (Buechley et al., 2019; Donázar et al., 2016; McClure et al., 2018). There are 61 raptor species recorded in Thailand (BCST, 2021), all of which are lawfully protected from trading and personal keeping. Of these 61 raptor species, 19 are considered resident species, 7 are resident or non-breeding visitors, 25 are non-breeding visitors, 5 are non-breeding visitors with a few records, 2 are spring and autumn passage migrants, and 3 were extirpated or probably extirpated from Thailand (white-rumped vulture, *Gyps bengalensis*; slender-billed vulture, *Gyps tenuirostris*, and red-headed vulture, *Sarcogyps calvus*).

The Kasetsart University Raptor Rehabilitation Unit (KURRU) has been established for the treatment of injured or orphaned raptorial birds (Accipitridae and Falconidae) and strigid owls (Tytonidae and Strigidae) (Pornpanom et al., 2019a) as well as veterinary education and research support. Since 2007, the year of establishment, there have been several studies on “hematology” and “hematozoa” that collected samples from admitted raptorial birds and strigid owls (Lertwatcharasarakul et al., 2021; Pornpanom et al., 2019a, 2019b; Salakij et al., 2012a, 2012b, 2015a, 2015b, 2018, 2019). This center currently directs its efforts towards treating injured raptors, promoting public conservation awareness, and supporting veterinary education and research.

Haemoproteus and *Plasmodium* spp. Have often been reported from this center (Pornpanom et al., 2019a; Salakij et al., 2012b, 2015a, 2018); however, there have been no reports on the molecular prevalence and genetic diversity of these parasites in raptorial birds. These parasites are vector-borne and transmitted by vectors as follows: biting midges and hippoboscid flies (*Haemoproteus* spp.) and *Culex*, *Aedes*, and *Culiseta* (*Plasmodium* spp.) (Valkiūnas, 2005). A previous report by Pornpanom et al. (2019a) showed the high prevalence and lineage diversity of *Haemoproteus* and *Plasmodium* spp. in 12 strigid owl species.

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In contrast to other studies (Lertwatcharasarakul et al., 2021; Pornpanom et al., 2019a, 2019b; Salakij et al., 2019), the present study focused on the molecular prevalence and genetic diversity of *Haemoproteus* and *Plasmodium* spp. in 22 raptor species admitted from February 2013 to September 2019. This study provides valuable information for the management of rehabilitation processes at the center. In addition, we provide information for further research on haemosporidian parasites in Thailand.

2. Materials and methods

2.1. Sample collection

One-milliliter blood samples were collected from diurnal raptors admitted to KURRU (14°1′N, 99°58′E), from February 2013 to September 2019. All raptors were physically restrained using a towel by an experienced assistant. Blood was collected from the right jugular vein of each bird, transferred into EDTA tubes, kept in an ice-box, and transported to the laboratory for molecular diagnostics.

2.2. Molecular diagnostics

Total DNA was extracted from 50 μL of EDTA-containing blood using the Blood Genomic DNA Extraction Mini Kit (FavorPrep, Pingtung, Taiwan). Nested-PCR amplification of the cytochrome *b* (cyt *b*) gene of *Haemoproteus* and *Plasmodium* spp. was performed as previously described (Bensch et al., 2000; Hellgren et al., 2004). The primers and thermocycling conditions used for the two parasites were similar. The HaemNFI and HaemNR3 primers were used for external nested PCR, and HaemF and HaemR2 primers were used for the internal nested-PCR reaction.

The thermocycling conditions for the external and internal reactions were as follows: pre-denaturation at 94 °C followed by 20 cycles (external reaction) or 35 cycles (internal reaction) at 94 °C for 30 s (denaturation), 50 °C for 45 s (annealing), 72 °C for 30 s (extension). and 72 °C for 10 min (final extension). The 20-μL PCR reaction mixture contained 2 μL of DNA template, 1 μL of each primer, 6 μL of water, and 10 μL of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Positive and non-template controls were used in all the reactions. The PCR product (479 bp) was analyzed using 1.5% agarose gel electrophoresis. The target band was then purified using the GEL/PCR purification Mini Kit (FavorPrep) and submitted to Apical Scientific (Selangor, Malaysia) for sequencing.

The blood samples were also tested for *Leucocytozoon* and *Trypanosoma* spp., following previously reported protocols (Hellgren et al., 2004; Valkiūnas et al., 2011). *Haemoproteus* and *Plasmodium* spp. positive samples were negative for *Leucocytozoon* and *Trypanosoma* spp. Some *Leucocytozoon* and *Trypanosoma* spp. positive samples were found in this bird population but were omitted in this study because they have been previously reported (Lertwatcharasarakul et al., 2021; Pornpanom et al., 2019b).

2.3. Sequences analysis

The quality of nucleotide sequences was determined using BioEdit software (Hall, 1999). *Haemoproteus-Plasmodium* co-infection was indicated by double peaks in the sequencing chromatogram (Dimitrov et al., 2016). All sequences from this study were single infections. Next, the forward and reverse sequences of each DNA sequence were used for lineage and phylogenetic analysis.

Our isolated sequences, with a consensus length of 479 nucleotides, were aligned with the cyt *b* sequences of *Haemoproteus* and *Plasmodium* spp. and deposited into the MalAvi database (Bensch et al., 2009) using BLAST to determine whether the isolated sequences were new lineages or not. Sequences with at least one nucleotide difference from the deposited sequences were considered a new lineage (Chagas et al., 2017; Ivanova et al., 2015). The new lineages were named following the MalAvi nomenclature (Bensch et al., 2009). The retrieved lineages were deposited in both the GenBank and MalAvi databases.

2.4. Phylogenetic analysis

Bayesian phylogenetic analysis of *Haemoproteus* and *Plasmodium* spp. was performed separately. All sequences isolated from diurnal raptors during the present study and other sequences retrieved from the MalAvi database were used for phylogenetic construction. The consensus length of cyt *b* was 479 nucleotides, and missing data in each lineage were replaced by "*N*" (Pornpanom et al., 2019a). The best-fit model for phylogenetic analysis of the cyt *b* gene of both *Haemoproteus* and *Plasmodium* spp. was the general time-reversible one with the invariant site and gamma distribution (GTR + I + G), selected using jModelTest2 software (Darriba et al., 2012).

A total of 92 cyt *b* sequences of *Haemoproteus* spp. were included in this study. Six sequences were isolated, and 86 sequences were retrieved from the MalAvi database: 20 sequences were undescribed species isolated from raptors, and 66 sequences were described species isolated from diverse avian host species. Phylogenetic analysis of *Plasmodium* spp. yielded 61 cyt *b* sequences of *Plasmodium* spp. Five sequences were isolated from the present study, and 56 sequences were retrieved from the MalAvi database: 30 sequences were undescribed species isolated from raptors, and 26 sequences were described species isolated from diverse avian host species. *Leucocytozoon* sp. SISKIN2 (AY393796) was used as a tree root for both *Haemoproteus* and *Plasmodium* spp. phylogenetics.

Bayesian phylogenetic analysis was performed using MrBayes version 3.2.6 (Ronquist and Huelsenbeck, 2003). The Markov chain Monte Carlo (MCMC) model was run for five million generations for both *Haemoproteus* and *Plasmodium* spp., with sampling at every 100 generations. The first 25% of trees were discarded as a "*burn-in*" step. Then, a consensus tree was constructed using the remaining trees. Posterior probabilities higher than 50% were presented at the nodes. The genetic distance between the different lineages was observed using the Jukes-Canter substitution model (all substitutions weighted equally) using MEGA7 (Kumar et al., 2016).

2.5. Giemsa-stained blood smears

Only two samples from Blyth's hawk-eagles (BHE; *Spizaetus alboniger*, KU549 and KU589) were prepared using Giemsa-stained blood smears. Blood smears were prepared immediately after blood collection and then allowed to dry using an electric fan. Staining was performed as previously reported (Valkiūnas et al., 2008). Thereafter, images were obtained using a BX53 light microscope equipped with a DP73 digital camera and cellSens Standard imaging software (Olympus, Tokyo, Japan). Morphological description and morphometric measurements were not performed because parasite morphology might be influenced by EDTA (Pornpanom et al., 2019a).

2.6. Statistical analysis

Descriptive statistics (percentages) were used to describe the prevalence of *Haemoproteus* and *Plasmodium* spp. based on molecular analysis results. The overall prevalence was calculated using data from all raptors. Only raptorial species with a number examined equal to or greater than 15 were used to calculate the prevalence of parasites within the species. The confidence intervals (CIs, 95%) were calculated using the "bionom.approx" function in R (R development core team, 2020).

Pearson's chi-square ($\chi 2$) was used to test the differences in haemosporidian parasite prevalence between resident and migratory birds. Based on the BCST (2021), the birds were grouped into three categories: resident, non-breeding visitor, and resident or non-breeding visitor birds. The $\chi 2$ was implemented using the "chisq.test" function in R (R

development core team, 2020).

3. Results

3.1. Molecular analysis and prevalence

There were 198 raptors admitted to KURRU, belonging to 22 raptor species (Table 1). The localities where the birds were caught included 30 provinces in Thailand (Fig. 1). The most common locality for raptors was Bangkok, where 102 raptors were found and admitted to KURRU, followed by Uthai Thani, where 10 raptors were caught. Other provinces recorded less than 10 raptors brought to KURRU.

Of these 198 raptors, 13 were singly positive for PCR detection of haemosporidian parasites *Haemoproteus* ($n = 8$) and *Plasmodium* spp. ($n = 5$). None of the 13 raptorial birds were positive for *Leucocytozoon* and *Trypanosoma* spp. Based on the molecular detection results, the prevalence of haemosporidian parasites in raptorial birds was very low [6.57% (95% CI: 3.11–10.01)]; for *Haemoproteus* spp. prevalence was 4.04% (95% CI: 1.29–6.78), and that for *Plasmodium* spp. was 2.53% (95% CI: 0.34–4.71) (Table 1). The prevalence of haemosporidian parasites in non-breeding visitors (21.05%, 4 out of 19 birds) was significantly higher than that of resident birds (5.23%, 8 out of 153 birds) and resident or non-breeding visitor birds (3.85%, 1 out of 26 birds) ($p = 0.027$).

Table 1
Prevalence of *Haemoproteus* and *Plasmodium* in diurnal raptorial birds in Thailand, during February 2013–September 2019.

Raptors	Number	Prevalence (%)		
		Haemoproteus	*Plasmodium*	Total
Black baza (*Aviceda leuphotes*)	2	0	0	0
Jerdon's baza (*Aviceda jerdoni*)	1	0	0	0
Black kite (*Milvus migrans*)	15	1 (6.67%)	0	1 (6.67%)
Brahminy kite (*Hiliastur indus*)	49	0	1 (2.04%)	1 (2.04%)
Black-shouldered kite (*Elanus caeruleus*)	47	0	0	0
Crested goshawk (*Accipiter trivigatus*)	5*	0	0	0
Eurasian kestrel (*Falco tinnunculus*)	1	0	0	0
Japanese sparrowhawk (*Accipiter gularis*)	1	0	0	0
Shikra (*Accipiter badius*)	16	1 (6.25%)	0	1 (6.25%)
Gray-faced buzzard (*Butastur indicus*)	2	1	0	1
Eurasian honey buzzard (*Pernis pilorhyncus*)	2	0	0	0
Rufous-winged buzzard (*Butastur liventer*)	17	2 (11.76%)	1 (5.88%)	3 (17.65%)
Crested serpent-eagle (*Spilornis cheela*)	5	0	0	0
Blyth's hawk-eagle (*Spizaetus alboniger*)	6	2	0	2
Changeable hawk-eagle (*Spizaetus cirrhatus*)	7	1	0	1
Mountain hawk-eagle (*Spizaetus nipalensis*)	3	0	0	0
White-bellied sea eagle (*Haliaeetus leucogaster*)	4	0	0	0
Eastern imperial eagle (*Aquila heliaca*)	3	0	0	0
Greater spotted-eagles (*Aquila clanga*)	1	0	0	0
Steppe eagles (*Aquila nipalensis*)	2	0	0	0
Cinerous vulture (*Aegypius monachus*)	1	0	1	1
Himalayan vultures (*Gyps himalayansis*)	8	0	2	2
Total	198	8 (4.04%)	5 (2.53%)	13 (6.57%)

* One sample was not performed nested-PCR.

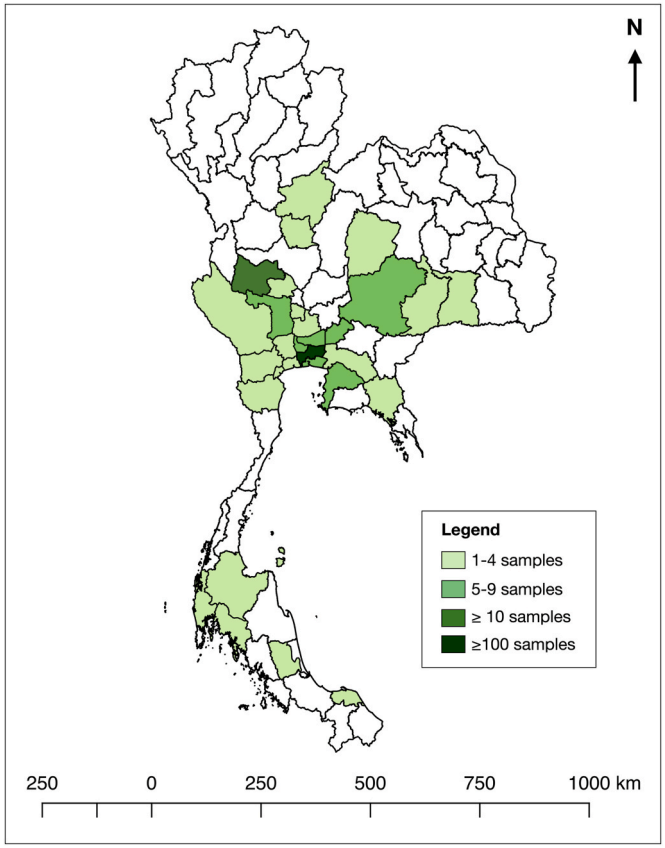

Fig. 1. Localities of the raptors included in this study. There were 30 provinces where the raptors are found and submitted into the Kasetsart University Raptor Rehabilitation Unit. These provinces are divided into four groups base on the number of raptors. Bangkok is the most common locality of raptor (n > 100).

3.2. Genetic diversity

Analysis of the sequences (with a consensus length of 479 nucleotides) revealed that 13 haemosporidians isolated from Thai raptors contained 11 haemosporidian lineages (Table 2). In addition, 4 out of the 11 lineages were recognized as new, including ACCBAD02, NISALB01, NISALB02, and AEGMO03.

For the other seven lineages, four lineages retrieved from this study belonged to lineages that originated from Strigiformes (TYTAL4, TYTAL6, GLACUC08, and OTUSCO02). Furthermore, based on the MalAvi database records, two lineages isolated in this study have been previously reported from more than three host orders: MILANS06 and ORW1.

MILANS06 has been reported from birds belonging to the orders Columbiformes, Coraciiformes, Accipitriformes, and Strigiformes. ORW1 has been reported in birds belonging to Coraciiformes, Accipitriformes, Passeriformes, Pelecaniformes, Piciformes, and Strigiformes. Another lineage, ACCBAD01, has been reported in birds belonging to Accipitriformes and Strigiformes.

Regarding the migratory status of the birds, the TYTAL6 lineage was found in both resident and non-breeding visitor birds (Table 2). Five lineages (NISALB01, NISALB02, OTUSCO02, TYTAL4, and GLACUC08) were found only in resident birds. AEGMO03 and MILANS06 were found only in non-breeding visitor birds. ACCBAD02 was found in resident and non-breeding visitor birds. The ACCBAD01 lineage was found in

Table 2
Haemoproteus and *Plasmodium* lineages isolated from diurnal raptors in Thailand, during February 2013–September 2019.

Lineages[a]	Isolates[b]	Parasite genus	Raptors information					GenBank
			Family	Scientific name	Common name	Locality	Status[C]	
ACCBAD02	KU219	*Haemoproteus*	Accipitridae	*Accipiter badius*	Shikra	Bangkok	R, N	MZ502244
NISALB01	KU549	*Haemoproteus*	Accipitridae	*Spizaetus alboniger*	Blyth's hawk-eagles	Chanthaburi	R	MZ502246
NISALB02	KU589	*Haemoproteus*	Accipitridae	*Spizaetus alboniger*	Blyth's hawk-eagles	Bangkok	R	MZ502247
OTUSCO02	R14	*Haemoproteus*	Accipitridae	*Butastur liventer*	Rufous-winged buzzard	N/A	R	MZ502239
TYTAL4	KU628	*Haemoproteus*	Accipitridae	*Milvus migrans*	Black kite	Suphan Buri	R	MZ502248
TYTAL6	R10	*Haemoproteus*	Accipitridae	*Butastur indicus*	Gray-faced buzzard	N/A	N	MZ502238
TYTAL6	KU306	*Haemoproteus*	Accipitridae	*Butastur liventer*	Rufous-winged buzzard	Bangkok	R	MZ502240
TYTAL6	KU245	*Haemoproteus*	Accipitridae	*Spizaetus cirrhatus*	Changeable hawk-eagles	Nonthaburi	R	MZ502245
ACCBAD01	KU372	*Plasmodium*	Accipitridae	*Haliastur indus*	Brahminy kite	Bangkok	R	MZ502250
AEGMO03	KU502	*Plasmodium*	Accipitridae	*Aegypius monachus*	Cinerous vulture	Nakhon Ratchasima	N	MZ502243
GLACUC08	KU410	*Plasmodium*	Accipitridae	*Butastur liventer*	Rufous-winged buzzard	Bangkok	R	MZ502241
MILANS06	KU247	*Plasmodium*	Accipitridae	*Gyps himalayansis*	Himalayan vulture	Chachoengsao	N	MZ502249
ORW1	KU686	*Plasmodium*	Accipitridae	*Gyps himalayansis*	Himalayan vulture	N/A	N	MZ502242

N/A = data not available.
[a] New lineage are given in bold.
[b] Isolates providing by KURRU.
[c] Migratory status provided by BCST (2021): R = resident and N = non-breeding visitor.

resident birds, but this lineage was also found in Thai shikra (*Accipiter badius*, GenBank: JN639001), which is considered a resident or non-breeding visitor bird. The ORW1 lineage was found in non-breeding visitor birds, but this lineage was also found in the Thai Asian barred owlet (*Glaucidium cuculoides*, GenBank: MK390836), which is considered a resident bird.

3.3. Phylogenetics analysis

Bayesian phylogenetic analysis of *Haemoproteus* spp. (Fig. 2) revealed that eight lineages isolated in this study were clustered into two clades (clades I and II). The lineage ACCBAD02 (isolate: KU219, GenBank: MZ502244) was clustered in clade I, with a 76% posterior probability. The highest genetic distance among the lineages within clade I was 0.06% (Fig. 3). *Haemoproteus* sp. ACCBAD02 (isolate: KU219) retrieved from *A. badius* was identical to *Haemoproteus* sp. AFR048 (GenBank: KM056451).

Other *Haemoproteus* lineages were clustered into clade II, with a 100% posterior probability. The highest genetic distance among lineages within clade II was 0.11%. *Haemoproteus* sp. OTUSCO02 (isolate: R14), NISALB02 (isolate: KU589), and TYTAL4 (isolate: KU628) were identical to *Haemoproteus* sp. OTUSCO01 (MT281465) retrieved from a raptor in China. Three TYTAL6 isolates, R10, KU245, and KU306, were identical to each other.

Bayesian phylogenetic analysis of *Plasmodium* spp. (Fig. 4) revealed that five lineages isolated in this study were clustered into three clades (clades I, II, and III). The lineage GLACUC08 (isolate: KU410, GenBank: MZ502241) and ORW1 (isolate: KU686, GenBank: MZ502242) were clustered into clade I, with a 62% posterior probability. The highest genetic distance among the lineages within clade I was 0.04% (Fig. 5).

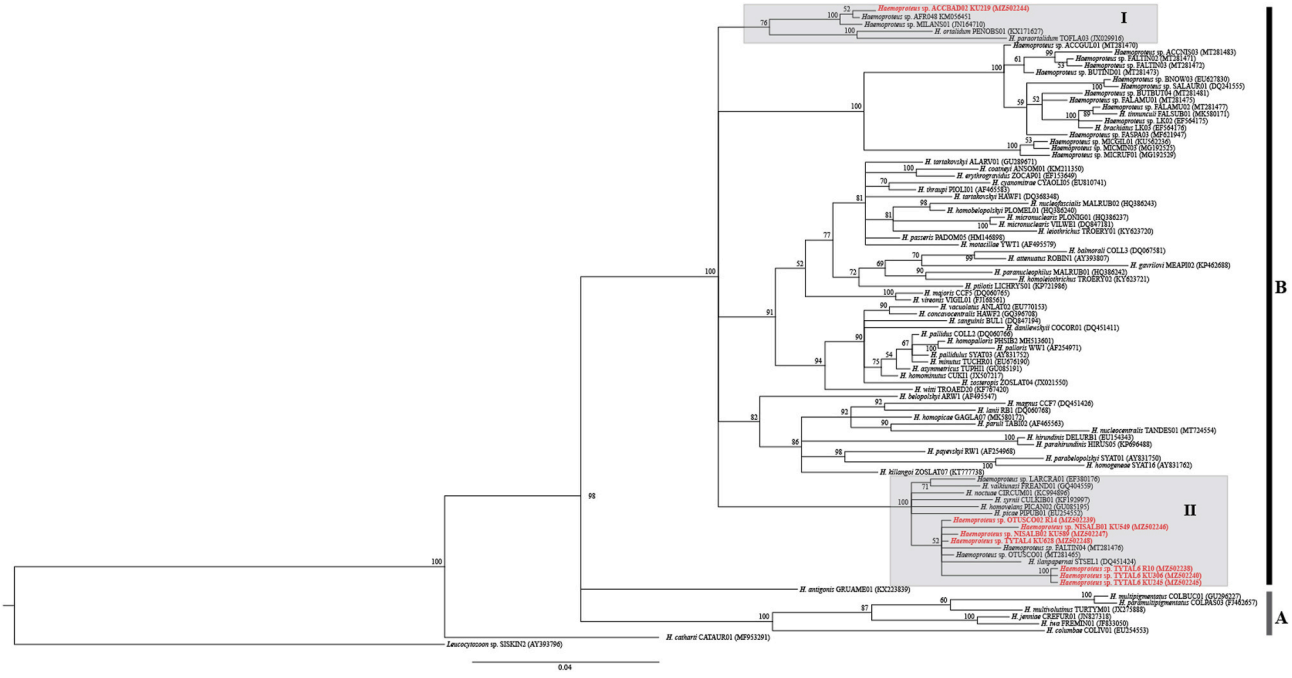

Fig. 2. Bayesian phylogeny based on partial cytochrome *b* gene (479 nucleotides) of *Haemoproteus* lineages. The lineages isolated in this study are given in red bold. MalAvi lineage codes and GenBank accession numbers are given after species names. Node values indicate percentages of posterior probabilities. Vertical bars indicate clades of subgenus *Haemoproteus* (**A**) and *Parahaemoproteus* (**B**) *Haemoproteus* isolated from this study are clustered into two clades (clade I and II). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

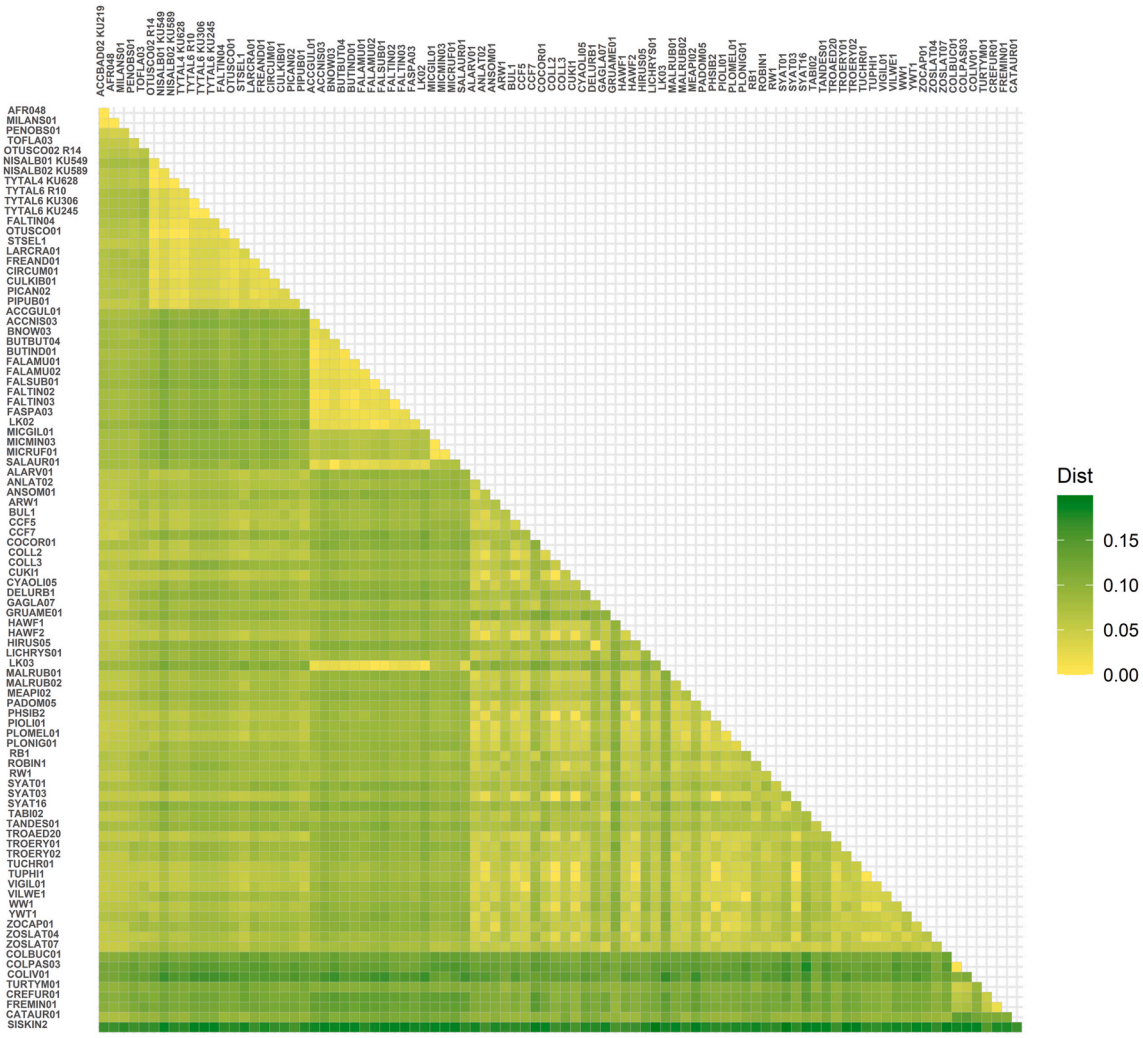

Fig. 3. Heatmap of pairwise genetic distances estimated from nucleotide sequences of the cytochrome *b* gene (479 nucleotides) of *Haemoproteus* spp. using the Jukes-Canter model.

Plasmodium spp. GLACUC08 (isolate KU410) and ORW1 (isolate KU686) were identical to *Plasmodium* sp. ORW1 (GenBank: AF254963) retrieved from a passerine bird (Oriental reed warbler, *Acrocephalus orientalis*).

The lineage AEGMO03 (isolate: KU502, GenBank: MZ502243) was clustered into clade II, with a 100% posterior probability. The highest genetic distance among lineages within clade II was 0.09%. *Plasmodium* sp. AEGMO03 (isolate: KU502) retrieved from a cinereous vulture (*Aegypius monachus*) in the present study was identical to *Plasmodium collidatum* FANTAIL01 (GenBank: AY714196) retrieved from rufous fantail (*Rhipidura rufifrons*) from Australia.

The lineage ACCBAD01 (isolate: KU372, GenBank: MZ502250) and MILANS06 (isolate: KU247, GenBank: MZ502249) were clustered into clade III, with 69% posterior probability. The highest genetic distance among lineages within clade III was 0.05%. *Plasmodium* sp. ACCBAD01 (isolate: KU372, GenBank: MZ502250) retrieved from a Brahminy kite (*Haliastur indus*) in the present study was identical to *Plasmodium* sp. ACCBAD01 (JN639001) retrieved from *A. badius* in Thailand. *Plasmodium* sp. MILANS06 (isolate: KU247, GenBank: MZ502249) retrieved from a Himalayan vulture (*Gyps himalayansis*) in Thailand was identical to *Plasmodium* sp. MILANS06 (JN164715) obtained from *Milvus migrans*.

3.4. Giemsa-stained blood smears

Giemsa-stained blood smears from BHE (KU549 and KU589) revealed young gametocytes, microgametocytes, and macrogametocytes of *Haemoproteus* spp. (Fig. 6). Parasitemia was low, with <1 gametocyte per 10,000 red blood cells. There was no morphological or morphometric information. No extracellular gametocytes were observed.

4. Discussion

According to a previous report, EDTA might interfere with the development of *Haemoproteus* and *Plasmodium*, resulting in extracellular gametocytes (Pornpanom et al., 2019a). In the present study, only two samples from BHE were prepared using Giemsa blood smears. Therefore, morphology and morphometry were not analyzed in this study. Extracellular gametocytes were not found in the blood smears of these two BHE, which differed from a report in owls (Pornpanom et al., 2019a). Palinauskas et al. (2013) reported *in vitro* exflagellation of *Haemoproteus tartakovskyi* by mixing blood containing gametocytes in 3.5% sodium citrate and allowing the blood to be exposed to air for 4 min. In addition, they inferred that exflagellation begins immediately if the blood contains mature gametocytes exposed to the air. In the present study, no extracellular gametocytes were found on the blood smears because they were prepared immediately after blood samples were collected.

The current study focused on genetic diversity and prevalence, based on the widely used PCR method (Cocumelli et al., 2021; Inumaru et al., 2017; Nourani et al., 2021; Scaglione et al., 2016; Schumm et al., 2021; Tasci et al., 2018; Win et al., 2020; Yang et al., 2021). The PCR method has been proven to be more sensitive than microscopic examination

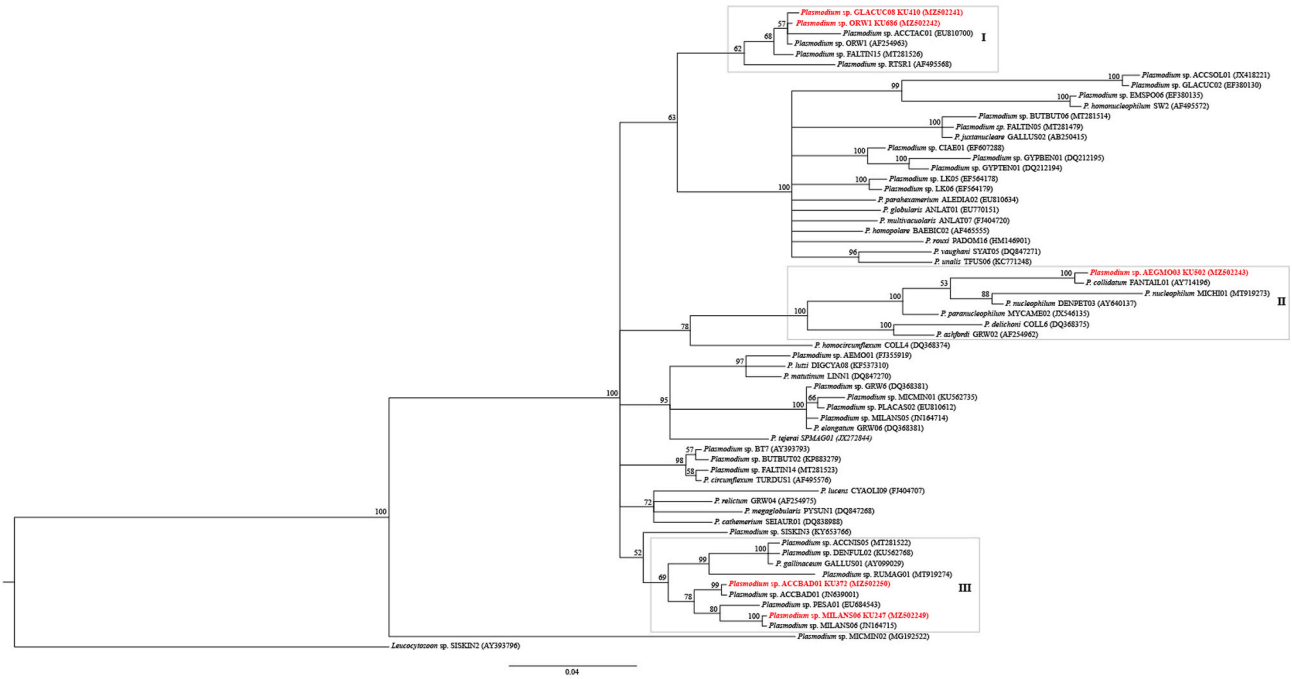

Fig. 4. Bayesian phylogeny based on the partial cytochrome *b* gene (479 base pairs) of *Plasmodium* lineages. The lineages isolated in this study are given in red bold. MalAvi lineage codes and GenBank accession numbers are given after species names. Node values indicate percentages of posterior probabilities. *Plasmodium* isolated from this study are clustered into three clades (clade I, II and II). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Valkiūnas et al., 2008). Hence, it is reasonable to report the prevalence of *Haemoproteus* and *Plasmodium* spp. based on molecular results.

The prevalence of *Haemoproteus* and *Plasmodium* spp. during the 6 years was very low, 4.04% (95% CI: 1.29–6.78) and 2.53% (95% CI: 0.34–4.71), respectively. Conversely, a previous report on prevalence in strigid owls from KURRU was relatively high, 24.6% (95% CI: 18.02–31.08) for *Haemoproteus* spp. and 9.0% (95% CI: 4.65–13.32) for *Plasmodium* spp. (Pornpanom et al., 2019a). These results indicated that diurnal raptors had lower sensitivity for infection with *Haemoproteus* and *Plasmodium* spp. than nocturnal raptors. This might be related to the activity of the vectors and avian hosts.

However, the information on prevalence of *Haemoproteus* and *Plasmodium* spp. in the present study and the aforementioned report on strigid owls (Pornpanom et al., 2019a) was gathered from only one rehabilitation center. This may have been biased by the population of the birds. Therefore, we suggest that to confirm the relationship between the activity of vectors and avian hosts, sample collection from the field in Thailand is required.

Diversity and phylogenetics revealed that both diurnal and nocturnal raptors might share the same species of *Haemoproteus* and *Plasmodium*. For example, *Haemoproteus* sp. OTUSCO02, TYTAL4, and TYTAL6 isolated from raptorial birds were first described in strigid owls. In addition, the *Plasmodium* GLACUC08 isolated from a rufous-winged buzzard (*Butastur liventer*) was first described in an Asian barred owlet (*Glaucidium cuculoides*) (Bensch et al., 2009). Thus, a screening process before admission of both raptorial birds and strigid owls is required to prevent haemosporidian transmission within the center.

Moreover, the phylogenetic tree of *Haemoproteus* and (Fig. 3) *Plasmodium* spp. (Fig. 4) and sequences retrieved from the MalAvi database (Bensch et al., 2009) showed that haemosporidian parasites isolated from other bird orders, such as Coraciiformes, Passeriformes, Pelecaniformes, Piciformes, and Strigiformes, were probably transmitted to the raptors. Conversely, haemosporidian parasites infecting raptors can also be transmitted to other avian hosts. In this case, there is evidence of *Plasmodium juxtanucleare*, which is typically specific to birds belonging to the order Galliformes (Valkiūnas, 2005), infecting passerine birds (Passeriformes) (Ferreira-Junior et al., 2018).

From the above information, the investigation of haemosporidian parasites in raptors or other wild birds was not only important in terms of conservation or diversity, but also from an economic perspective, as these parasites can affect industrial poultry farms. The average temperature of Thailand is over 20 °C (Pornpanom et al., 2019a), suitable for the development of *Haemoproteus* and *Plasmodium* spp. in vectors (Bukauskaitė et al., 2015; LaPointe et al., 2010). Thus, a combination of microscopic and molecular analysis of haemosporidian parasites using blood samples of wild birds in the field might reveal a number of overlooked haemosporidian parasites in Thailand and nearby countries. Moreover, experimental research focusing on the treatment of blood parasites specific to raptorial birds and strigid owls is needed in the future.

5. Conclusion

Here, we identified 13 lineages of haemosporidian parasites in diurnal raptors in Thailand. This was the first and largest *Haemoproteus* and *Plasmodium* spp. study ever performed in diurnal raptors in Thailand. Of the 13 identified lineages, four were recognized as new lineages globally (ACCBAD02, NISALB01, NISALB02, and AEGMO03). Data from the present study indicated cross-infection of parasites between raptorial birds and strigid owls. This information can be applied to improve the rehabilitation process at the rehabilitation center, for example, by screening for blood parasites before admission and preventing insect vectors within the center. In addition, the findings on lineage diversity and phylogenetics can support future studies on *Haemoproteus* and *Plasmodium* spp. in Thailand and nearby countries.

Ethics approval

This study was approved by the Institutional Laboratory Animal Care and Use Committee of Kasetsart University, Thailand (Approval no. ACKU 01560).

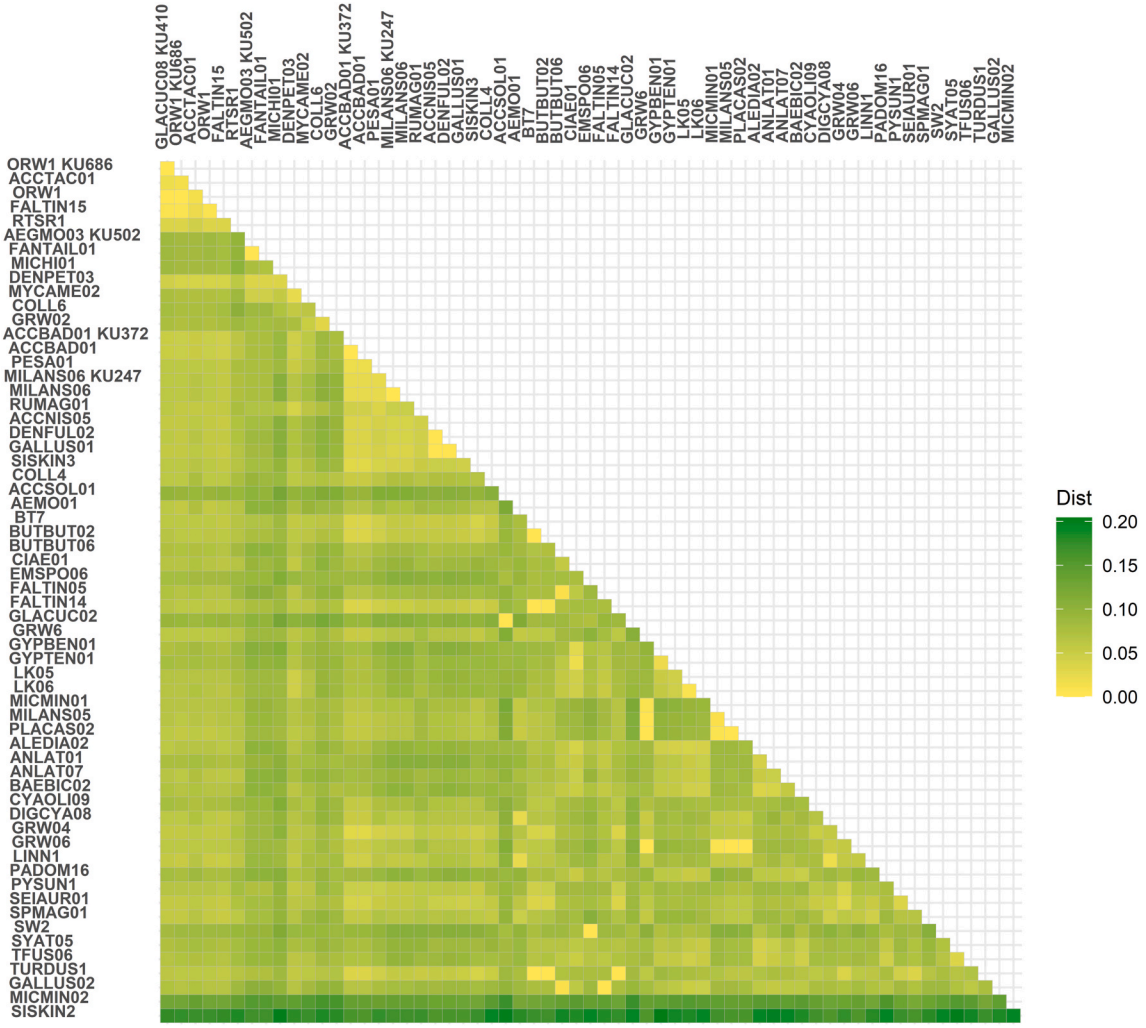

Fig. 5. Heatmap of pairwise genetic distances estimated from nucleotide sequences of the cytochrome *b* gene (479 nucleotides) of *Plasmodium* spp. using the Jukes-Canter model.

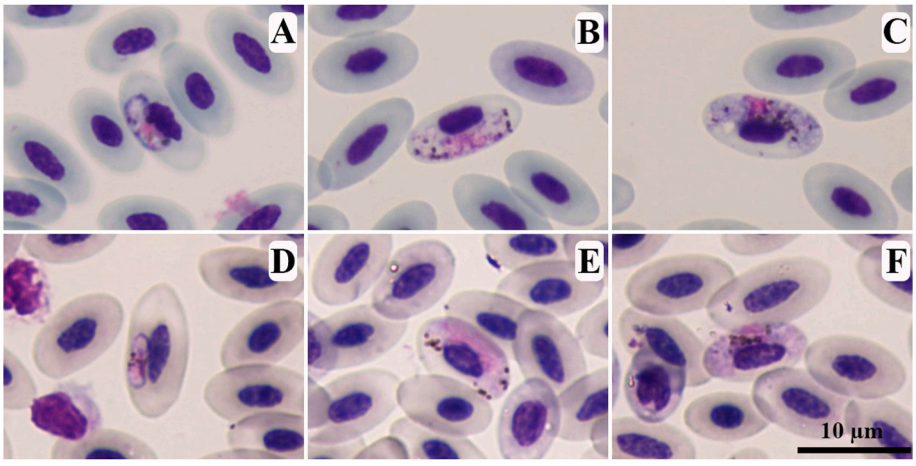

Fig. 6. *Haemoproteus* spp. infected in Blyth's hawk-eagles (*Spizaetus alboniger*), KU549 (**A-C**) and KU589 (**D-F**). Young gametocytes (**A&D**), microgametocytes (**B&E**) and macrogametocytes (**C&F**). Giemsa staining.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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number 55.61), Thailand.

References

BCST, 2021. Checklist Thai Birds 2020. The Bird Conservation Society of Thailand, Nonthaburi, Thailand.

Bensch, S., Hellgren, O., Pérez-Tris, J., 2009. MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. Mol. Ecol. Resour. 9, 1353–1358. https://doi.org/10.1111/j.1755-0998.2009.02692.x.

Bensch, S., Stjerman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proc. Roy. Soc. Lond. B 267, 1583–1589. https://doi.org/10.1098/rspb.2000.1181.

Buechley, E.R., Santangeli, A., Girardello, M., Neate-Clegg, M.H.C., Oleyar, D., McClure, C.J.W., Şekercioğlu, Ç.H., 2019. Global raptor research and conservation priorities: tropical raptors fall prey to knowledge gaps. Divers. Distrib. 25, 856–869.

Bukauskaitė, D., Ziegyte, R., Palinauskas, V., Iezhova, T.A., Dimitrov, D., Ilgunas, M., Bernotiene, R., Markovets, M.Y., Valkiunas, G., 2015. Biting midges (Culicoides, Diptera) transmit Haemoproteus parasites of owls: evidence from sporogony and molecular phylogeny. Parasites Vectors 8, 303. https://doi.org/10.1186/s13071-015-0910-6.

Chagas, C.R.F., Valkiūnas, G., Guimarães, L.O., Monteiro, E.F., Guida, F.J.V., Simões, R. F., Rodrigues, P.T., Luna, E.J.A., Kirchgatter, K., 2017. Diversity and distribution of avian malaria and related haemosporidian parasitesin captive birds from a Brazilian megalopolis. Malar. J. 16, 83. https://doi.org/10.1186/s12936-017-1729-8.

Cocumelli, C., Iurescia, M., Diaconu, E.L., Galietta, V., Raso, C., Buccella, C., Stravino, F., Grande, F., Fiorucci, L., De Liberato, C., Caprioli, A., Battisti, A., 2021. Plasmodium matutinum causing avian malaria in Lovebirds (Agapornis roseicollis) hosted in an Italian zoo. Microorganisms 9, 1356. https://doi.org/10.3390/microorganisms9071356.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

Dimitrov, D., Iezhova, T.A., Zehtindjiev, P., Bobeva, A., Ilieva, M., Kirilova, M., Bedev, K., Sjöholm, C., Valkiūnas, G., 2016. Molecular characterisation of three avian haemoproteids (Haemosporida, Haemoproteidae), with the description of Haemoproteus (Parahaemoproteus) palloris n. sp. Syst. Parasitol. 93, 431–449. https://doi.org/10.1007/s11230-016-9638-8.

Donázar, J.A., Cortés-Avizanda, A., Fargallo, J.A., Margalida, A., Moleón, M., Morales-Reyes, Z., Moreno-Opo, R., Pérez-García, J.M., Sánchez-Zapata, J.A., Zuberogoitia, I., Serrano, D., 2016. Roles of raptors in a changing world: from flagships to providers of key ecosystem services. ARDEOLA 63, 181–234.

Ferreira-Junior, F.C., de Angeli Dutra, D., Silveira, P., Pacheco, R.C., Witter, R., de Souza Ramos, D.G., Pacheco, M.A., Escalante, A.A., Braga, É.M., 2018. A new pathogen spillover from domestic to wild animals: Plasmodium juxtanucleare infects free-living passerines in Brazil. Parasitology 145, 1949–1958. https://doi:10.1017/S003118201800077X.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hellgren, O., Waldenström, J., Bensch, S., 2004. A new PCR assay for simultaneous studies of Leucocytozoon, Plasmodium, and Haemoproteus from avain blood. J. Parasitol. 90, 797–802. https://doi.org/10.1645/GE-184R1.

Inumaru, M., Murata, K., Sato, Y., 2017. Prevalence of avian haemosporidia among injured wild birds in Tokyo and environs, Japan. Int. J. Parasitol. Parasites. Wild. 6, 299–309. https://doi.org/10.1016/j.ijppaw.2017.09.007.

Ivanova, K., Zehtindjiev, P., Mariaux, J., Georgiev, B.B., 2015. Genetic diversity of avian haemosporidians in Malaysia: cytochrome b lineages of the genera Plasmodium and Haemoproteus (Haemosporida) from Selangor. Infect. Genet. Evol. 31, 33–39. https://doi.org/10.1016/j.meegid.2015.01.004.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.

LaPointe, D.A., Goff, M.L., Atkinson, C.T., 2010. Thermal constraints to the sporogonic development and altitudinal aistribution of avian malaria Plasmodium relictum in Hawai'i. J. Parasitol. 96, 318–324. https://doi.org/10.1645/GE-2290.1.

Lertwatcharasarakul, P., Salakij, C., Prasopsom, P., Kasorndorkbua, C., Jakthong, P., Santavakul, M., Suwanasaeng, P., Ploypan, R., 2021. Molecular and morphological analyses of Leucocytozoon parasites (Haemosporida: leucocytozoidae) in raptors from Thailand. Acta Parasitol. https://doi.org/10.1007/s11686-021-00403-6.

McClure, C.J.W., Westrip, J.R.S., Johnson, J.A., Schulwitz, S.E., Virani, M.Z., Davies, R., Symes, A., Wheatley, H., Thorstrom, R., Amar, A., Buij, R., Jones, V.R., Williams, N. P., Buechley, E.R., Butchart, S.H.M., 2018. State of the world's raptors: distributions, threats, and conservation recommendations. Biol. Conserv. 227, 390–402.

Nourani, L., Baghkheirati, A.A., Zargar, M., Karimi, V., Djadid, N.D., 2021. Haemoproteosis and avian malaria in columbidae and corvidaefrom Iran. Vet. Med. Sci. https://doi:10.1002/vms3.549.

Palinauskas, V., Križanauskienė, A., Iezhova, T.A., Bolshakov, C.V., Jönsson, J., Bensch, S., Valkinas, G., 2013. A new method for isolation of purified genomic DNA from haemosporidian parasites inhabiting nucleated red blood cells. Exp. Parasitol. 133, 275–280. https://doi.org/10.1016/j.exppara.2012.12.003.

Pornpanom, P., Chagas, C.R.F., Lertwatcharasarakul, P., Kasorndorkbua, C., Valkiūnas, G., Salakij, C., 2019a. Molecular prevalence and phylogenetic relationship of Haemoproteus and Plasmodium parasites of owls in Thailand: data from a rehabilitation centre. Int. J. Parasitol. Parasites. Wild. 9, 248–257. https://doi.org/10.1016/j.ijppaw.2019.06.002.

Pornpanom, P., Salakij, C., Prasopsom, P., Lertwatcharasarakul, P., Kasorndorkbua, C., Santavakul, M., 2019b. Morphological and molecular characterization of avian trypanosomes in raptors from Thailand. Parasitol. Res. 118, 2419–2429. https://doi:10.1007/s00436-019-06379-7.

R development core team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical computing, Vienna, Austria.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574. https://doi.org/10.1093/bioinformatics/btg180.

Salakij, C., Kasorndorkbua, C., Lertwatcharasarakul, P., Salakij, J., 2012a. Hematology, molecular phylogeny and ultra-structure of Trypanosoma corvi in a Shikra. Comp. Clin. Pathol. 21, 1757–1761. https://doi.org/10.1007/s00580-012-1612-5.

Salakij, C., Kasorndorkbua, C., Lertwatcharasarakul, P., Salakij, J., 2015a. Ultra-structure of blood cells and molecular characteristics of Haemoproteus sp. in Blyth's hawk eagle. Comp. Clin. Pathol. 24, 1293–1299. https://doi.org/10.1007/s00580-015-2161-5.

Salakij, C., Kasorndorkbua, C., Pornpanom, P., Salakij, J., Jakthong, P., 2019. Quantitative and qualitative characteristics of blood cells in black-shouldered, Brahminy, and black kites. Vet. Clin. Pathol. 1–12. https://doi.org/10.1111/vcp.12691.

Salakij, C., Kasorndorkbua, C., Salakij, J., Suwannasaeng, P., Jakthong, P., 2015b. Quantitative and qualitative morphologic, cytochemical and ultrastructural characteristics of blood cells in the Crested Serpent eagle and Shikra. Jpn. J. Vet. Res. 63, 95–105. https://doi.org/10.14943/jjvr.63.3.95.

Salakij, C., Pornpanom, P., Lertwatcharasarakul, P., Kasorndorkbua, C., Salakij, J., 2018. Haemoproteus in barn and collared scops owls from Thailand. J. Vet. Sci. 19, 280–289. https://doi.org/10.4142/jvs.2018.19.2.280.

Salakij, J., Lertwatcharasarakul, P., Kasorndorkbua, C., Salakij, C., 2012b. Plasmodium circumflexum in a Shikra (Accipiter badius): phylogeny and ultra-structure of the haematozoa. Jpn. J. Vet. Res. 60, 105–109.

Scaglione, F.E., Cannizzo, E.T., Chiappino, L., Sereno, A., Ripepi, M., Salamida, S., Manuali, E., Bollo, E., 2016. Plasmodium spp. in a captive raptor collection of a safaripark in northwest Italy. Res. Vet. Sci. 104, 123–125. https://doi.org/10.1016/j.rvsc.2015.12.010.

Schumm, Y.R., Bakaloudis, D., Barboutis, C., Cecere, J.G., Eraud, C., Fischer, D., Hering, J., Hillerich, K., Lormée, H., Mader, V., Masello, J.F., Metzger, B., Rocha, G., Spina, F., Quillfeldt, P., 2021. Prevalence and genetic diversity of avian haemosporidian parasites in wild bird species of the order Columbiformes. Parasitol. Res. 120, 1405–1420. https://doi.org/10.1007/s00436-021-07053-7.

Tasci, G.T., Olmez, N., N, P.A., Akca, A., Sari, B., Arslan, M.O., Mor, N., Vatansever, Z., 2018. Prevalence and molecular characterization of haemosporidians in domestic geese: a new focus of haemosporidian parasites, Kars province, Northeastern Turkey. Isr. J. Vet. Med. 73, 33–42.

Valkiūnas, G., 2005. Avian Malaria Parasites and Other Haemosporidia. CRC Press, Boca Raton.

Valkiūnas, G., Iezhova, T.A., Carlson, J.S., Sehgal, R.N.M., 2011. Two New Trypanosoma Species from African birds, with notes on the taxonomy of avian trypanosomes. J. Parasitol. 97, 924–930.

Valkiūnas, G., Iezhova, T.A., Križanauskienė, A., Palinauskas, V., Sehgal, R.N.M., Bensch, S., 2008. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. J. Parasitol. 94, 1395–1401. https://doi.org/10.1645/GE-1570.1.

Win, S.Y., Chel, H.M., Hmoon, M.M., Htun, L.L., Bawm, S., Win, M.M., Murata, S., Nonaka, N., Nakao, R., Katakura, K., 2020. Detection and molecular identification of Leucocytozoon and Plasmodium species from village chickens in different areas of Myanmar Acta. Trop 212. https://doi.org/10.1016/j.actatropica.2020.105719.

Yang, G., He, H., Zhang, G., Zhao, W., Zhou, J., Qian, Y., Huang, X., Dong, L., 2021. Neglected parasite reservoirs in wetlands: prevalence and diversity of avian haemosporidians in waterbird communities in Northeast China. Int. J. Parasitol. Parasites. Wild. 15, 177–183. https://doi.org/10.1016/j.ijppaw.2021.04.013.