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Molecular Prevalence and Haematological Assessments of Avian Malaria in Wild Raptors of Thailand

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Simple Summary: Avian malaria, caused by *Plasmodium*, is considered to be a major vector-borne disease in raptors. Most infected raptors present no clinical manifestations, which are from subclinical to various morbidity. There are occasional reports of *Plasmodium* infected in raptors in Thailand. However, the information of parasite impact on infected raptors is limited. The objectives of this study are to investigate the prevalence in wild raptor populations by using active surveillance in several localities of Thailand and evaluate the impact of avian malaria in raptors' health. The authors found that Southern Thailand has higher prevalence of avian malaria than the other country's regions. This suggested that the raptors in Southern Thailand, which has humid environment, are more vulnerable to the malarial infection. Additionally, most tested raptors could tolerate the infection. The results from this study can be used for development of strategies for management of endangered raptor species, especially in rehabilitation institutes.

Abstract: Raptors (Accipitriformes, Falconiformes and Strigiformes) are important for ecological niches as bioindicators and an apex predator; however, their global populations have continuously decreased due to human activities, habitat loss and contagious diseases. Avian malaria that may cause the negative impact on raptors' health may also contribute to the declining of raptor populations. This study reported malaria's molecular prevalence and genetic diversity in wild-caught and rehabilitated raptors in the Kasetsart University Raptor Rehabilitation Unit. In total, 109 raptors from 18 provinces of Thailand were classified into two groups, which included 78 diurnal raptors (DIRs) in Accipitriformes and 31 nocturnal raptors (NORs) in Strigiformes. Each ethylenediaminetetraacetic (EDTA) blood sample (0.5–1 mL) was tested through haematological analyses and polymerase chain reaction (PCR)-based detection to assess parasites' health impacts. Amplicons of PCR positive samples were analysed for a nucleotide sequencing and phylogenetic relationships. The overall prevalence of avian malaria was low at 3.67% (4/109) (95% CI: 1.44–9.06%), with a prevalence of 3.86% (3/78) (95% CI: 1.32–10.70%) in DIRs and 3.23% (1/31) (95% CI: 1.32–10.70%) in NORs. Most of the infected samples were from southern Thailand. This suggested that the raptors in humid habitats are more vulnerable to the malarial infection, which was likely associated with vector and parasite abundance.

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Clinical appearances and haematological examinations demonstrated that raptors could tolerate the infection and only became asymptomatic and subclinically infected. This study is the first report of the infection of avian malaria in Cinerous Vulture, Himalayan Vulture and Barred Eagle Owl in Thailand, providing baseline information in preparedness for the disease diagnostic and further study of avian malaria in some endangered raptor species.

Keywords: haematology; phylogeny; *Plasmodium*; birds of prey; KURU

1. Background

Raptors, which are taxonomically classified into the orders Accipitriformes, Falconiformes and Strigiformes, are considered ecological bioindicators. They are distributed in various types of habitats (i.e., rainforests, wetlands and urban areas) [1]. There are 73 raptor species that have been recorded in Thailand and have been legally protected. Of these 73 raptor species, 36 are resident species and 31 are non-breeding visitors [2,3]. Particularly, Himalayan Vulture, (*Gyps himalayensis*), and Cinereous Vulture, (*Aegypius monachus*), (non-breeding visitors) that are threatened by several factors, such as the decline in natural food sources, poaching for ornamental purposes and toxic contamination in their prey (using of non-steroidal anti-inflammatory drugs in livestock). Thus, their global populations tend to be decreasing [4]. In addition to anthropogenic threats, infectious diseases in tropical regions, such as haemosporidiosis, negatively affect vulnerable and endangered raptor species, thus increasing pressure on these predatorial species toward extinction [5].

Avian malaria is caused by protozoa (order: Haemosporida, genus: *Plasmodium* sp.) and is still considered a major vector-borne disease in avian species. The disease is transmitted by blood-sucking vectors, including Culicidae mosquitoes and Ceratopogonidae midges. *Plasmodium* sp. undergoes intracellular merogony to cause intravascular haemolysis, resulting in anaemia and weakness, especially in immunocompromised birds [6]. Most infected raptors present various clinical appearances, from subclinical signs to severe morbidity and eventually mortality. In addition, age might not be the only factor causing higher susceptibility to infection [7], but any kinds of stress, such as emaciation or opportunistic infection, could also worsen the severity of the disease [8].

An introduction of the disease via migratory avian species or activities of humans invading the territory of non-adaptive hosts could substantially increase its virulence, as reported in native Hawaiian bird species (i.e., Maui Parrotbill, (*Pseudonestor xanthophores*), Akikiki, (*Oreomystis bairdi*), and Akekee, (*Loxops caeruleirostris*)), resulting in the vanishing of these endemic species due to their first exposure to the disease [9]. These occurrences suggest that the virulence of parasites depends on host–parasite interactions, including co-evolutions, host susceptibility and the current immune status of the hosts [10,11].

Approximately 55 species of *Plasmodium* sp. have been described in birds. The main species associated with Accipitriformes are *Plasmodium circumflexum*, *P. elongatum*, *P. fallax*, *P. lophurae*, *P. relictum*, *P. vaughani* and *P. polare*, and those associated with Strigiformes are *P. subpraecox*, *P. fallax*, *P. gundersi*, *P. hexamerium* and *P. elongatum* [6,12,13]. These parasite species have been reported to infect a wide range of avian species. Thus, raptors or other immunocompetent hosts may transmit the disease as carriers to other susceptible avian populations [14]. Additionally, the abundance of vectors promoted by environmental factors (temperature and larval vector habitat) is considered a risk for the persistence of the diversity of parasitic species [15].

Currently, polymerase chain reaction (PCR)-based detection is more effective than microscopic examination of the blood for the diagnosis of haemosporidian infections. In addition, genetic sequencing could reveal parasites at the lineage level [16]. Although there are some reports of *Plasmodium* infected in Thai raptors admitted to the Kasetsart University Raptor Rehabilitation Unit [17,18], investigation in the wild population is lacking. Additionally, there is no information of the disease's impact on Thai raptors

in the wild. Thus, this study aimed to investigate the prevalence in wild raptor populations by using active surveillance in several localities of Thailand, as well as to evaluate the health impact of malarial infection and to determine the genetic diversity of *Plasmodium* spp. We hypothesized that habitat quality will influence on parasite infection. We predicted that Southern Thailand with grater habitat diversity have higher prevalence of avian malaria than the other regions in Thailand. Additionally, the infected raptor may show the specific haemogram. Thus, the results from this study may be valuable information for the development of management strategies for raptors, some of which are endangered species.

2. Methods

2.1. Sample Collection

From February 2020 to May 2022, 0.5–1 mL of EDTA blood was immediately collected via jugular venipuncture from diurnal (Accipitriformes) raptors (DIRs) and nocturnal (Strigiformes) raptors (NORs) that were either injured or orphan individuals admitted to the Kasetsart University Raptor Rehabilitation Unit (KURU) in Nakhon Pathom, Thailand (14°1′ N, 99°58′ E) for rehabilitation and eventual release. In the case of vultures, blood samples were collected from the medial metatarsal vein. These blood samples collected at KURU were designated as KU, and those being wild-caught, free-ranging raptors were designated as R. Free-ranging raptors were caught by mist-netting or Bal-chatri's trap [19]. In total, 109 individuals belonging to 24 raptor species/taxa were classified and composed of 78 DIRs and 31 NORs (Table 1). Of these 109 birds, 56 birds were admitted to the KURU (KU samples) and the other 53 birds were captured from the wild (R samples).

Table 1. Prevalence of *Plasmodium* spp. in raptors of Thailand (February 2020–May 2022), and haematological analyses in raptors and their reference values in parentheses ( ); the data are represented as the mean ± SD.

Species [a]	No.	Prevalence (%)	Haematological Analyses [b]		
			PCV (%)	RBCs ($\times 10^{12}$/L)	WBCs ($\times 10^{9}$/L)
Diurnal raptors (Accipitriformes)	78	3.85% (3/78)			
Shikra (*Accipiter badius*)	6	14.3% (1/7)	40.7 ± 3.6 (38.0 ± 0.0) [c]	4.41 ± 0.4 (2.86 ± 0.35) [c]	3.53 ± 2.0 (17.3 ± 4.2) [c]
Crested Goshawk (*Accipiter trivirgatus*)	1	0	45	3.15	4.18
Cinereous Vulture (*Aegypius monachus*)	1	100% (1/1)	(43.0 ± 3) [c]	(2.39 ± 0.17) [c]	(19.26 ± 3.09) [c]
Steppe Eagle (*Aquila nipalensis*)	1	0	34	2.51	5.43
Booted Eagle (*Hieraaetus pennatus*)	1	0	34	2.25	5.35
Black Baza (*Aviceda leuphotes*)	2	0	38.5 ± 3.84	2.0 ± 0.65	2.5 ± 2.81
Grey-faced Buzzard (*Butastur indicus*)	2	0	42.5 ± 0.41	2.72 ± 0.23	13.5 ± 4.89
Rufous-winged Buzzard (*Butastur liventer*)	2	0	34 ± 1.41	2.71 ± 0.13	2.03 ± 0.12
Himalayan Buzzard (*Buteo refectus*)	1	0	43	2.55	3.85
Pied Harrier (*Circus melanoleucos*)	24	0	41.1 ± 3.31	3.02 ± 0.61	6.64 ± 4.58
Peregrine Falcon (*Falco peregrinus*)	2	0	41.5 ± 0.71	3.30 ± 0.21	4.48 ± 0.25
Himalayan Vulture (*Gyps himalayensis*)	2	50% (1/2)	28 (39.1 ± 3.3) [d]	2.24 (3.4 ± 1.0) [d]	7.5 (11.1 ± 4.3) [d]
Brahminy Kite (*Haliastur indus*)	1	0	42	1.84	7.8
Black Kite (*Milvus migrans govinda*)	13	0	38.1 ± 5.12	5.48 ± 0.86	2.81 ± 1.92
Black-eared Kite (*Milvus migrans lineatus*)	13	0	35.8 ± 3.61	2.06 ± 0.68	4.10 ± 1.32
Crested Honey Buzzard (*Pernis ptilorhynchus orientalis*)	2	0	38 ± 2.83	2.21 ± 0.13	2.40 ± 0.07
Red-headed Vulture (*Sarcogyps calvus*)	2	0	46.5 ± 2.12	NT	NT
Crested Serpent Eagle (*Spilornis cheela burmanicus*)	1	0	45	3.93	4.25
Nocturnal raptors (Strigiformes)	31	3.23% (1/31)			
Spotted Owlet (*Athene brama*)	2	0	41 ± 1.41	2.71 ± 0.06	5.5 ± 1.41

Table 1. *Cont.*

Species [a]	No.	Prevalence (%)	Haematological Analyses [b]		
			PCV (%)	RBCs ($\times 10^{12}$/L)	WBCs ($\times 10^{9}$/L)
Barred Eagle Owl (*Ketupa sumatrana*)	3	33.3% (1/3)	38.0 ± 1.0 (39 ± 5.6) [e]	2.17 ± 0.49 (3.3 ± 0.4) [e]	3.01 ± 2.16 (9.7 ± 2.6) [e]
Buffy Fish Owl (*Ketupa ketupu*)	1	0	34	1.87	4.05
Collared Scops Owl (*Otus lettia*)	6	0	39.2 ± 4.61	2.72 ± 0.90	4.787 ± 3.74
Brown Wood Owl (*Strix leptogrammica*)	1	0	38.5	1.63	2.85
Eastern Barn Owl (*Tyto javanica*)	18	0	39.6 ± 4.48	2.68 ± 0.87	4.50 ± 3.54
Total	**109**	3.67% (4/109)			

Abbreviations: PCV = packed cell volume; RBCs = red blood cell counts; WBCs = white blood cell counts. NT = not tested due to inadequate samples. [a] Species' names are presented according to the IOC World Bird List [20]. [b] Haematologic values are calculated from non-parasitized raptors. [c] Reference values for each haematological parameter are presented in parentheses as the mean ± SD [21,22]. [d] Haematologic reference values calculated from 10 healthy Himalayan Vultures at KURU, Thailand (unpublished). [e] Haematologic reference values calculated from 3 healthy Barred Eagle Owls at KURU, Thailand (unpublished).

These raptors were obtained from 18 provinces of Thailand (Figure 1 and Table 2) covering 6 regions, with 65 individuals from the Central region, in which most of the samples were obtained from Nakhon Pathom and Nakhon Nayok provinces (n = 28 and 24 individuals, respectively), 26 individuals from the Northern region, in which most of the samples were from Chiang Rai province (n = 24), and 9 individuals from Nakhon Ratchasima province in the Northeastern region. In the Eastern (Prachin Buri), Western (Kanchanaburi, Phetchaburi, and Ratchaburi) and Southern (Ranong, Surat Thani and Yala) regions, blood samples were obtained from fewer than five individuals from each region.

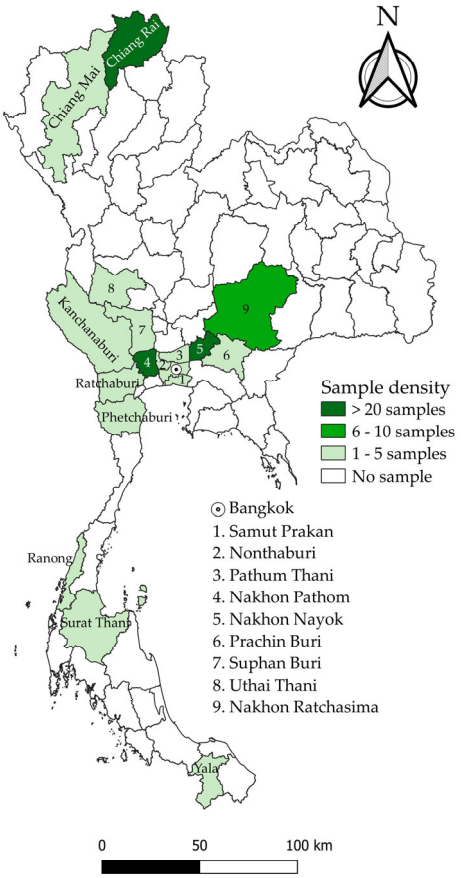

Figure 1. The localities of raptors included in this study comprised 18 provinces. Raptors were obtained by either being caught in the wild or admitted to KURU.

Table 2. Regional prevalence of *Plasmodium* sp. in raptors of Thailand (February 2020–May 2022).

Locality	Species	No.	Prevalence [a] (%)	Isolate	Lineage [b]	GenBank
Northern region		26	0			
Chiang Mai (*n* = 2)	Himalayan Buzzard (*Buteo refectus*)	1 [c]				
	Buffy Fish Owl (*Ketupa ketupu*)	1 [c]				
Chiang Rai (*n* = 24)	Pied Harrier (*Circus melanoleucos*)	24 [d]				
Northeastern region		9	0			
Nakhon Ratchasima (*n* = 9)	Crested Serpent Eagle (*Spilornis cheela burmanicus*)	1 [c]				
	Shikra (*Accipiter badius*)	4 [c]				
	Eastern Barn Owl (*Tyto javanica*)	2 [c]				
	Crested Honey Buzzard (*Pernis ptilorhynchus orientalis*)	2 [c]				
Eastern region		1	0			
Prachin Buri (*n* = 1)	Himalayan Vulture (*Gyps himalayensis*)	1 [c]				
Central region		65	1.56% (1/65)			
Bangkok (*n* = 3)	Black Baza (*Aviceda leuphotes*)	1 [c]				
	Peregrine Falcon (*Falco peregrinus*)	1 [c]				
	Black Kite (*Milvus migrans govinda*)	1 [c]				
Nonthaburi (*n* = 2)	Eastern Barn Owl (*Tyto javanica*)	2 [c]				
Nakhon Nayok (*n* = 24)	Black Kite (*Milvus migrans govinda*)	11 [d]				
	Black-eared Kite (*Milvus migrans lineatus*)	13 [d]				
Nakhon Pathom (*n* = 28)	Shikra (*Accipiter badius*)	3	3.57% (1/28)	R191	MILANS06	OR066213
	Crested Goshawk (*Accipiter trivirgatus*)	1 [c]				
	Steppe Eagle (*Aquila nipalensis*)	1 [c]				
	Spotted Owlet (*Athene brama*)	2 [c]				
	Black Baza (*Aviceda leuphotes*)	1 [d]				
	Grey-faced Buzzard (*Butastur indicus*)	2 [d]				
	Rufous-winged Buzzard (*Butastur liventer*)	2 [d]				
	Peregrine Falcon (*Falco peregrinus*)	1 [c]				
	Black Kite (*Milvus migrans govinda*)	1 [c]				
	Collared Scops Owl (*Otus lettia*)	2 [c]				
	Eastern Barn Owl (*Tyto javanica*)	12 [c]				
Pathum Thani (*n* = 1)	Brahminy Kite (*Haliastur indus*)	1 [c]				
Samut Prakan (*n* = 1)	Collared Scops Owl (*Otus lettia*)	1 [c]				
Suphan Buri (*n* = 4)	Collared Scops Owl (*Otus lettia*)	1 [c]				
	Eastern Barn Owl (*Tyto javanica*)	2 [c]				
	Barred Eagle Owl (*Ketupa sumatrana*)	1 [c]				
Uthai Thani (*n* = 2)	Red-headed vulture (*Sarcogyps calvus*)	2 [c]				
Western region		3	0			

Table 2. *Cont.*

Locality	Species	No.	Prevalence [a] (%)	Isolate	Lineage [b]	GenBank
Ratchaburi (*n* = 1)	Collared Scops Owl (*Otus lettia*)	1 [c]				
Kanchanaburi (*n* = 1)	Collared Scops Owl (*Otus lettia*)	1 [c]				
Phetchaburi (*n* = 1)	Booted eagle (*Hieraaetus pennatus*)	1 [c]				
Southern region		5	60% (3/5)			
Yala (*n* = 3)	Barred Eagle Owl (*Ketupa sumatrana*)	2 [c]	33.3% (1/3)	KU729	ORW1	OR066210
	Brown Wood Owl (*Strix leptogrammica*)	1 [c]				
Surat Thani (*n* = 1)	Cinereous Vulture (*Aegypius monachus*)	1 [c]	100% (1/1)	KU851	MILANS05	OR066211
Ranong (*n* = 1)	Himalayan Vulture (*Gyps himalayensis*)	1 [c]	100% (1/1)	KU852	ORW1	OR066212
Total		109	3.67% (4/109)			

[a] The prevalence of *Plasmodium* sp. in each region and province is shown as a percentage with its proportion in parentheses. [b] Lineage nomenclature provided by the MalAvi database [16], [c] KU samples, [d] R samples.

2.2. Complete Blood Count (CBC) and Microscopic Examination

Five µL of each EDTA blood sample was mixed with 995 µL of Natt and Herrick's solution to obtain the total red blood cell count (RBCs) and white blood cell count (WBCs). Packed cell volume (PCV) was determined by using a microhaematocrit centrifuge at 12,000× *g* for 3 min. Two blood smears from each sample were prepared from EDTA blood and stained with Wright's stain before performing microscopic examinations. The parasitaemia level and parasite identification were simultaneously performed by screening at least 10,000 red blood cells [23]. Then, the rest of each blood sample was stored at 4 °C until DNA extraction.

2.3. DNA Extraction, PCR Amplification and DNA Sequencing

Total DNA was extracted from 20 µL of EDTA blood by using a Blood Genomic DNA Extraction Mini Kit (FavorPrep; Pingtung, Taiwan). Nested PCR was performed according to the procedure [24], with the first reaction using HaemNF1 (5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) primers. The amplicon from the first reaction was then used as a template in the second reaction with HaemF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (5′-GCATTATCTGGATGTGATAATGGT-3′) primers to amplify the 478 base pairs (excluded primers) of the cytochrome b (*cyt b*) gene.

All PCR reactions were prepared with a total of 20 µL, which included 2 µL of DNA template, 10 µL of DreamTaq Green PCR Mastermix (2X) (ThermoScientific; Waltham, MA, USA), 0.2 µL of each primer at a concentration of 10 µM and 7.6 µL of water. Positive and non-template controls were prepared for all reactions. PCR amplification of both reactions was initiated by using pre-heated denaturation at 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. Then, the final extension ended at 72 °C for 5 min. Amplicons were electrophoresed on a 1.5% agarose gel at 100 V for 20 min to detect specific bands of the parasite under ultraviolet light.

Malaria-positive amplicons were subjected to DNA sequencing by using Celemics' BT Sequence (Geumcheon-gu, Seoul, Korea). All sequences were screened, and the quality of each sequence was checked by using the BioEdit program [25].

2.4. Sequence Analysis and Phylogenetics

The consensus length of our sequences generated by BT sequencing (Geumcheon-gu, Seoul, Korea) was 472 bp. Thus, we did not identify lineage following the MalAvi nomencla-

ture [16]. However, these four sequences (*Plasmodium* sp. KU729, KU851, KU852 and R191) were included for Bayesian phylogenetic analysis. Bayesian phylogeny was constructed by using *Plasmodium* sp. sequences from the current study and other *Plasmodium* sp. and *Haemoproteus* sp. sequences obtained from the MalAvi database [16]. The *Leucocytozoon* sp. lineage SISKIN2 (GenBank accession no. AY393796) was used as the tree root. The consensus length of the sequence was 479 bp, some missing data (gap position) in each sequence were coded as "*N*". MrBayes version 3.2.3 was used to construct a phylogenetic tree. The best-fit model was a general time-reversible model (GTR), selected based on hierarchical likelihood ratio test (hLRT). Markov chain Monte Carlo (MCMC) was run for three million generations, with sampling every 100 generations. The first 25% of tree were discarded as a "*burn-in*" step. The genetic distance between the different lineages was observed by using the Jukes–Canter substitution model (all substitutions were weighted equally) [26].

2.5. Statistical Analyses

Descriptive statistics (mean, standard deviation (SD)) were used to describe the complete blood count (CBC) parameters in healthy and infected raptors in each species/taxon and then compared to their references. The prevalence and 95% confidence interval of avian malaria in each region (Northern, Northestern, Eastern, Central, Western and Southern) were calculated by the function bionom.approx in the R program [27].

3. Results

3.1. Molecular Prevalence and Disease Distribution

The prevalence of avian malaria (*Plasmodium* spp.) was determined based on PCR analysis with *cyt b*-specific primers. In total, 4 of the 109 raptors were positive, with an overall prevalence of 3.67% (95% CI: 1.44–9.06%) (Table 1). Regarding the groups of raptors, three of the 78 DIRs (*Accipitriformes*) were infected, resulting in a prevalence of 3.86% (95% CI: 1.32–10.70%); this included isolates from a Shikra (R191), a Cinereous Vulture (KU851) and a Himalayan Vulture (KU852). In addition, 1 of the 31 NORs (*Strigiformes*) was infected—a Barred Eagle Owl (KU729)—resulting in a prevalence of 3.23% (95% CI: 1.32–10.70%). Thus, there was no significant difference in the prevalence between the two groups of raptors ($p > 0.05$).

Regarding the origins of the positive samples, 75% (3/4) of the infected raptors were from different provinces of the southern region (Table 2) including a Cinereous Vulture from Surat Thani (9° 8′ 24″ N, 99° 19′ 48″ E), a Himalayan Vulture from Ranong (9° 58′ 12″ N, 98° 37′ 48″ E) and a Barred Eagle Owl from Yala (6° 33′ 0″ N, 101° 17′ 24″ E). The infected Shikra was from Nakhon Pathom (13° 55′ 0″ N, 100° 7′ 0″ E) in the central region. It was noted that the southern region had a high regional prevalence at 60%.

3.2. Phylogenetic Analyses

Bayesian phylogeny (Figure 2) revealed that *Plasmodium* sp. KU729 isolated from the Barred Eagle Owl (OR066210) was grouped together with *Plasmodium* sp. KU852 isolated from Himalayan Vulture (OR066212), with 100% similarity. Plasmodium sp. KU851 isolated from Cinereous Vulture (OR066211) was grouped together with *Plasmodium elongatum* lineage GRW06 isolated from Great Reed Warbler (*Acrocephalus arundinaceus*) (DQ368381) and *P. elongatum* lineage ERIRUB01 isolated from European robin (*Erithacus rubecula*) (KT282462); the percentages of similarity were 99.28% and 99.64%, respectively. *Plasmodium* sp. R191 isolated from Shikra (OR066213) was grouped together with *Plasmodium* isolated from Thai raptors: *Plasmodium* sp. lineage GLACUC06 isolated from Asian-barred Owlet (*Glaucidium cuculoides*, MK390825), *Plasmodium* sp. lineage NISCU2 isolated from Brown Hawk Owl (*Ninox scutulata*, MK390833) and *Plasmodium* sp. lineage ACCBAD01 isolated from Shikra (JN639001), with the percentages of similarity ranging from 97.45% to 98.19%. However, this clade contained one *Plasmodium* sp. isolated red junglefowl (*Plasmodium gallinaceum* lineage GALLUS01 (AY099029)).

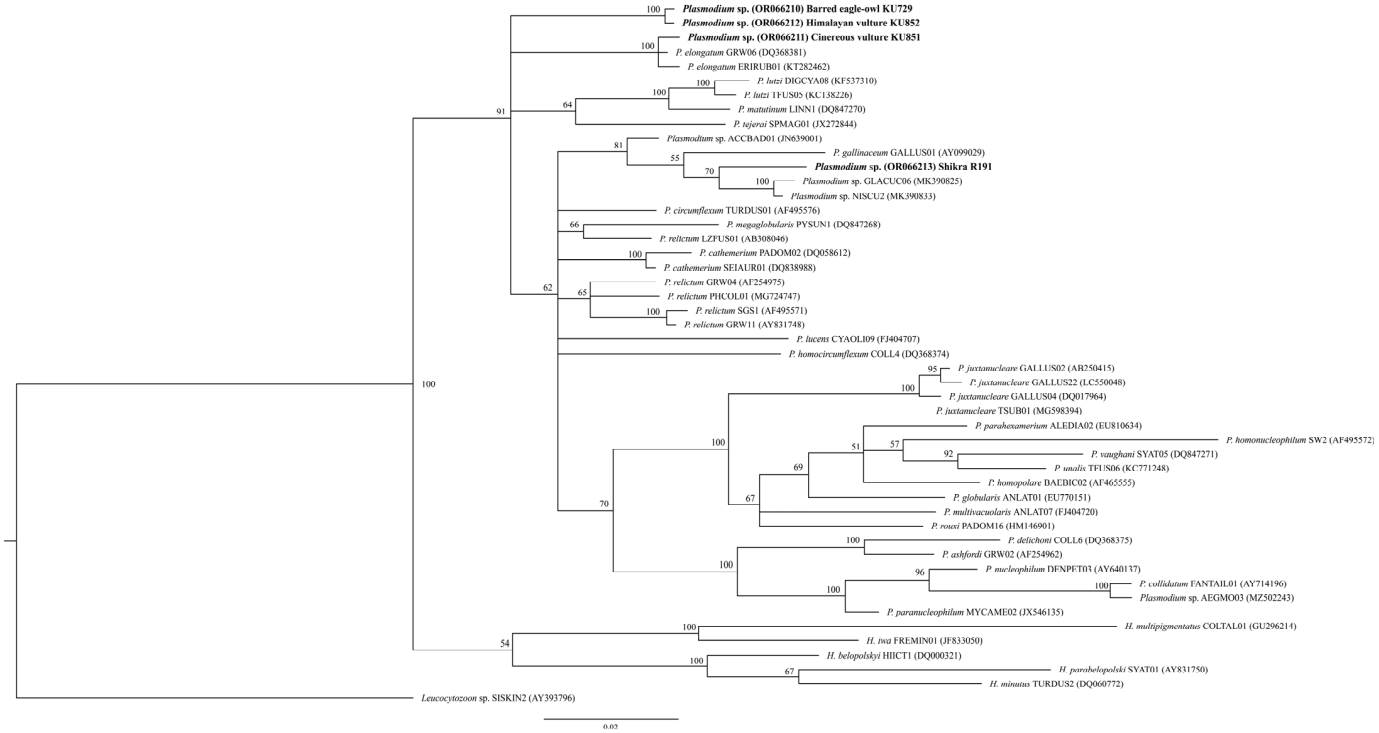

Figure 2. Bayesian phylogeny based on the partial cytochrome b (*cyt b*) gene (479 bp) of *Plasmodium* sequences. The four *Plasmodium* sp. sequences isolated from the current study are highlighted in bold, presented with their scientific names, and shown with the isolates provided by KURU. MalAvi lineage names and all GenBank accession numbers in parentheses are given after the species' names. Node values indicate percentages of posterior probabilities. The *Leucocytozoon* sp. lineage SISKIN2 was set as the tree root.

3.3. Haematological and Microscopic Examinations

Overall, the haematological parameters in infected raptors were not greatly different from the reference range. Pack cell volume (PCV), the major screening parameter for determining of anaemia, in infected Barred Eagle Owl, Cinereous Vulture, Himalayan Vulture and Shikra were 39%, 45%, 37% and 40%, respectively. These values were in the normal range. Red blood cell counts (RBCs) should be associated with the PCV. In this study, only the infected Cinereous Vulture and Barred Eagle Owl had RBCs values lower than the reference range (1.1×10^{12}/L and 2.75×10^{12}/L), whereas RBCs values in the infected Himalayan Vulture and Shikra were within the reference range (2.59×10^{12}/L and 3.85×10^{12}/L).

Regarding the white blood cell counts (WBCs), which indicate either immune status or inflammatory process, we found that WBCs values in infected Barred Eagle Owl (6.9×10^{9}/L) and Shikra (4.15×10^{9}/L) were lower than the reference range (Table 1), but that in infected Cinereous Vulture (21.9×10^{9}/L) and Himalayan Vulture (7.7×10^{9}/L) were within the range (Table 1).

Additionally, this study calculated the haematologic values from other non-parasitized birds (Table 1). We highlighted that these were the birds with sample size larger than 10 (which can be used as the reference range), including Pied Harrier, Black Kite, Black-eared Kite and Eastern Barn Owl. Furthermore, one Himalayan Vulture (KU852) showed low PCV (28%) without the infection or evidence of iron deficiency.

Microscopically, Wright's-stained blood smears from the Cinereous Vulture (KU851) and Himalayan Vulture (KU852) revealed intracellular and extracellular gametocytes of *Plasmodium* sp. The parasitemia was low, with less than five gametocytes per 10,000 red blood cells. However, the gametocytes were not found in the blood smears from the Barred Eagle Owl (KU729) and Shikra (R191), although the PCR results were positive.

4. Discussion

In the current study, two air-dried blood smears from each sample were prepared by using Wright's staining. Extracellular gametocytes were found in the blood smears from both the Cinereous and the Himalayan Vulture. According to a previous report, EDTA might interfere the gametocyte development [17]. Additionally, another anticoagulant (sodium citrate) together with air exposure was also induce the exflagellation of *Haemoproteus Tartakovskyi* [28]. Thus, using anticoagulant for investigation of haemosporidian parasites is not recommended. However, EDTA blood smear was important for routine haematological examination. It can provide the information of blood cell morphology that might reveal underlying pathologic conditions [29]. Furthermore, this study found one Himalayan Vulture with a low PCV and the blood examinations revealed information for excluding of any blood parasite infection and iron deficiency.

This study presented the prevalence and genetic diversity of *Plasmodium* spp. in raptors obtained from wild populations based on PCR analysis. The overall prevalence in DIRs was as low as the prevalence previously reported [18]. The prevalence in NORs in this study was also low. However, a previous study demonstrated a higher prevalence in NORs, which was related to the biology of the infected birds [17]. Most NORs (e.g., Barred Eagle Owl, Collared Scops Owl and Brown Hawk Owl) may live in woodlands or dark hollows of a tree and be active during nightfall, thus increasing their likelihood of exposure to vectors. In addition, this could indicate that the epidemiology of malaria in DIRs has been constant at a low level, which may be due to their biology of actively hunting during the daytime [18]. Furthermore, the defensive behaviour of birds (foot stomping, head and wing movements and tail shaking) [30] that prevent mosquito bites might be related to prevalence differences in DIRs and NORs.

The prevalence of *Plasmodium* in southern Thailand was high. Southern Thailand is mostly covered with evergreen rainforest; the year-round climate is humid with heavy rainfalls and the optimal temperature is beneficial for the abundance of vectors and parasites [31]. Additionally, wild-caught raptors including Pied Harrier, and sympatric Black Kite and Black-eared Kite (n = 24, 11, 13, respectively; Table 2.) did not have any malaria infection reflecting that lowland Northern and Central Thailand, which predominates with rice fields, grasslands, public parks and urban areas, could sustain a low occurrence of disease compared to southern Thailand [32]. It may be inferred that the locality promotes the persistence of this disease in such an ecosystem. Further study should investigate more samples in these regions that comprise various types of habitats to further quantify disease outbreaks in the wild.

The Himalayan Vulture (KU852), a non-breeding/winter visitor, and the Barred Eagle Owl (KU729), a sedentary forest species, were obtained from different provinces—Ranong and Yala, respectively—in Southern Thailand. The phylogenetic analyses revealed that *Plasmodium* infected in both Himalayan Vulture and Eagle Owl were identical to each other. This shared lineage in both migratory and sedentary raptors suggested that the infection could have occurred as a local infection in the region where vectors and parasites are abundant [31], thus increasing the frequency of being bitten by the vectors in those areas. There is likely no host specificity in this *Plasmodium* sp., so any species of raptors—both DIRs and NORs—can be susceptible to malarial infection. Since the environment in Southern Thailand may promote the abundance of parasites and vectors, future studies should investigate haemosporidian parasites in Southern Thailand.

The Cinereous Vulture (KU851), a non-breeding/winter visitor, which was also obtained from Surat Thani province in Southern Thailand, was infected with *Plasmodium* sp. that closely related to *P. elongatum* lineage GRW06 (DQ368381) and ERIRUB01 (KT282462), as revealed by the Bayesian phylogenetic analysis. These two lineages were isolated from the Great Reed Warbler and European Robin, respectively. The Great Reed Warbler lives in temperate regions (Eastern Asia/Eurasia) that are sympatric to the Cinereous Vulture [1,4]. This suggests that the infection in this vulture may occur at its natal site or during its migration [11]. Moreover, it can be assumed that either parasite-driven selection or an immune

mechanism works against the severity of chronic infection in such vulture species [33,34]. This would also suggest that migratory species play a potential role in the global distribution of the parasite species, resulting in a shared diversity of parasites with other avian species during migration [13].

Shikra (R191), which can be classified as either a resident or non-breeding visitor or the so-called partial migrant [35], was infected with the *Plasmodium* sp. that closely related with *Plasmodium* sp. isolated from Thai raptors, lineage GLACUC06 (MK390825), NISCU2 (MK390833) and ACCBAD01 (JN639001). Additionally, this *Plasmodium* sp. is related to the *P. gallinaceum* lineage GALLUS01 (AY099029) isolated from Red Junglefowl (*Gallus gallus*). It might be an undescribed *Plasmodium*, which has the genetic characteristics closely similar to the *P. gallinaceum*. However, further investigations using combined microscopic and molecular techniques were needed.

Although *Plasmodium* sp. is considered a haemoparasite that is pathogenic in avian hosts, unlike other haemosporidians [6], all infected raptors in the current study remained asymptomatic. Haematological assessments revealed that the infected raptors did not present an anaemic state (Table 1). The severity of the infection could likely be related to the intensity of parasitaemia level within the host [5], so haematological examination is important when evaluating the clinical impact of the disease [36]. Raptors may have co-evolved with the parasites, so they have developed certain mechanisms of their immune system that reduce the lethal severity of the disease or even eliminate the parasite [14]. Some migratory raptors that have certain illnesses, such as stress or emaciation, could become immunocompromised and susceptible to infection, resulting in a clinically severe disease [8].

5. Conclusion

The molecular prevalence of avian malaria in raptors discovered in Thailand was notably low, and the genetic diversity was polyphyletic. Haematological assessments revealed its low pathogenicity, which may be associated with the species of the parasite. Southern Thailand, with its humid rainforest ecological niche, had the highest prevalence and diversity of *Plasmodium* spp. Further investigation is required to determine the epidemiology of haemosporidian parasites in the region and to identify risk factors for such infections in raptors with different habitats. Additionally, further research should investigate to understand whether or not *Plasmodium* is a contributing factor in the decline of endangered vultures, especially the migratory species, and other endangered raptors in general. This study provides valuable insights into the prevalence and genetic diversity of *Plasmodium* spp. in raptors in Thailand and offers crucial information for effective disease management strategies for endangered species, such as the development of routine health assessment protocol in the rehabilitation process and the standardized laboratory analysis.

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