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PREVALENCE AND PHYLOGENETIC ANALYSIS OF AVIAN HAEMOSPORIDIA IN WILD BIRDS IN THE REPUBLIC OF KOREA

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ABSTRACT: Avian blood parasites, including *Plasmodium* spp. and *Haemoproteus* spp., are found worldwide but only limited information of their occurrence is available in the Republic of Korea (ROK). We determined the prevalence of *Plasmodium* and *Haemoproteus* and their phylogenetic characteristics in wild birds in ROK. Blood samples were collected from 118 wild birds of 27 species in the Chonbuk Province, ROK. While 43 (36%) were positive for avian haemosporidia on microscopic examination of blood smears, 53 (45%) were positive by PCR targeting the cytochrome *b* gene. By direct sequencing of PCR amplicons, 47 (89%) were identified as *Haemoproteus* spp. and 6 (11%) as *Plasmodium* spp. Phylogenetic analysis using the cytochrome *b* gene revealed that resident and migrant birds have very similar genetic lineages of both parasites in ROK, suggesting the possibility that migrant birds may act as a mediator for the parasite among Asian countries.

Key words: Avian haemosporidia, *Haemoproteus*, phylogenetic analysis *Plasmodium*, wild bird.

INTRODUCTION

Avian haemosporidia, including *Plasmodium* and *Haemoproteus* species, is common worldwide and is transmitted by biting arthropods of the genera *Culex*, *Aedes*, *Hippobosca*, and *Culicoides* (Valkiūnas 2004; Ishtiaq et al. 2007; Tanigawa et al. 2013). Migratory birds and a climate suitable for vector populations are key factors of transmission and distribution of many vector-borne diseases, including avian malaria (Rogers and Randolph 2006; Ishtiaq et al. 2007). Prevalence and level of infection in a geographic area are closely related to correlations among hosts, vectors, and parasites (Knowles et al. 2011). The genera *Plasmodium* and *Haemoproteus* have been well studied, and it is known that species of *Haemoproteus* exhibit greater host specificity than do species of *Plasmodium* (Beadell et al. 2004).

Clinical signs of avian haemosporidia include anemia, emaciation, loss of appetite, difficulty in breathing, lameness, reduced reproductivity, and death; there may also be no evident clinical signs (Atkinson et al. 2000; Merino et al. 2000; Knowles et al. 2010). Pathologic gross lesions may show enlargement of the liver, spleen, and kidneys (Valkiūnas 2004). Species of *Haemoproteus* are believed to be less pathogenic than *Plasmodium* (Campbell 2015). If birds survive from the infection, they are usually chronically infected or exposed several times to haemosporidia, and most of them do not exhibit any clinical signs (Valkiūnas 2004; Palinauskas et al. 2008). Studies have shown high mortality in acute infections or in native wild or captive birds through accidental introductions of avian malaria (Atkinson et al. 2000; Donovan et al. 2008; Cellier-Holzem et al. 2010).

The Korea peninsula is located in northeast Asia and is central to mainland China, Russia, and Japan. The country has seas bordering three sides and many mountains, rivers, and wetlands, so many migratory birds (around 1.58 million birds) visit Korea annually for breeding, overwintering, or to rest (Lee et al. 2000). It has a temperate climate although the temperature difference between seasons is significant, ranging from –10 to 30 C. Around 390 species of resident and migrating birds live in Korea and, in summer and winter, 87% of them are migratory (Lee et al. 2000; Ishtiaq et al. 2007). A recent study in Japan indicates a high prevalence of avian haemosporidian parasites in winter migrating birds, suggesting

their role in moving the infection between countries (Inumaru et al. 2017). Although many migratory birds come to the Republic of Korea (ROK) from southern areas where vector insects live year-round, there are only two reports of wild birds that were infected by avian haemosporidia in ROK (Ishtiaq et al. 2007; Jang et al. 2017).

Because avian haemosporidia have been identified frequently during blood examination of wild birds in Chonbuk province, this study aimed to determine the prevalence of avian haemosporidia and to perform phylogenetic analyses in order to determine the infection status and the relationship between migratory and resident birds in Chonbuk Province.

MATERIALS AND METHODS

Case selection

From January to December 2016, blood samples were collected from rescued wild birds at the Chonbuk Wildlife Center, ROK (35°56′32″N, 126°57′37″E). A total of 493 birds were rescued; however 38.1% (188/493) birds were dead on arrival to the center or were euthanized after a basic physical examination because of substantial and irreparable injuries. In addition, 38.3% (189/493) of birds were nestlings or too small for collecting blood, so a total of 118 birds from 27 species was included in this study. We obtained the following information: species, suspected age, reason for distress, and results of physical and radiographic examinations.

Laboratory examination of blood samples

Blood samples were collected from the jugular or cutaneous ulnar vein using a 21- or 24-ga needle. The collected blood was transferred to ethylenediaminetetraacetic acid (EDTA)- and heparin-treated tubes. A manual complete blood count was performed to determine red blood cell (RBC) and white blood cell (WBC) counts, differential count of WBC, packed cell volume, and hemoglobin concentration. Wintrobe's indexes (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were calculated based on the measured values of RBC count, packed cell volume, and hemoglobin concentration. Clinical chemistry profiles were analyzed using a VetScan Avian/Reptilian Profile Plus (Abaxis, Union City, California, USA) analyzer.

Peripheral blood smears were prepared immediately after blood collection. Slides were air-dried and fixed in methanol followed by Diff-Quik (Sysmex, Kobe, Japan) or Wright-Giemsa (MUTO, Tokyo, Japan) staining. On every slide, more than 100 fields and 50,000 erythrocytes were observed with 1,000× magnification (Godfrey et al. 1987). Parasitemia and its severity were confirmed and graded (1–4) by microscopic examination (Campbell 2015). After laboratory examination, the remaining blood samples were stored in a freezer at –27 C for subsequent molecular diagnosis.

Polymerase chain reaction

Genomic DNA was extracted from the EDTA-treated whole blood (20 μL) using a Qiagen DNA mini kit® (Valencia, California, USA) following the manufacturer's guidelines. For the detection of avian haemosporidia and the phylogenetic analyses, four universal primer pairs targeting common sequences of mitochondrial cytochrome *b* gene of *Plasmodium* and *Haemoproteus* species were used (Beadell et al. 2004; Ishtiaq et al. 2007; Table 1). The first reaction was run using the Universal 1 primer pair (3760F/4292Rw2). If the fragment did not amplify, follow-up runs were attempted with smaller fragments, using primers Universal 2, 3, and 4, in order (Beadell et al. 2004; Ishtiaq et al. 2007).

The PCRs were performed in volumes of 20 μL with AccuPower® GoldHotStart Taq PCR PreMix (Bioneer, Daejeon, Republic of Korea) containing GoldHotstart Taq DNA polymerase (1 U), dNTP mixtures (250 μM each), a reaction buffer with 1.5 mM $MgCl_2$ (a stabilizer), and a tracking dye. The concentration of each primer was 10 pmol/μL. The reaction profile consisted of 5 min of denaturation at 95 C followed by 40 cycles each of 30 s at 95 C, 30 s at 51 C, and 30 s at 72 C. A final extension was run 3 min at 72 C, and all reactions were performed in an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, California, USA). Every reaction was run twice with a positive control. All amplified products were visualized on 1.8% agarose tris-acetate-EDTA gels using Redsafe® (INtRON, Sungnam, Republic of Korea) under ultraviolet light. The PCR products were sent to a commercial laboratory (Cosmogenetech, Seoul, Republic of Korea) and sequenced bidirectionally using an ABI 3730XL (Applied Biosystems) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After sequencing the amplicons, the sequences were merged and then homology of the deduced nucleotide sequences for the genus *Plasmodium* or *Haemoproteus* was analyzed with the GenBank database BLAST (National Center for Biotechnology Information

TABLE 1. Information on the primer pairs used to amplify the common sequences of genera *Plasmodium* and *Haemoproteus* for the detection of avian haemosporidia schizonts in wild birds of the Republic of Korea and the phylogenetic analyses used.

Primers	Sequence (5′→3′)	Product size (base pairs)
3760F	GAG TGG ATG GTG TTT TAG AT	533
4292Rw2	TGG AAC AAT ATG TAR AGG AGT	
F1	CAAT ATT TAC CTT TAT CAT GGA T	433
4292Rw2	TGG AAC AAT ATG TAR AGG AGT	
F3	CCA GGA CTT GTT TCA TGG AT	295
4292Rw2	TGG AAC AAT ATG TAR AGG AGT	
3760F	GAG TGG ATG GTG TTT TAG AT	256
R1	CTT TTT AAG GTT GGG TGA CTT	

2016). Clinical isolates of *Plasmodium* or *Haemoproteus* species, confirmed by PCR using species-specific primers (Beadell 2004), were used as the positive controls.

Phylogenetic analysis

Phylogenetic analyses of identified sequences of *Plasmodium* and *Haemoproteus* species were performed for the identification of species from these genera and for evaluating the genetic relationship with the sequences that were reported from other countries. All PCR-positive samples that were cytochrome *b* sequences with 214–533 base pairs were evaluated. Two sequences of *Plasmodium* spp. were used as an outgroup. For genus *Plasmodium*, 25 sequences were included and for genus *Haemoproteus* 28 sequences were included.

Sequences of the genera *Plasmodium* or *Haemoproteus* identified in this study were aligned using CLUSTAL X (Han et al. 2009), with previously reported sequences that were collected from the National Center for Biotechnology Information Nucleotide database. Neighbor-joining phylogenetic analyses with 1,000 bootstrap values were performed for the sequence data with MEGA v7 software (Kumar et al. 2016).

RESULTS

Prevalence of genera *Plasmodium* and *Haemoproteus*

On microscopic examination of the blood smears, 36% (43/118) were identified as positive for avian haemosporidia. Several stages of the *Plasmodium* or *Haemoproteus* protozoa were seen in RBCs (Fig. 1). Some trophozoites and pigments of *Plasmodium* species were seen in WBCs and platelets. Heterophils from 82% (97/118) of the birds with avian haemosporidia showed toxic changes (grades 2–3), and their monocytes and lymphocytes had reactive forms. The grade of toxic change was similar in mild to severe infections.

The PCR method with four sets of primers revealed that 45% (53/118) from 18 species were positive for the genera *Plasmodium* or *Haemoproteus*. Analysis by PCR detected 10 more cases than those detected on blood smears. Using direct sequencing and sequence comparison with a BLAST search, 89% (47/53) infections were confirmed as genus *Haemoproteus* and 11% (6/53) were identified as genus *Plasmodium*. Prevalence of the genus *Plasmodium* was 5% (6/118), and that of the genus *Haemoproteus* was 40% (47/118).

Plasmodium infection was found in Eurasian Magpie (*Pica pica*), Oriental Turtledove (*Streptopelia orientalis*), Brown Hawk Owl (*Ninox scutulata*), and Common Kestrel (*Falco tinnunculus*). *Haemoproteus* infection was found in Eurasian Magpie, Chinese Oriole (*Oriolus chinensis*), Common Pheasant (*Phasianus colchicus*), Yellow Bittern (*Ixobrychus sinensis*), Common Buzzard (*Buteo buteo*), Eurasian Hobby (*Falco subbuteo*), Scops Owl (*Otus scops*), Brown Hawk Owl, Eurasian Eagle Owl (*Bubo bubo*), Intermediate Egret (*Egretta intermedia*), Domestic Pigeon (*Columba livia* var. *domestica*), North-

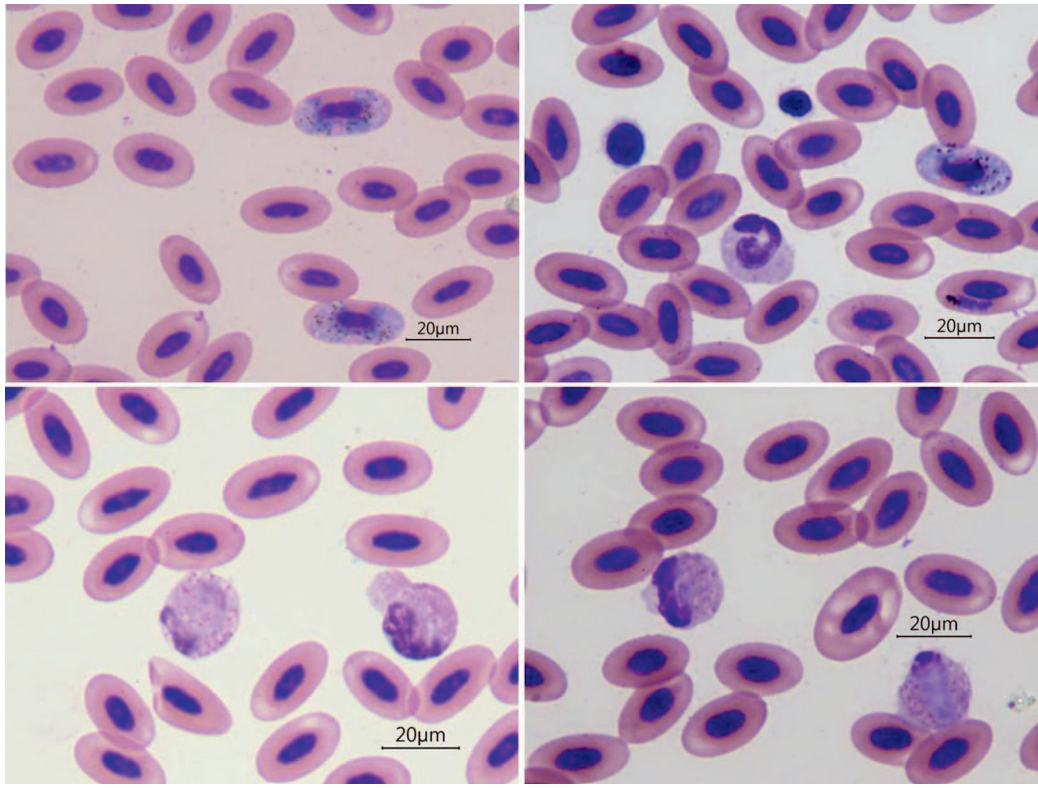

FIGURE 1. Red blood cells and white blood cells of wild birds of the Republic of Korea that were infected by (upper left) *Plasmodium* or (upper right) *Haemoproteus* species on microscopic examination of the blood smears. Several stages of the *Plasmodium* or *Haemoproteus* protozoa are seen in red blood cells. Some trophozoites and pigments of *Plasmodium* species are seen in white blood cells and platelets. Note that the heterophils show (lower left) clumping of the granules and (lower right) cytoplasmic vacuolation, suggesting toxic changes.

ern Goshawk (*Accipiter gentilis*), Oriental Dollarbird (*Eurystomus orientalis*), Black-crowned Night Heron (*Nycticorax nycticorax*), Scaly Thrush (*Zoothera dauma*), Common Kestrel, and Indian Spot-billed Duck (*Anas poecilorhyncha*).

To assess the bird species that were sensitive to haemosporidian parasite infection, we screened the 27 species that had more than five individual birds that were infected (Table 2). Species with more than four individuals infected included Scops Owl (7/9) and Brown Hawk Owl (19/23). The Eurasian Magpie sample consisted of only three individuals, but all were infected. The prevalence of haemosporidia among the 10 resident species, 13 summer migrant species, and five winter migrant species were compared. A total of 33% (15/45) resident species, 63% (34/54) summer migrant species, and 21% (4/19) winter migrant species were infected.

Laboratory examination

Laboratory examination was performed in 47 out of 53 birds infected with haemosporidian parasites because insufficient samples were available from the rest of the birds. Anemia in the complete blood count was observed in only 10 birds. While one of the anemic birds had *Plasmodium* infection, four showed *Haemoproteus* infection. Not all of the birds had the causative diseases, or conditions such as massive internal or external bleeding for anemia, regardless of the type of the parasite.

TABLE 2. Information and number of parasite-positive birds sampled to assess the prevalence of haemosporidia schizonts in wild birds of the Republic of Korea.

Birds sampled				Migration status[a]	No. sampled	No. infected (PCR)	
Order	Family	Scientific name	Common name			*Plasmodium*	*Haemoproteus*
Accipitriformes	Accipitridae	*Accipiter gentilis*	Northern Goshawk	WV/PM	1		1
		Aegypius monachus	Cinereous Vulture	WV	1		
		Buteo buteo	Common Buzzard	WV/PM	6		2
Anseriformes	Anatidae	*Anas poecilorhyncha*	Indian Spot-billed Duck	WV/R	10		1
		Cygnus cygnus	Whooper Swan	WV	1		
Charadriiformes	Laridae	*Larus crassirostris*	Black-tailed Gull	R	1		
Columbiformes	Columbidae	*Columba livia* var. *domestica*	Domestic Pigeon	R	8		1
		Streptopelia orientalis	Oriental Turtle Dove	R	3	1	
Coraciiformes	Coraciidae	*Eurystomus orientalis*	Oriental Dollarbird	SV	1		1
Falconiformes	Falconidae	*Falco subbuteo*	Eurasian Hobby	SV	2		2
		Falco tinnunculus	Common Kestrel	R	15	1	3
Galliformes	Phasianidae	*Phasianus colchicus*	Common Pheasant	R	2		2
Passeriformes	Corvidae	*Cyanopica cyanus*	Azure-winged Magpie	R	1		
		Garrulus glandarius	Eurasian Jay	R	1		
		Pica pica	Eurasian Magpie	R	3	1	2
	Oriolidae	*Oriolus chinensis*	Chinese Oriole	SV	1		1
	Turdidae	*Zoothera dauma*	Scaly Thrush	SV/R	1		1
Pelecaniformes	Ardeidae	*Ardea cinerea*	Grey Heron	SV/R	4		
		Bubulcus ibis	Eastern Cattle Egret	SV	3		
		Egretta alba modesta	Great Egret	SV/R	1		
		Egretta garzetta	Little Egret	SV/R	2		
		Egretta intermedia	Intermediate Egret	SV/R	1		1
		Ixobrychus sinensis	Yellow Bittern	SV	1		1
		Nycticorax nycticorax	Black-crowned Night Heron	SV/R	5		1
Strigiformes	Strigidae	*Bubo bubo*	Eurasian Eagle Owl	R	11		4
		Ninox scutulata	Brown Hawk-owl	SV/PM	23	3	16
		Otus scops	Scops Owl	SV/PM	9		7
Total					118	6	47

[a] WV = winter visitants; PM = passage migrants; R = residents; SV = summer visitants.

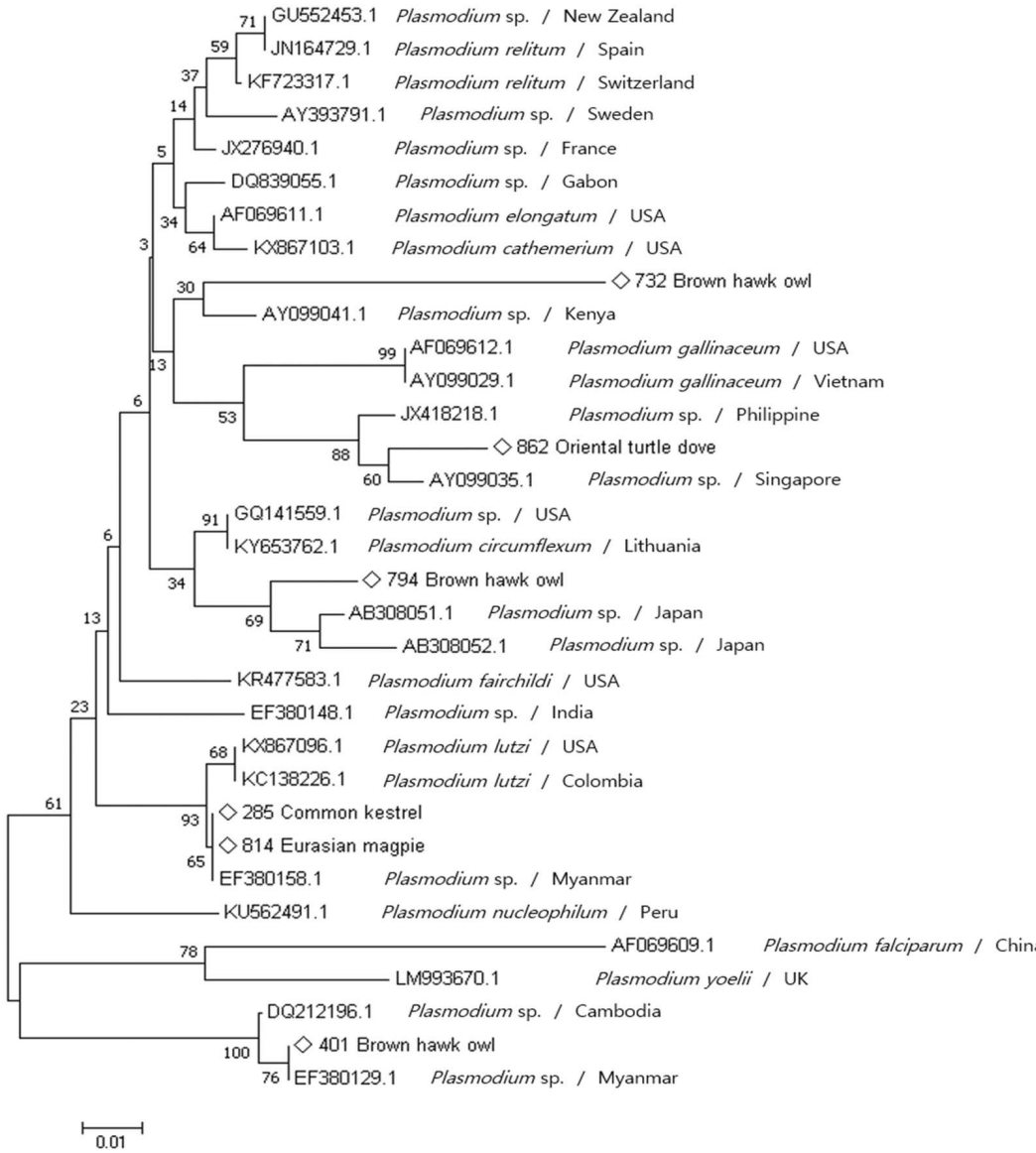

FIGURE 2. Phylogenetic analysis of genus *Plasmodium* in wild birds of the Republic of Korea. Sequences identified in this study (six individuals) are marked with diamonds. Previously reported sequences of *Plasmodium* spp. (n=25) from wild birds were included and the sequences from human (AF069609.1) and mammalian (LM993670.1) were used as an outgroup. Neighbor-joining phylogenetic analysis was performed by MEGA v7. Numbers on branches indicate bootstrap values based on 1,000 replicates.

Phylogenetic analysis

All identified cytochrome *b* gene sequences (n=53) in this study were included in the phylogenetic analyses. In the phylogenetic tree for *Plasmodium* spp., various genotypes were identified in ROK (Fig. 2). Three sequences detected in the Brown Hawk Owl, a summer migratory bird, showed three lineages; one was identical to the sequence from Brown Hawk Owls from Myanmar (case no. 401), and the other two were most similar to the sequences from Brown Hawk Owls from Kenya (732) or Japan (794), suggesting that this migratory bird may carry various

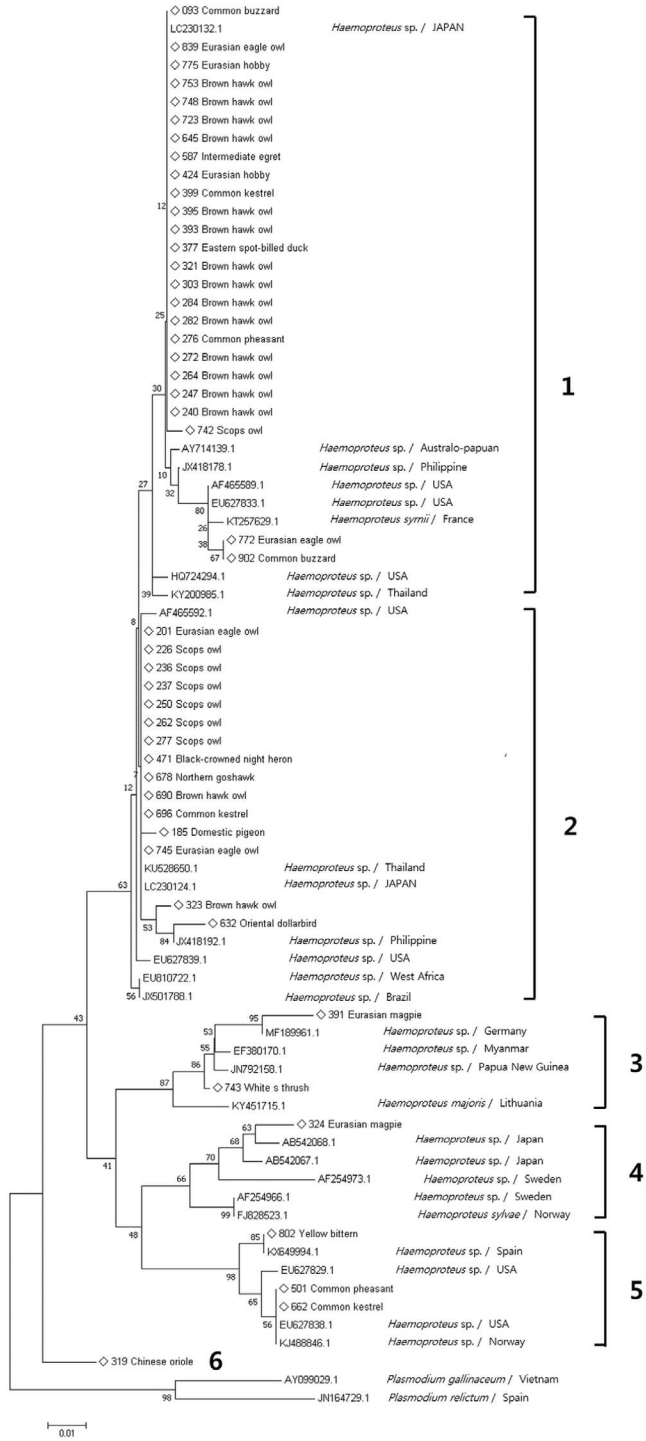

FIGURE 3. Phylogenetic analysis of genus *Haemoproteus* in wild birds of the Republic of Korea. Sequences identified in this study (47 individuals) are marked with diamonds. Previously reported sequences of *Haemoproteus* spp. (n=28) from wild birds were included and two *Plasmodium* species (AY099029.1 and JN164729.1) were used as an outgroup. Neighbor-joining phylogenetic analysis was performed by MEGA v7. Numbers on branches indicate bootstrap values based on 1,000 replicates.

Plasmodium spp. to ROK. Sequences from Oriental Turtledove, Common Kestrel, and Eurasian Magpie, which are resident birds in Korea, belonged to two different clades in the tree: one is similar to the sequence from Singapore (862) and the other two are similar to those from Myanmar (285 and 814).

In the phylogenetic tree for *Haemoproteus* species, 47 sequences belonged to six different clades, regardless of the natural habitats of the host (Fig. 3). Of the six clades, the largest included 23 sequences (one Common Buzzard [93], one Eurasian Eagle Owl [839], two Eurasian Hobby [775 and 424], 14 Brown Hawk Owl [753, 748, 723, 645, 395, 393, 321, 303, 282, 264, 240, 247, 272, and 284], one Common Kestrel [399], one Intermediate Egret [587], one Eastern Spot-billed Duck [377], and one Common Pheasant [276]) and was identical to the sequence of *Haemoproteus* spp. found in Japan (LC230132.1). A shared lineage with *Haemoproteus* spp. from Thailand (KU528650.1) and Japan (LC230124.1) was also found in 12 birds: two Eurasian Eagle Owls (201 and 745), six Scops Owls (226, 236, 237, 250, 262, and 277), one Black-crowned Night Heron (471), one Northern Goshawk (678), one Brown Hawk Owl (690), and one Common Kestrel (696). The sequence from one Eurasian Eagle Owl (772) lineage was same as one from a Common Buzzard (902). A *Haemoproteus* species from Spain (KX649994.1) was identical to that from a Yellow Bittern (802). One Common Pheasant (501) and one Common Kestrel (662) had the same sequences of *Haemoproteus* spp. as have been found in the US and Norway (EU627838.1, KJ488859.1).

DISCUSSION

Several migratory birds pass through Korea and could transmit various pathogens from one location to another. This process, however, has not been well studied for infectious diseases except avian influenza. Haemosporidia infections, including avian malaria, have been observed in considerable numbers of wild birds in the Chonbuk Wildlife Center, especially in summer migrants (63%). Summer migratory birds come to Korea from April to June for breeding and leave for regions in lower latitudes for wintering when autumn comes, around September and October. The most typical summer migrants that come to Korea include Scops Owl, Brown Hawk Owl, and Oriental Dollarbird; residents include Eurasian Magpie, Eurasian Eagle Owl, and Common Kestrel. One third (33%) of the resident birds and 21% of the winter migratory birds were infected. Although there are many birds whose blood samples were not collected, the prevalence of avian haemosporidia was found to be more than 40% in the sample studied. The prevalence of avian malaria in the sample was 5%, but it would have been higher if the young and vulnerable birds that died earlier were included. This survey was done with rescued birds from a wildlife center and more than 70% of the rescued birds were excluded, so a further study including other regions and rescue centers is necessary to investigate an accurate prevalence in Korea.

Among investigated species that were represented by more than five individuals, species that had more than four individuals infected with both genera, *Plasmodium* and *Haemoproteus*, were Scops Owl, Brown Hawk Owl, Eurasian Eagle Owl, and Common Kestrel. All Eurasian Magpie (n=3) and Eurasian Hobby (n=2) were infected. Of the four species that are common raptors of Korea (families Strigidae and Falconidae), the Scops Owl and Brown Hawk Owl are summer migrants and the Eurasian Eagle Owl and Common Kestrel are residents. In summer, the two migrants breed in China, the Russian Far East, Japan, Taiwan, and Korea and in winter in the Malaysia and Indonesia regions.

Haemoproteus only infects erythrocytes but *Plasmodium* can penetrate leukocytes or platelet as well (Campbell 2015). However, if only erythrocytes are infected, pigmented granules and the shape and location of the nuclei are diagnostic features of *Plasmodium* (Campbell 2015). Multiple schizonts inside and outside of the erythrocytes, and pigmented granules, were observed on blood smears,

but it is difficult to identify the two genera, *Plasmodium* and *Haemoproteus*, especially in chronic or mild infections. In addition, microscopic examination of blood smears is affected by the level of infection and the skill of the investigator (Waldenström et al. 2004; Smith et al. 2015). The use of PCR and genetic sequencing has made various studies possible, including the ability to determine precise identifications and prevalence and to quickly perform phylogenetic analyses with large numbers of samples (Bensch et al. 2000; Ricklefs and Fallon 2002; Hellgren et al. 2007). However, molecular diagnostics can generate false-negative results due to the failure of DNA extraction or to an insufficient concentration of DNA in the sample (Richard et al. 2002). For this reason, we performed PCR and sequence analysis to differentiate between species from the two genera.

In 10 birds, examination of blood smears failed to detect the parasitemia. Regardless of the observation of infected blood cells on the blood smears, 82% of the infected birds showed a toxic change in heterophils. This could have been caused by trauma or inflammation in the injured birds, but avian haemosporidia could also be responsible. Haemosporidia were observed to be present in 43 of the birds using both the blood smear and PCR methods. Many reports have revealed that the PCR is much-more sensitive than the blood smear examination, especially in cases of low parasitemia (Valkiūnas et al. 2008). However, as mixed infections and failure of PCR are also possible, it is beneficial to use both methods for detection (Jarvi et al. 2002; Valkiūnas et al. 2006).

To evaluate the genetic relationship of the parasites between domestic resident birds and migratory birds in Chonbuk province, ROK, we compared identified sequences of avian haemosporidia from this study with sequences from known species using phylogenetic analysis. As in earlier reports (Waldenström et al. 2002), cross-infection between wild birds, including transmission from migrants to residents, were observed. Multiple lineages were identical to those which have been reported in other countries. One migrant had a sequence lineage identical to one from Myanmar and two residents shared another lineage identical to one from Myanmar. Therefore, parasites from resident birds could have been transmitted by migrants in Korea. One sequence from an Oriental Turtledove was most similar to that of a *Plasmodium* sp. from Singapore, and they were both in same clade as *Plasmodium gallinaceum* from Vietnam and the US. *Plasmodium gallinaceum* is a pathogenic agent in poultry, so it could negatively affect domestic poultry.

One lineage of *Haemoproteus* spp. was shared by 23 birds, suggesting active transmission during the warm seasons in Korea. Like *Plasmodium* spp., various *Haemoproteus* spp. were observed. Host shifts by blood-sucking insects were revealed among many birds in Korea. In Japan, avian haemosporidial DNA in mosquitoes was detected by PCR (Tanigawa et al. 2013). More studies for avian haemosporidia infection in arthropods of Korea, and accompanying phylogenetic analyses, are needed.

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