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RESEARCH ARTICLE

Molecular Detection of Two New *Haemoproteus* Mitochondrial Cytb Lineages in Ciconiform and Charadriiform Migratory Birds in Turkey

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A B S T R A C T

Although some molecular epidemiological surveys on avian haemosporidian protozoa have been conducted in Turkey, it is of great importance to increase the number of studies on the subject in this country, which has important bird migration routes between Europe and Africa. Hatay province, which is the intersection point of many bird migration routes in the intercontinental crossing, is a very suitable geographical location for the investigation of avian haemosporidian protozoa in a wide variety of bird species that use it as a route. Therefore, this study was planned to survey *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp. in migratory birds sampled from this province. The animal material of the study consisted of *Ciconia ciconia* (Ciconiformes, n:45), *Ichthyaetus melanocephalus* (Charadriiformes, n:2), *Pelecanus onocrotalus* (Pelecaniformes, n:5) and *Pelecanus crispus* (Pelecaniformes, n:1). Microscopic examinations revealed *Haemoproteus* spp. gametocytes (5.66%) in one white stork and two Mediterranean gulls. On the other hand, nested PCR assay targeting the mitochondrial cytochrome b (Cytb) gene revealed the presence of *Plasmodium*/*Haemoproteus* spp. in 11.3% (6/53) of migratory birds. *Leucocytozoon* spp. was not detected by either microscopy or PCR. PCR-positive products were sequenced bi-directionally, and the GenBank and MalAvi databases were used for phylogenetic analysis and lineage identification of the isolates. This study revealed H-MYCAME08 lineage (OR227579, OR227580, OR227581, OR227582) in *Ciconia ciconia* and H-LARCRA01 [*H.* (*Parahaemoproteus*) *larae*] lineage (OR227577, OR227578) in *Ichthyaetus melanocephalus*, respectively. There are no studies investigating the haemosporidian parasites of these birds in Turkey, and studies on haemosporidian parasites of these species in different countries are also limited. Although the *Haemoproteus* MYCAME08 Cytb lineage has been demonstrated in ciconiform birds (Ciconidae) and the LARCRA01 lineage in charadriiform birds (Laridae), this study is unique in terms of reporting these lineages in different species in the same families. Inspired by this, we envisage that the expanding host range of Cytb lineages of haemosporidian protozoa is an indication that there is still a long way to go with avian haemosporidian protozoa.

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INTRODUCTION

Migratory birds that make long-distance flights each year encounter various arthropod vectors and infectious agents including parasites harbored by them in multiple ecosystems (Emmenegger *et al*., 2018; Ciloglu *et al*., 2020; Subedi *et al*., 2024), while resident bird species interact with parasites and their vectors only in the

ecosystem where they reside (Møller and Erriyzøe, 1998). Migrating birds interact more with parasitic agents than resident birds, which leads to an increase in the parasite burden they carry (Hub´alek, 2004). Birds infected with haemosporidian parasites migrating between continents transmit these parasites to new hosts through vectors in the regions they visit, causing an increase in the prevalence of haemosporidian parasites and thus diseases caused by them (Aghayan, 2012; Gutierrez-Lopez *et al*., 2015; Ramey *et al*., 2015; Ramey *et al*., 2016; Hahn *et al*., 2018).

Situated in the Western Palearctic region with a subtropical climate, Turkey hosts various birds migrating to Europe, Africa, and Asia due to its diverse ecosystems and appropriate wetlands (Magnin *et al*., 2000; Turan and Arıkan, 2011; Inci *et al*., 2016). Hatay province, where migratory bird activity is high, has a critical location where migrating birds arrive to the country in warmer seasons and depart in colder seasons (Turan and Arıkan, 2011). White storks (*Ciconia ciconia*), a common Palearctic bird species and an indicator species of an ecologically balanced environment, are one of the most common migratory birds frequently seen in the region (Valkiūnas *et al*., 2016). Among the other bird species, the Dalmatian pelican and Great white pelican are generally seen in the summer months and during migration periods while the Mediterranean gull is observed in wetlands and seashores, and rarely in inland areas (Atahan *et al*., 2008).

White storks are long-distance migratory birds in most parts of Europe, northwest Africa, and southwest Asia. Turkey is thought to have one of the largest populations of white storks in Europe (Girisgin *et al*., 2017). The Northern European population of white storks wintering in Africa spends most of the year in Africa, where they are exposed to many parasitic infections. It remains unclear whether these bird species are infected with haemoparasites on their African breeding grounds and whether they transfer these agents to their European breeding grounds (Valkiūnas *et al*., 2016). Although several parasite species, including cestodes, trematodes, nematodes, and protozoa, have been reported in free-living white storks in Europe (Höfle *et al*., 2003; Cabez´on *et al*., 2011; Sitko and Heneberg, 2015; Michalczyk *et al*., 2020; Meister *et al*., 2022), information on hemosporidian infections in these birds is very limited, except for a few studies in some countries (Valkiūnas *et al*., 2016; Meister *et al*., 2023). Although microscopic (Özmen *et al*., 2009; Balkaya *et al*., 2016) and molecular (Marzal and Albayrak, 2012; Ciloglu *et al*., 2016; Zerek *et al*., 2023a; Zerek *et al*., 2023b) investigations concerning the occurrence of avian haemosporidian protozoa in wild birds other than pigeons have been conducted in Turkey, no study has been performed to investigate haemosporidian parasites of white storks.

This study aimed to investigate the prevalence of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites in some migratory birds from the Hatay province, Turkey by morphological and molecular techniques and give a substantial contribution to the relevant literature by determining the lineages of the detected haemosporidian parasites.

MATERIALS AND METHODS

Study area and migratory birds: The sampling was undertaken in the southernmost province of Turkey, Hatay province (36°23'40.6"N 36°23'22.7"E), which borders Syria and is located on one of the most critical migratory bird routes worldwide. Hatay Mustafa Kemal University, Wildlife Rescue and Rehabilitation Centre provides emergency care, rehabilitation, treatment, and intensive care services for migratory birds that are sick or injured due to various causes such as trauma and firearms. The center supports the protection and conservation of wildlife through the provision of care for injured and sick migratory birds. In total, 53 migrating birds from 4 different bird species belonging to 3 different orders and families brought to this center were included in the study. Most of the material was collected from white storks (*Ciconia ciconia*, Ciconiiformes, Ciconiidae, n: 45) and a smaller portion was collected from Great white pelicans (*Pelecanus onocrotalus*, Pelecaniformes, Pelecanidae, n:5), Dalmatian pelican (*Pelecanus crispus*, Pelecaniformes, Pelecanidae, n:1) and Mediterranean gulls (*Ichthyaetus melanocephalus*, Charadriiformes, Laridae, n:2). The species identification of sampled birds was performed by using the relevant pieces of literature (Porter *et al*., 2009; Svensson *et al*., 2010; Kiziroglu, 2013).

Blood material: Fifty-three blood samples were obtained from migratory birds belonging to 4 distinct species in the orders Ciconiiformes (n:45), Pelecaniformes (n:6), and Charadriiformes (n:2) (Table 1). Blood samples were taken from the underwing veins of each migratory bird, and 0,5-2 mL of blood was drawn into an ethylenediaminetetraacetic acid (EDTA) containing tube from each bird. After sampling, the blood materials were taken to the parasitology laboratory for subsequent examinations.

Microscopy: After the thin blood smear preparation from each blood sample, the smears were stained in 10% Giemsa, and the gametocytes of avian haemosporidia were investigated under Olympus CX-31 (Tokyo, Japan) light microscope equipped with a digital camera with an imaging software (cellSens standard v.1.13, Olympus, Tokyo, Japan) by using relevant kinds of literature (Valkiūnas, 2005; Valkiūnas and Iezhova, 2018; 2022). A minimum of one hundred microscopy fields were screened under x400 or x1000 magnification for 30-45 minutes. The parasitemia level was determined following the criteria described by Godfrey *et al*. (1987). For the isolation of genomic DNA, the rest of the blood materials were kept at -20°C.

Genomic DNA isolation: From each blood sample of the migrating bird, genomic DNA was extracted by using a commercial extraction kit (High Pure PCR Template Preparation Kit, Roche, Germany) under the manufacturer's instructions. The eluted DNA concentration was quantified by using Colibri, Titertek Berthold nanodrop spectrophotometer. The isolated genomic DNA samples were kept at -20ºC until molecular analysis.

PCR assay and sequencing: Nested PCR and sequencing were used for the definitive diagnosis of avian haemosporidian protozoa in birds. In this study, a nested PCR procedure targeting the amplification of mitochondrial cytochrome b gene fragment of *Haemoproteus, Plasmodium,* and *Leucocytozoon* spp. was employed. Following the previously described PCR protocols (Bensch *et al*., 2000; Hellgren *et al*., 2004), a mitochondrial cytochrome b gene fragment of avian haemosporidia (478 bp) was amplified to detect parasites. As the starting primers for the initial PCR, HaemNF1 (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') were used to amplify a Cytb gene fragment (617 bp). HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') were utilized as the second PCR primers for *Haemoproteus* and *Plasmodium*; HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') were as the primers of the second PCR for *Leucocytozoon* (Hellgren *et al*., 2004). Each PCR amplification was conducted in a final volume of 20 µL, consisting of 12.5 µL ultrapure water (PCR grade), 4 µL master mix (5 x FIREPol®, Solis BioDyne), 0.5 µL each of forward and reverse primers (10 pmol), and 2.5 µl template DNA (100 ng/µL). 2.5 µL of the first PCR amplicon was used as template DNA in the second PCR reaction mixture. Except for the number of cycles, all PCR amplification steps were the same. Thermocycling conditions were established as follows; 95°C for 3 minutes (initial denaturation), 20 cycles for the first PCR, and 35 cycles for the second PCR (94°C for 30 s, 50°C for 30 s, 72°C for 45 s), and 72°C for 10 minutes. Amplicons were then subjected to electrophoresis using a 1.5% agarose gel in TAE buffer. The gel was stained with ethidium bromide after electrophoresis. It was then visualized in UVP High-Performance UV transilluminator. As positive and negative controls, sequence-confirmed DNA from our previous study (Zerek *et al*., 2023a) and UltrapureTM Distilled Water (Invitrogen, DNAse/RNAse-Free) were utilized, respectively. PCR amplicon concentrations found to be adequate (min. 20 ng/µl) with an evident NanoDrop curve were forwarded to a commercial company for sequence analysis.

Phylogenetic analysis: The resulting forward and reverse sequence chromatograms were assembled by using the Geneious version 7.1.3 (Bomatters, "http://www.geneious.comww") (Kearse *et al*., 2012). DNA sequences were deposited in the Genbank (OR227577, OR227578, OR227579, OR227580, OR227581 and OR227582) and MalAvi databases (H-PARV-5, H-PARV-8, H-PARV-16, H-PARV-17, H-PARV-23 and H-PARV-48). Cytb gene phylogenetic analyses were conducted including avian haemosporidian lineages from MalAvi databases (http://mbio-serv2.mbioekol.lu.se/Malavi/, accessed on August 2023) and National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on August 2023) (Zhang *et al*., 2000). The sequences were aligned with Geneious version 7.1.3 using the ClustalW method (Larkin *et al*., 2007). As recommended by multi-gene analysis, *Leucocytozoon* was included as an out-group (Borner *et al*., 2016; Galen *et al*., 2018). Bayesian inference (BI) and maximum likelihood (ML) analyses using MrBayes 3.2.2 and RAxML 7.2.8 respectively were used to infer phylogenetic relationships (Huelsenbeck and Ronquist, 2001; Stamatakis, 2014). To identify the best evolutionary model for the ML method, JModelTest v.2.1.10 was used (Darriba *et al*., 2012). On the basis of the Akaike information criterion (AIC), the transition model with a discrete gamma distribution (TVM+G) was preferred (Posada, 2003). Phylogenetic analysis was performed using PhyML, bootstrapped using 1000 replicates (>50%) (Guindon and Gascuel, 2003). MrBayes, implemented from the CIPRES computational resource, was used for the BI analysis (Miller *et al*., 2010). Two Markov chains were simultaneously run for 5 million generations that were sampled every 1000 generations, with the first 1250 trees (25%) discarded as a burn-in step, and the consensus trees were estimated using the remaining trees. Bayesian Posterior Probabilities cut-off was considered >50%. A pairwise distance matrix was used to compare the aligned sequences of the avian *Haemoproteus* lineages.

RESULTS

Morphological examination: The developmental stages of avian haemosporidian protozoa were investigated microscopically in Giemsa-stained smears prepared from 53 migratory birds of four migratory bird species in the orders Ciconiiformes (n: 45), Pelecaniformes (n: 6) and Charadriiformes (n: 2) in this study. Microscopic analysis revealed the presence of *Haemoproteus* spp. gametocytes in one *Ciconia ciconia* and two *Ichtyaetus melanocephalus* (Fig. 1) included in the study. No positivity was detected for *Plasmodium* and *Leucocytozoon* spp. The level of parasitemia in *Haemoproteus* spp. positive samples was quite low (<0.1%). Details of the microscopy results are indicated in Table 1.

Nested PCR: PCR assay targeting the amplification of the 478 bp Cytb gene fragment of *Haemoproteus*/*Plasmodium* spp. revealed the molecular prevalence as 11.3% (6/53) in migratory birds. Taken individually, molecular positivity of *Haemoproteus*/*Plasmodium* spp. was detected in four *Ciconia ciconia* and two *Ichtyaetus melanocephalus* species of Ciconiiform and Chradriiform birds, respectively. Molecular positivity for *Haemoproteus*/*Plasmodium* spp. was detected in three white stork blood samples that were microscopically negative. For all other samples, microscopy and nested PCR results were consistent. *Leucocytozoon* spp. could not be detected by nested PCR as well as microscopy. Table 1 provides detailed information concerning molecular data.

Table 1: Microscopy and nested PCR results with CytB lineages and their accession numbers registered in GenBank

Order	Bird (n)	Positive (microscopy)					Positive (nPCR)	Lineages and accession numbers
		P	H	L	Total	P/H	L	
Ciconiiformes								H- MYCAME08 (OR227579)
	Ciconia ciconia						-	H- MYCAME08 (OR227582)
	(White stork) (45)	-	1	-	1	4		H- MYCAME08 (OR227580)
								H- MYCAME08 (OR227581)
Pelecaniformes	*Pelecanus onocrotalus*						-	-
	(Great white pelican) (5)	-	-	-	-	-		
	Pelecanus crispus (Dalmatian pelican) (1)	-	-	-	-	-	-	-
Charadriiformes	*Ichthyaetus melanocephalus*						-	H- LARCRA01 (OR227577)
	(Mediterranean gull) (2)	-	2	-	2	2		H- LARCRA01 (OR227578)
Total	53	-	3	-	3	6	-	

P: *Plasmodium* spp., H: *Haemoproteus* spp., L: *Leucocytozoon* spp., P/H: *Plasmodium*/*Haemoproteus* spp.

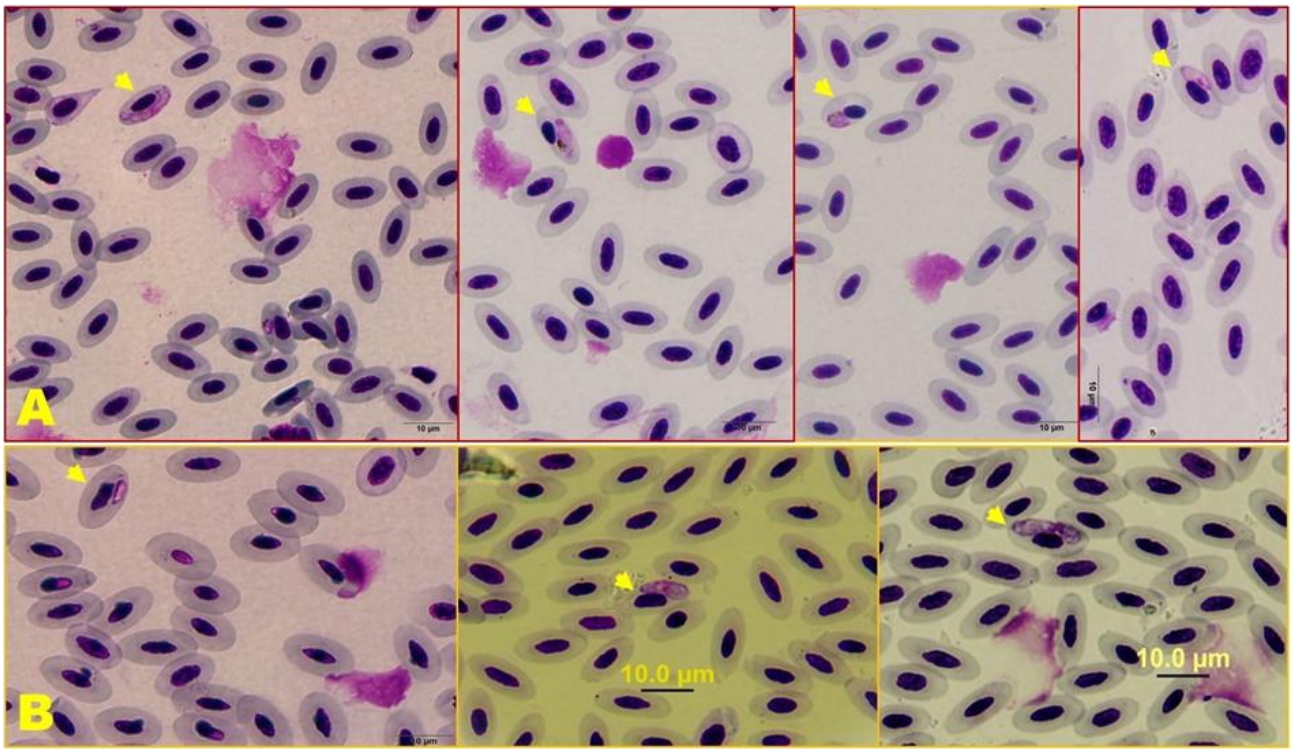

Fig. 1: Giemsa-stained thin blood smears prepared from white storks and Mediterranean gulls (x1000 magnification, scale bar = 10 µm). (A). Gametocytes of *Haemoproteus* spp. (MYCAME08 lineage from *Ciconia ciconia*) (B). Gametocytes of *Haemoproteus* spp. (LARCRA01 lineage from *Ichtyaetus melanocephalus*) Gametocytes are marked with a yellow arrow.

Phylogenetic analysis: Two Cytb lineages (MYCAME08 and LARCRA01) of *Haemoproteus* were found in this study. The lineage MYCAME08 was identified in four sequences (OR227579, OR227580, OR227581 and OR227582), all from *Ciconia ciconia*, known as white stork. In addition, the gene similarity ranged from 97.76% to 100 % among them (Table 2). The other lineage, LARCRA01, was found in two *Ichthyaetus melanocephaus*, known as Mediterranean gull, with 99.44% gene similarity among them (Table 2).

In regards to phylogenetic data, the concatenated BI/ML tree has shown identical topology with two main clades observed (Fig. 2). The first one subdivided in two subclades (*Haemoproteus* subclade and *Plasmodium* subclade), with well-supported branches. The second main clade comprises only *Haemoproteus* sequences. The lineages from this study grouped in both main clades, being the LARCRA01 sequences located at the first main clade and the MYCAME08 sequences located at the second main clade.

In the first main clade, the two sequences from this study grouped into a branch composed by five lineages (LARCRA01, EUDRUB01, CIRCUM01, TANGAL01 and DENAUT01), within the LARCRA01 branch. In this branch, the sequences from Poland and Turkey were closer to the sequences from this study, being all from closely related hosts (*Laridae Family*, Charadriiformes Order). In the second main clade, only *Haemoproteus* sequences from four lineages (MYCAME08, BUTVER01, CATAUR01 and PSOOCH01) were observed, with the four sequences from this study within the MYCAME08 branch. This branch comprises sequences from America and Africa, being all from Pelicaniformes and Ciconiiformes hosts.

DISCUSSION

Turkey is an important destination for migratory birds and especially Hatay province, which is located in a place where these birds enter and exit Turkey, has a strategic geographical position in terms of studying the epidemiology, lineage diversity, and host-parasite interactions of avian haemosporidian parasites. While resident birds are only infected with haemosporidian

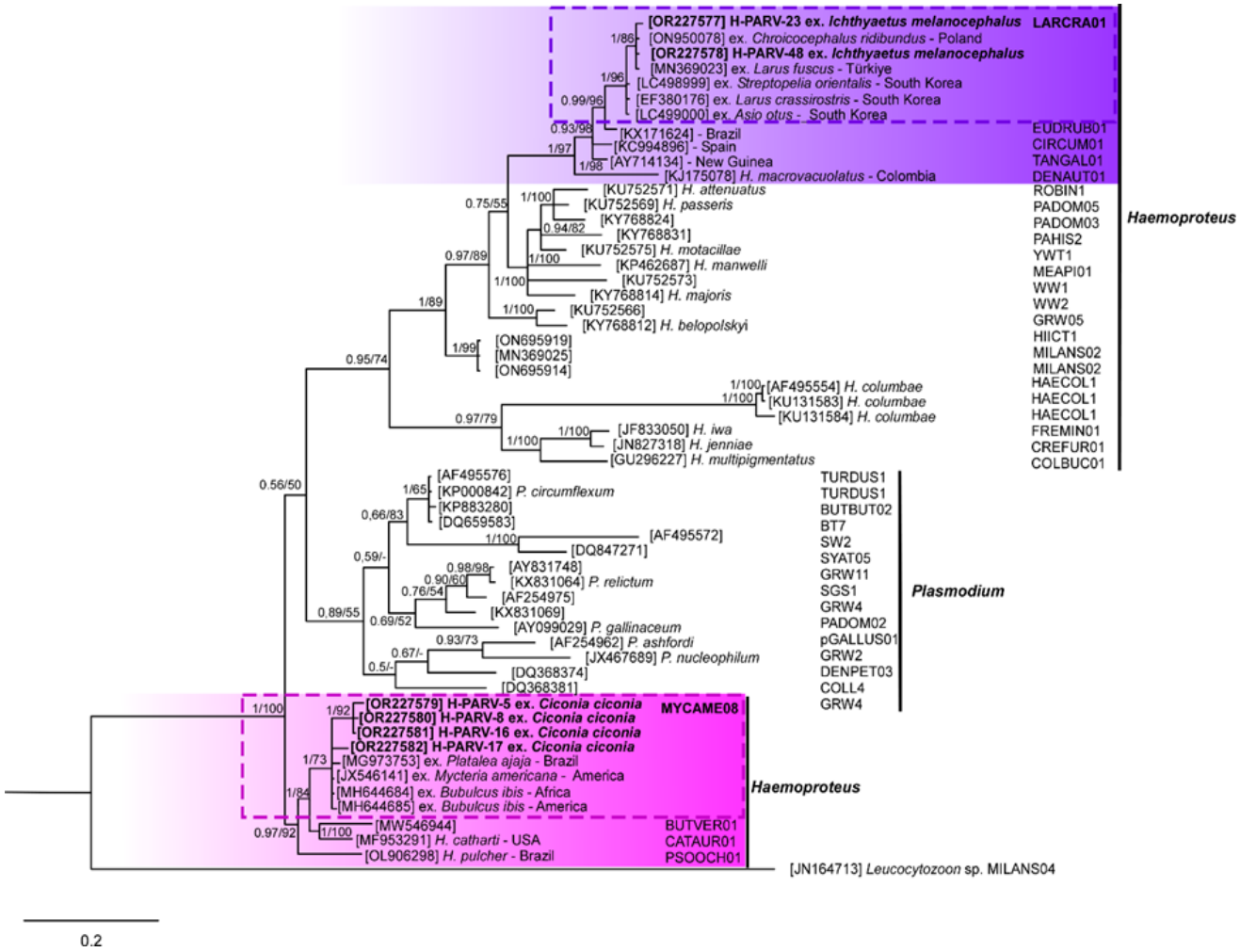

Fig. 2: Consensus phylogram of Avian haemosporidians (*Plasmodium* and *Haemoproteus*) based on mitochondrial cytochrome b gene. The topology trees using Bayesian inference and Maximum likelihood analyses were identical (represented by the BI tree). The values associated with the branches are related to the bootstrap values (BI/ML). The scale bar represents 0.02 nucleotide substitutions per site. *Leucocytozoon* spp. MILANS04 [JN164713] was used as an outgroup.

Table 2: The shaded matrix (lower) indicates the pair-wise distance and the non-shaded matrix (upper) indicates the percentage of similarity (%) of the nucleotide sequences among the avian *Haemoproteus* lineages found in this study and available in the GenBank (446 nt)

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.
1. H-PARV-23 [LARCRA01]*		99.44%	99.22%	99.22%	99.22%	99.44%	99.44%	90.02%	90.25%	90.47%	90.81%	90.79%	90.77%	90.77%	90.54%
2. H-PARV-48 [LARCRA01]*	0.00		99.77%	99.77%	99.77%	100%	100%	90.09%	90.32%	90.54	90.99%	90.97%	90.95%	90.95%	90.72%
3. LC498999 [LARCRA01]	0.00	0.00		100%	100%	99.78%	99.78%	89.91%	90.13%	90.36%	90.81%	90.79%	90.77%	90.77%	90.54%
4. EF380176 [LARCRA01]	0.00	0.00	0.00		100%	99.78%	99.78%	89.91%	90.13%	96.36%	90.81%	90.79%	90.77%	90.77%	90.54%
5. LC499000 [LARCRA01]	0.00	0.00	0.00	0.00		99.78%	99.78%/	89.91%	90.13%	90.36%	90.81%	90.79%	90.77%	90.77%	90.54%
6. ON950078 [LARCRA01]	0.00	0.00	0.00	0.00	0.00		100%	90.13%	90.36%	90.58%	91.03%	91.01%	90.99%	90.99%	90.77%
7. MN369023 [LARCRA01]	0.00	0.00	0.00	0.00	0.00	0.00		90.11%	90.34%	90.56%	90.01%	90.99%	90.97%	90.97%	90.74%
8. H-PARV-05 [MYCAME08]*	0.10	0.10	0.10	0.10	0.10	0.10	0.10		99.10%	99.33%	98.21%	97.98%	98.42%	98.42%	98.20%
9. H-PARV-08 [MYCAME08]*	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.01		99.78%	97.76%	97.98%	98.42%	98.42%	98.20%
10. H-PARV-16 [MYCAME08]*	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	0.00		97.98%	98.20%	98.65%	98.65%	98.42%
11. H-PARV17 [MYCAME08]*	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.01	0.02	0.01		98.43%	98.87%	98.87%	98.65%
12. MG973753 [MYCAME08]	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.02	0.01	0.01	0.02		99.55%	99.55%	99.32%
13. JX546141 [MYCAME08]	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.01	0.01	0.01	0.01	0.00		100%	99.77%
14. MH644684 [MYCAME08]	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.01	0.01	0.01	0.01	0.00	0.00		99.77%
15. MH644685 [MYCAME08]	0.10	0.09	0.10	0.10	0.10	0.09	0.09	0.01	0.01	0.01	0.01	0.01	0.00	0.00	

*Sequences from this study.

protozoa in the ecosystem in which they live, birds migrating between breeding and wintering areas are likely to be infected with a much wider variety of avian haemosporidian parasites as they pass through different ecosystems (Waldenström *et al*., 2002; Walther *et al*., 2016). In Turkey, which is located on the flyway of many birds, birds stop at some focal points during migration and face the risk of being infected with various haemosporidian parasites and lineages in the avian haemosporidian cycle in the ecosystems of the stopover sites. On the other hand, haemosporidian parasites harbored by migrating birds can infect resident birds living in the same ecosystem when the vector arthropods exist. To obtain more detailed data on all these, it is recommended to investigate haemosporidian parasites of birds captured for various reasons, especially on migratory bird flyways, and to update these data periodically to observe differences. To date, there are some studies investigating avian haemosporidian protozoa of

many species in the orders Acciptriformes, Anseriformes, Bucerotiformes, Columbiformes, Coraciiformes, Falconiformes, Galliformes, Passeriformes and Piciformes in Turkey (Özmen *et al*., 2005; Balkaya *et al*., 2016; Ciloglu *et al*., 2016; Tasci *et al*., 2018; Ciloglu *et al*., 2019; Ciloglu *et al*., 2020; Ciloglu *et al*., 2022; Zerek *et al*., 2023a, 2023b). Although the majority of the studies are on songbirds (Passeriformes), there are a few studies in which avian haemosporidian parasites have been investigated in birds of the families Ardeidae (Pelecaniformes), Ciconiidae (Ciconiiformes) and Scolopacidae (Charadriformes) in a small number of migratory birds (Ciloglu *et al*., 2020; Zerek *et al*., 2023b). In the current study, the presence of haemosporidian parasites in *C. ciconia* (Ciconiidae: Ciconiformes), *I. melanocephalus* (Laridae: Charadriiformes), *P. onocrotalus* and *P. crispus* (Pelecanidae: Pelecaniformes) were investigated by morphological and molecular methods. *Haemoproteus* spp. gametocytes were detected in thin blood smears prepared from one white stork (2.2%) and two Mediterranean gulls (100%), and nPCR analysis confirmed the presence of *Haemoproteus* spp. in four white storks (8.9%) and two Mediterranean gulls (100%) in the study. In addition, the Cytb lineages of *Haemoproteus* isolates were also investigated by sequence and phylogenetic analyses, and the *Haemoproteus* Cytb lineages H-MYCAME08 in white storks and H-LARCRA01 in Mediterranean gulls were identified for the first time in Turkey.

Although some *Haemoproteus* species, such as *H. crumenium* and *H. ciconiae*, have been identified in storks of the order Ciconiiformes, there is limited information on the diversity of the *Haemoproteus* lineages (Valkiūnas *et al*., 2016; Valkiūnas and Iezhova, 2022). The *Haemoproteus* MYCAME08 Cytb lineage was detected in 8.9% of the migratory storks of *C. cinonia*, which represents a significant part of the material of this study. The H-MYCAME08 *Haemoproteus* sp. Cytb lineage, previously known as MYCAMH1, has been reported in wood storks (*Mycteria americana*; Ciconiiformes: Ciconiidae) in central Brazil and the USA (Villar *et al*., 2013; Vanstreels *et al*., 2022), but has not been reported in storks or other bird species elsewhere. Phylogenetic analyses by Vanstreels *et al*. (2022) indicated that the H-MYCAME08 *Haemoproteus* sp. Cytb lineage has a phylogenetic proximity to *H. pulcher* from red-legged seriema (*Cariama cristata*), *H. catharti* from a vulture (*Cathartes aura*), and *Haemosporidia* sp. PSOOCH01 from pale-winged trumpeter (*Psophia leucoptera*), and is related to *Haemocystidium* spp. and *Plasmodium* spp. in addition to the known *Haemoproteus* species. They also pointed out that these haemosporidian parasites in the same cluster are likely to represent a genus or sub-genus that has not yet been described. Another study (Villar Couto *et al*., 2019) of avian haemosporidian parasites in Cattle Egrets (*Bubulcus ibis*) found that the BULIBH1 and BULIBH2 *Haemoproteus* Cytb lineages formed a well-supported clade separated from the *Parahaemoproteus* group with *H. catharti* from New World vultures (Cathartidae) and MYCAMH1 *Haemoproteus* Cytb lineage from Wood Stork (Villar *et al*., 2013; Yabsley *et al*., 2018). Remarkably, this *Haemoproteus* group, clearly separated from other *Haemoproteus* species, is closer to *Plasmodium* species on the phylogenetic tree (Villar Couto *et al*., 2019). In the present study, our four *Haemoproteus* Cytb lineages from white storks formed a well-supported MYCAME08 branch with the aforementioned BULIBH1 from Africa (MH644684), BULIBH2 from America (MH644685), MYCAMH1 from America (JX546141), PLATAjH1 (MG973753), a new *Haemoproteus* haplotype found in Roseate spoonbills (*Platalea ajaja*) from Brazil, and BUTVER01 from *Burhinus vermiculatus* (MW546944), *H. catharti* CATAUR01 lineage from *Cathartes aura* (MF953291) and *H. pulcher* PSOOCH01 lineage from *Cariama cristata* (OL906298) were also included in the clade in which this branch was found. Mitochondrial Cytb gene analysis revealed the presence of H-MYCAME08 *Haemoproteus* sp. lineage in ciconiform migratory birds in Turkey for the first time, and this study is expected to promote to the fauna of Turkey in terms of *Haemoproteus* sp. Cytb lineage diversity. Furthermore, this study will enrich molecular phylogenetic studies of avian haemosporidia in ciconiform or migratory birds worldwide. In order to elucidate the complexity of its phylogeny, further investigation of the *Haemoproteus* sp. MYCAME08 Cytb lineage in ciconiform birds is advocated.

Two Mediterranean gulls examined in the study were microscopically and molecularly positive for avian haemosporidia. Sequence results showed that both isolates were *Haemoproteus* sp. LARCRA01 Cytb lineage [*H.* (*Parahaemoproteus*) *larae*] and this lineage was reported for the first time in *I. melanocephalus* charadriiform larid bird species in the present study. The literature has revealed that the *H.* (*Parahaemoproteus*) *larae* LARCRA01 Cytb lineage is usually found in charadriiform birds of the Laridae family (Ishtiaq *et al*., 2007; Inumaru *et al*., 2017; Inumaru *et al*., 2020; Wlodarczyk *et al*., 2022). Until the present day, *Haemoproteus* LARCRA01 lineages from *Larus* spp. belonging to the Charadriiformes order, Laridae family have been reported in some bird species such as common gull (*Larus canus*) from Japan, Chiba (Inumaru *et al*., 2017), Black-tailed gull (*Larus crassiostris*) from South Korea and Japan (Ishtiaq *et al*., 2007; Inumaru *et al*., 2017), Black-headed gull (*Larus ridibundus*) from Poland (Wlodarczyk *et al*., 2022) and Caspian gull (*Larus cachinnans*) from Spain (Ricklefs and Fallon, 2002; Inumaru *et al*., 2020). This study reports the presence of *Haemoproteus* LARCRA01 Cytb lineage in the genus *Ichthyaetus* in the Laridae family for the first time. Phylogenetic analyses revealed that this lineage isolated from *I. melanocephalus* clustered together with *Haemoproteus* LARCRA01 Cytb lineages from *Chroicocephalus ridibundus* in Poland (ON950078) (Wlodarczyk *et al*., 2022), *Larus fuscus* in Turkey (MN369023) (https://www.ncbi.nlm.nih.gov/nuccore/MN369023) and *Larus crassiostris* in South Korea (EF380176) (Ishtiaq *et al*., 2007) with 99.22-100% similarity rate. The clustering of the *Haemoproteus* sp. LARCRA01 Cytb lineage in the phylogenetic tree with different LARCRA01 Cytb lineages isolated from columbiform birds such as the oriental turtle dove, *Streptopelia orientalis* (LC498999) from South Korea, or strigiform birds such as the long-eared owl, *Asio otus* (LC499000) from South Korea, with 99.22-99.77%

similarity rate suggests that the host range and low host specificity of this lineage warrants further investigations.

The present study revealed that *Leucocytozoon* spp. was not detected in sampled migratory bird species by molecular and microscopic analyses. To the best of our knowledge, although there are some molecular and microscopic reports of *Leucocytozoon* spp. in birds in Turkey (Marzal and Albayrak, 2012; Ciloglu *et al*., 2016; Ciloglu *et al*., 2020; Zerek *et al*., 2023b), no data shows that it has been reported in storks, pelicans, and gulls, except for *Ixobrychus minutus* belonging to the Ardeidae family (Pelecaniformes) (Ciloglu *et al*., 2020). The majority of the birds in which *Leucocytozoon* spp. have been reported in Turkey are birds of prey and songbirds (Ciloglu *et al*., 2016; Ciloglu *et al*., 2020; Zerek *et al*., 2023b). In a study conducted in Turkey, birds of prey and a small number of ciconiform birds were examined together, and *Leucocytozoon* spp. was detected in *Clanga pomarina* and *Circaetus gallicus* bird species. However, this protozoon was not detected in white storks (Zerek *et al*., 2023b). The reason why *Leucocytozoon* spp. was not found in storks, pelicans, and gulls in the study may be related to the habitats where these birds live and its vector simuliid blackfly populations in those habitats.

Conclusion: In conclusion, it is thought that *Haemoproteus* sp. MYCAME08 and LARCRA01 Cytb lineages, which were reported for the first time in migratory birds of *C. ciconia* and *I. melanocephalus* species in Turkey, respectively, will contribute to the lineage diversity of avian haemosporidian protozoa. Further studies on the prevalence and lineage diversity of haemosporidian protozoa of birds in Turkey, which has significant bird migration routes between Europe and Africa, will continue to contribute to the subject. For this reason, periodic observations of many different bird species for haemoparasites, especially in intersection foci such as Hatay during migration times, will support the ever-expanding avian haemosporidian protozoon diversity.

Author contribution: A.Z., O.C., and M.Y. conceptualized the study. A.Z., F.N.S., and M.E.A., performed the field works and collected blood materials. A.Z., O.C., I.E., and M.Y. conducted laboratory analysis. O.C., L.P.U., and M.S. conducted phylogenetic analysis. A.Z. and O.C. prepared the original draft. All of the authors have reviewed and approved the current version of the manuscript.

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Data availability: The data sets obtained and/or analyzed in the present study are available by contacting the corresponding author with a reasonable request.

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