Prevalence and Molecular Characterization of Haemosporidians in Domestic Geese: A New Focus of Haemosporidian Parasites, Kars Province, Northeastern Turkey

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ABSTRACT

Leucocytozoon, Haemoproteus and Plasmodium spp. are vector-borne protozoan blood parasites of domestic and wild birds and called avian haemosporidians (Apicomplexa: *Haemosporida*). There have been many studies on the haemosporidians of wild birds, especially in the Passeriformes order around the world. However to the best knowledge of the authors no data has beenpublished about the prevalence and characterization of haemosporidians that infect domestic geese (*Anser anser domesticus*). In the present study, a total of 400 domestic geese blood samples from Kars Province, Northeastern Turkey were investigated for the presence of avian haemosporidians using traditional microscopic and molecular methods. From the microscopic examination, a total of 58 (14.5%), 48 (12%) and 94 (23.5%) samples were found positive for *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp., respectively. Molecular analyses revealed that 145 (36.25%) and 148 (37%) of the examined blood samples from the domestic geese were infected with *Haemoproteus/ Plasmodium* and *Leucocytozoon* spp., respectively. Sequence analysis of the isolates also revealed two new lineages within *Haemoproteus* and *Plasmodium* spp. which were found to be close to the lineages reported from the avian species in Anceriformes order. The obtained sequences from *Leucocytozoon* isolates were identical to the TUSW04 lineage which was reported from some *Anas* species in Anceriformes order. To the best of our knowledge this study has provided the first data on the prevalence and molecular characterization of the avian haemosporidians that infect domestic geese.

Keywords: Anceriformes; Haemosporidian Parasites; Molecular Characterization; Northeastern Turkey; Prevalence.

INTRODUCTION

Avian haemosporidians (Apicomplexa: *Haemosporida*), including *Plasmodium, Haemoproteus* and *Leucocytozoon* sare vector-borne parasites of domestic and wild birds transmitted by biting midges, louse flies, black flies and mosquitoes. These parasites have an economic importance especially in domestic avian species by causing anemia, hypoxia, fever, leukocytosis, hepato-spleenomegaly, respiratory distress, anorexia and death (1-7).

Protozoan blood parasite infections in avian species have been studied for more than a century, and representative species of parasites from the genera *Haemoproteus, Plasmodium*,

and *Leucocytozoon* have been detected on every continent, except Antarctica (4, 8). More than 250 species by morphological characteristics of parasite blood stages, and over 2000 unique genetic lineages of avian haemosporidians by molecular studies have been recently identified (4, 9-11). In addition, there have been many studies carried out to determine the prevalence of haemosporidians in wild birds, especially on Passeriformes around the world. Studies have shown that these haematozoa infections can have adverse effects on fitness in certain avian species. Host populations that are restricted to islands, or host species that have not previously been exposed to haematozoa infection being particularly vulnerable to pathogenic effects of these parasites (2, 11-13).

In recent years, haemosporidian parasite DNA sequences have been facilitated by molecular-based screening techniques (9, 14-16). Especially *cytochrome b* gene region, which has conserved regions, has been found to be suitable for detection and identification of haemosporidian lineages (15). Nonetheless, because of the complexity of molecular analysis of haemosporidian parasites, it is generally recommended to be incorporated with morphological examination of parasite under a microscope (3, 17-20).

In Turkey, there are few studies concerning avian haemosporidians. The most detailed surveys were conducted by Inci *et al.* (21-24) and Ciloglu *et al.* (11, 25), and several haemosporidian lineages from both avian and vector insect species were reported in Sultan Marshes, Central Anatolia Region of Turkey. Leucocytozooniosis was reported in wild birds (26, 27) and *Plasmodium* species were detected in mosquitoes (21, 22) and raptor birds (11). And also, *Haemoproteus* infections were encountered in wild pigeon, dove, sparrow and owl (28, 29). In a study, 7 of 9 sparrowhawks were found infected with haemosporidian parasites (30). In another study (31), haemosporidians were researched on the krüper's nuthatch (*Sitta krueperi* Pelzeln). However, there has been no data regarding the presence and the prevalence of haemosporidian parasites in domestic geese (*Anser anser domesticus*).

Kars province is one of the most common places where domestic geese farming is carried out, with the highest amount in Turkey. Moreover, goose breeding is an important source of income for the locals, especially housewives. Therefore, the diagnosis and the treatment of avian haemosporidian infections are essential for the local people.

In the present study, we aimed to investigate the prevalence of haemosporidian parasites in domestic geese (*Anser anser domesticus*) by using both microscopic and molecular-based techniques in the Kars province and surroundings, in the Northeastern Region of Turkey. The molecular characterization of the haemosporidian lineages were also revealed with the study.

MATERIAL AND METHODS

Kars province is one of the most common places where geese farming is carried out and consitutes the highest number of geese in Turkey. The number of geese was over 50,000 in the study area (40˚36'04.82"N, 43˚05'50.83"E), 280,000 in Kars and surroundings, and nearly 900,000 in whole Turkey. Mean temperature of Kars during November can reach 14.9˚C and the altitude of study area is 1768 m above the sea level.

A total of 400 blood samples were collected into two seperate tubes (one for blood smears, one for DNA extraction) from the domestic geese (*Anser anser domesticus*) during slaughtering which were grown for nutrition purposes, during November 2015.

The blood smears were prepared when domestic geese (*Anser anser domesticus*) were slaughtered. Two blood smears were prepared from each goose, air-dried within 5-10 seconds after preparation and brought to the laboratory. Smears were fixed in 100% methanol and then stained with 10% solution of Giemsa for 1 hour. One hundred fields were examined under a light microscope (Nikon Eclipso 600 equipped with Nikon P5100 digital camera) at 400x and 1000x magnification in each blood smears for detection of parasites.

DNA extraction was performed by using a commercial DNeasy kit protocol (Zymo Research, Quick-gDNA Blood Mini Prep, USA). The isolated total genomic DNA (gDNA) was stored at -20˚C until analysis. DNA concentrations of the samples were measured (Qubit Fluorometric Quantitation, Invitrogen, Life Technologies) to optimize the amount of gDNA used in the PCR mastermix. For genetic analysis of mitochondrial *cytochrome b* gene *(cyt b)* of haemosporidian parasites of geese, extracted DNA was used in nested PCR with genus spesific primers. The primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') (I: inosine, a universal base) and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') were used to amplify parasite mtDNA from all haemosporidians in the first step of nested PCR (3, 15, 19, 32-34). A total volume of 25µl reaction mixture was composed of 8.5µl nuclease-free

water, 12,5µl master miks (MyTaq™, Bioline), 1µl of each primer (10 µM concentration) and 2µl of template DNA (50ng). PCR reaction conditions were as follows: denaturation at 94˚C for 3 min, 20 cycles at 94˚C for 30 s, 50˚C for 30 s, 72˚C for 45 s and final extension at 72˚C for 10 min in Thermal Cycler (Biometra, Analytik Jena, USA).

For the second PCR, HaemF (5'-ATGGTGCTTT CGATATATGCATG-3') and HaemR2 (5'-GCATTATCT GGATGTGATAATGGT-3') primers were used for *Haemoproteus* / *Plasmodium* species and HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGG IGC-3') primers were used for *Leucocytozoon* species (3, 15, 19, 32-34). Two µL of the first PCR product was used as a template for the second PCR. The reaction conditions were the same as first PCR, however, it was now performed over 35 cycles instead of 20 cycles. Negative (nuclease-free water) and positive controls (positive samples were provided by Department of Parasitology, Faculty of Veterinary Medicine, Erciyes University, Kayseri/Turkey and Prof. Dr. Gediminas Valkiunas, Institude of Ecology, Nature Research Center, Vilnius/Lithuania) were used. The PCR products were analyzed on a 1.5% agarose gel using 0.5X TBE and visualized by an ethidium bromide (0.5 µg/ml) stain under ultraviolet light.

A total of 5 and 3 PCR products from nested PCR of *Haemoproteus*/*Plasmodium* spp. and *Leucocytozoon* spp. were further purified from agarose gel, using a commercial kit (High Pure PCR Product Purification Kit, Roche Life Science). The amplified *cyt-b* target fragments were sequenced in both strands using HAEMF/HAEMR2 primers for species of *Plasmodium* and *Haemoproteus* spp. and HaemFL/HaemR2L primers for species of *Leucocytozoon* in an Automatic Sequencer at Sentegen Company (Turkey). Paired nucleotide sequences were oriented, edited, and aligned with Geneious 10.2.3 software (35) to produce a single consensus sequence.

The obtained sequences compared at 479 bp with sequences in the GenBank database using the Basic Local Alignment Search Tool (NCBI website) (36) and sequences in the MalAvi database (9, 16). The lineages were identified and named following the nomenclature in the MalAvi database and deposited in GenBank with the accessions MG593840-42. Phylogenetic reconstructions were performed by Bayesian (BA) inference. The best-fit DNA-substitution model for BA analyses based on the Akaike information criterion (AIC) algorithm was selected as TrN+G for all trees by using jModel test v.0.1.1 (37). The BA analyses were run in MrBayes version 3.2.6 (38) through the plugin available with Geneious 10.2.3 software (35).

RESULTS

Development stages of *Haemoproteus, Plasmodium* spp. and *Leucocytozoon* spp., were identified (Fig. 1) in the smears of 58 (14.5%), 48 (12%) and 94 (23.5%) out of 400 samples, respectively. The microscopic prevalence of the haemosporidian parasites with mix infections were summarized in Table 1. *Leucocytozoon* spp. was the most common parasite and this

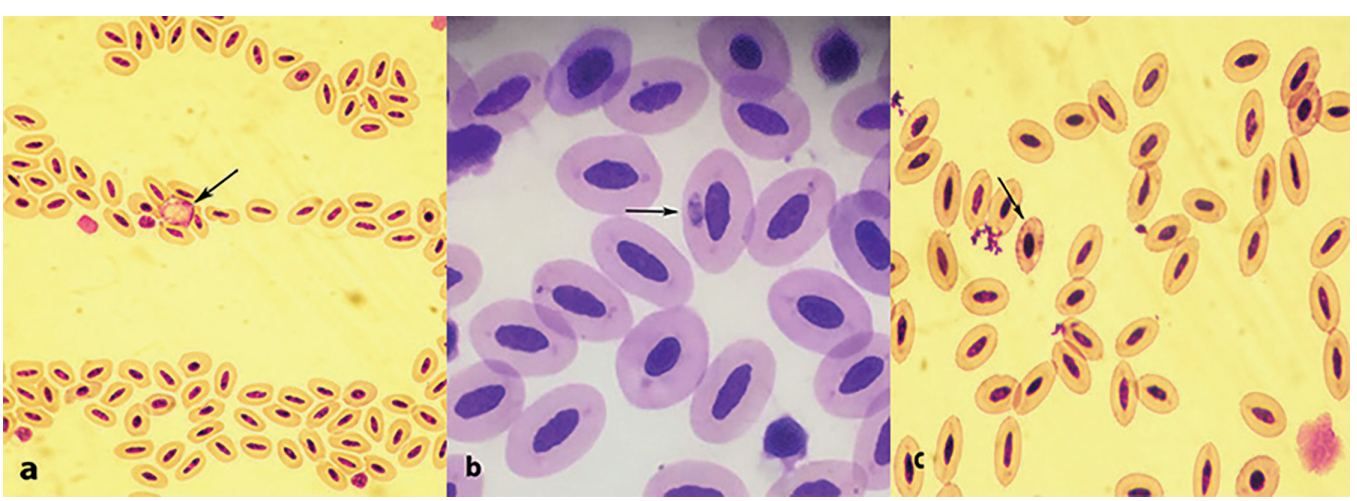

Figure 1. Microscopic images of haemosporidians in Giemsa-stained thin blood films of erythrocytic stages in geese blood. a. *Leucocytozoon* spp. b. *Plasmodium* spp. c. *Haemoproteus* spp. Original magnification: X1000.

Table 1. Prevalence of *Plasmodium, Haemoproteus* and *Leucocytozoon* species by microscopic examination.

Method	*P*		*H* spp.		*L*		Mix Infection with *P*/*H* and *L*		Mix Infection with *P* and *H*		Mix Infection with *P* and *L*		Mix Infection with *H* and *L*	
	+	%	+	%	+	%	+	%	+	%	+	%	+	%
Microscopy	48	12	58	14.5	94	23.5	8	2	9	2.25	14	3.5	23	5.75

P: Plasmodium, H: Haemoproteus, L: Leucocytozoon, +: positive

Table 2. Prevalence of *Plasmodium, Haemoproteus* and *Leucocytozoon* species by PCR.

Method	*Plasmodium / Haemoproteus* spp.		*Leucocytozoon* spp.		Mix Infection with *Plasmodium/ Haemoproteus* and *Leucocytozoon* spp.	
	Positive	%	Positive	%	Positive	%
PCR	145	36.25	148	37	86	21.5

was followed by *Haemoproteus* and *Plasmodium* spp. in the examined geese.

The results of the nested PCR analyses were given in Table 2. A total of 145 (36.25%) and 148 (37%) out of 400 samples were found positive for *Haemoproteus*/*Plasmodium* spp. and *Leucocytozoon* spp., respectively. Sequence analysis of the PCR-products confirmed the PCR results.

The mt-cytb sequences of the 5 isolates from the *Haemoproteus*/*Plasmodium* nested PCR positive geese revealed two new lineages (KVHmp and KVPlm) within *Haemoproteus* (3 isolates named as KVHmp1-3) and *Plasmodium* (2 isolates named as KVPlm1-2) genera. The BA phylogeny of the characterized lineages of *Haemoproteus* and *Plasmodium* along with the related sequences selected from GenBank and MalAvi database were shown in Fig. 2 and Fig. 3, respectively. The KVHmp lineage showed highest identity (99.6%) to ANSIND01 lineage (GenBank accession: KJ577824) reported from *Anser indicus* (bar-headed goose) in Mongolia and clustered together (Fig. 2). The KVPlm lineage exhibited highest identities (99.4% and 99.2%) with the lineages reported from *Anas acuta* (northern pintail) in USA (GenBank accession: KJ776820 and KC409132) and clustered together in the phylogenetic tree (Fig. 3). The three *Leucocytozoon* isolates (KVLeu1-3) showed 100% identity to each other and were characterized as TUSW04 lineage which was already reported from *Anser indicus* (bar-headed goose) in Mongolia (GenBank accession: KJ577823) and, *Anas discors* (blue-winged teal) (GenBank accession: KU363716), *Anas platyrhynchos* (Common Mallard) (GenBank accession: KU295418) and *Anas* sp. (GenBank accession: KM386327) in USA (Fig. 4).

DISCUSSION

A large majority of the occupation and income in Kars province is animal breeding, in Northeastern Region of Turkey. One of the breeding animals is geese. Kars province is popular area where geese farming is carried out. In Turkey, the highest number of geese are in Kars. Moreover, goose breeding is an income source for locals, especially housewives. As a result of this, the most celebrated meal to be served to guests is geese meat that attracts the interest of local and foreign tourists. The goose meat, liver, feathers and eggs are sold outside of the province of Kars. These products are also exported abroad. However, geese breeding is done by traditional methods and no medications are administered. Disease outbreaks are encountered and as a consequence of mortality, however the cause of death is not always elucidated. In this study, it was attempted to determine whether there was a relationship of protozoan diseases with the mortality cases in domestic geese.

Blood parasites belong to *Haemoproteus, Plamodium* and *Leucocytozoon* genus appear to be ubiquitous in avians (12, 13). However, there is no data about haemosporidians of domestic geese around the world. This study provides first initial findings on the prevalence of haemosporidians in domestic geese in ther Kars Region of Turkey. On the other hand, there have been few studies (11, 21-31) that reported several *Haemoproteus, Plamodium* and *Leucocytozoon* infections in the avian species belonging to Passeriformes, Accipitriformes and Falconiformes orders. All these studies indicate that Turkey has favourable conditions for development and dissemination of avian haemosporidian diseases.

In the present study, both microscopic examination and molecular analyses revealed a high haemosporidian prevalence among the geese population in the region of Kars with

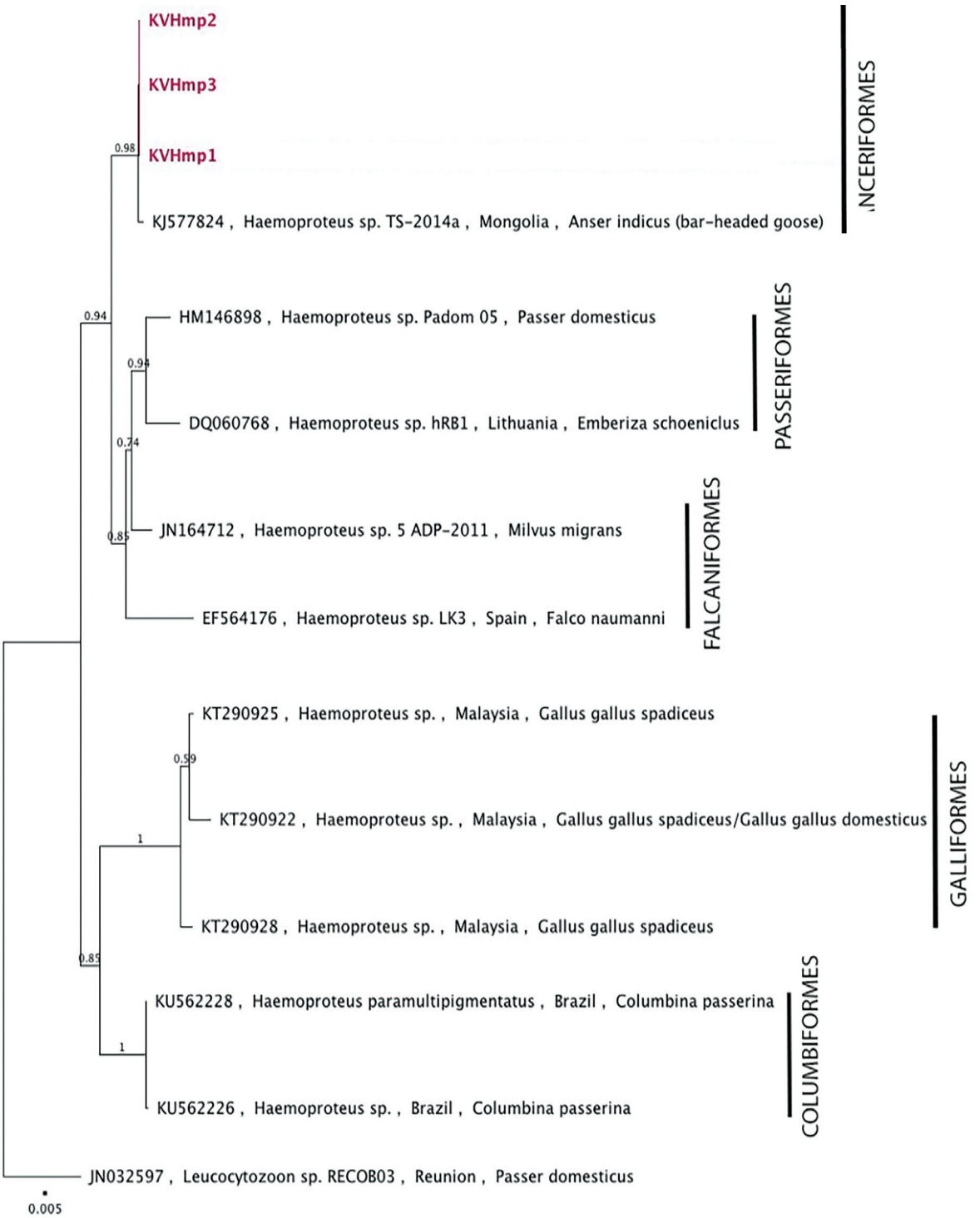

Figure 2. BA tree based on mt-cytb sequences deposited in GenBank and our original data (bold red character) for *Haemoproteus* lineages. BA posterior probability values are shown before the nodes. *Leucocytozoon* spp. was used as outgroup taxa. Bars represent 0.1 substitutions per site.

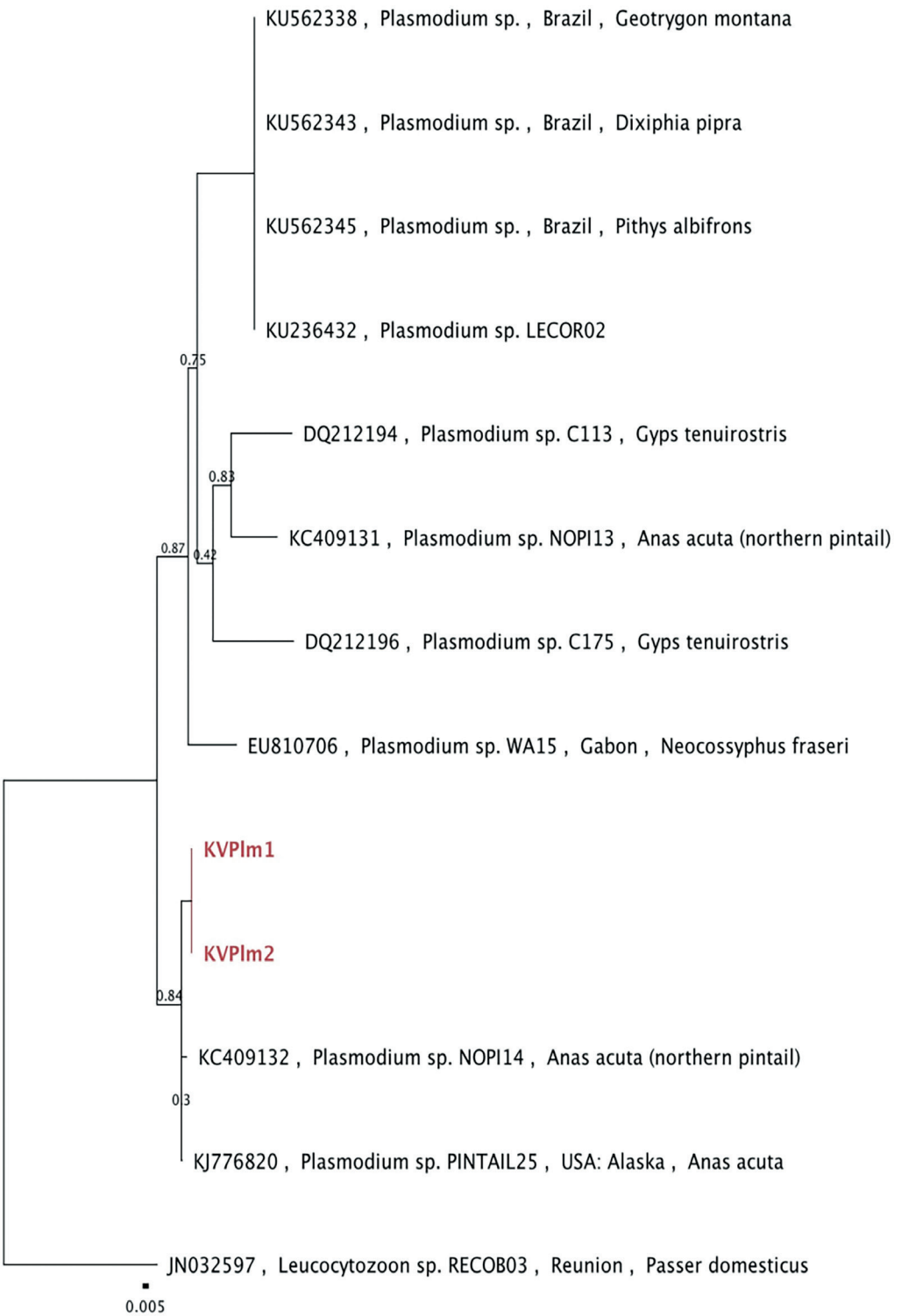

Figure 3. BA tree based on mt-cytb sequences deposited in GenBank and our original data (bold red character) for *Plasmodium* lineages. BA posterior probability values are shown before the nodes. *Leucocytozoon* sp. was used as outgroup taxa. Bars represent 0.1 substitutions per site.

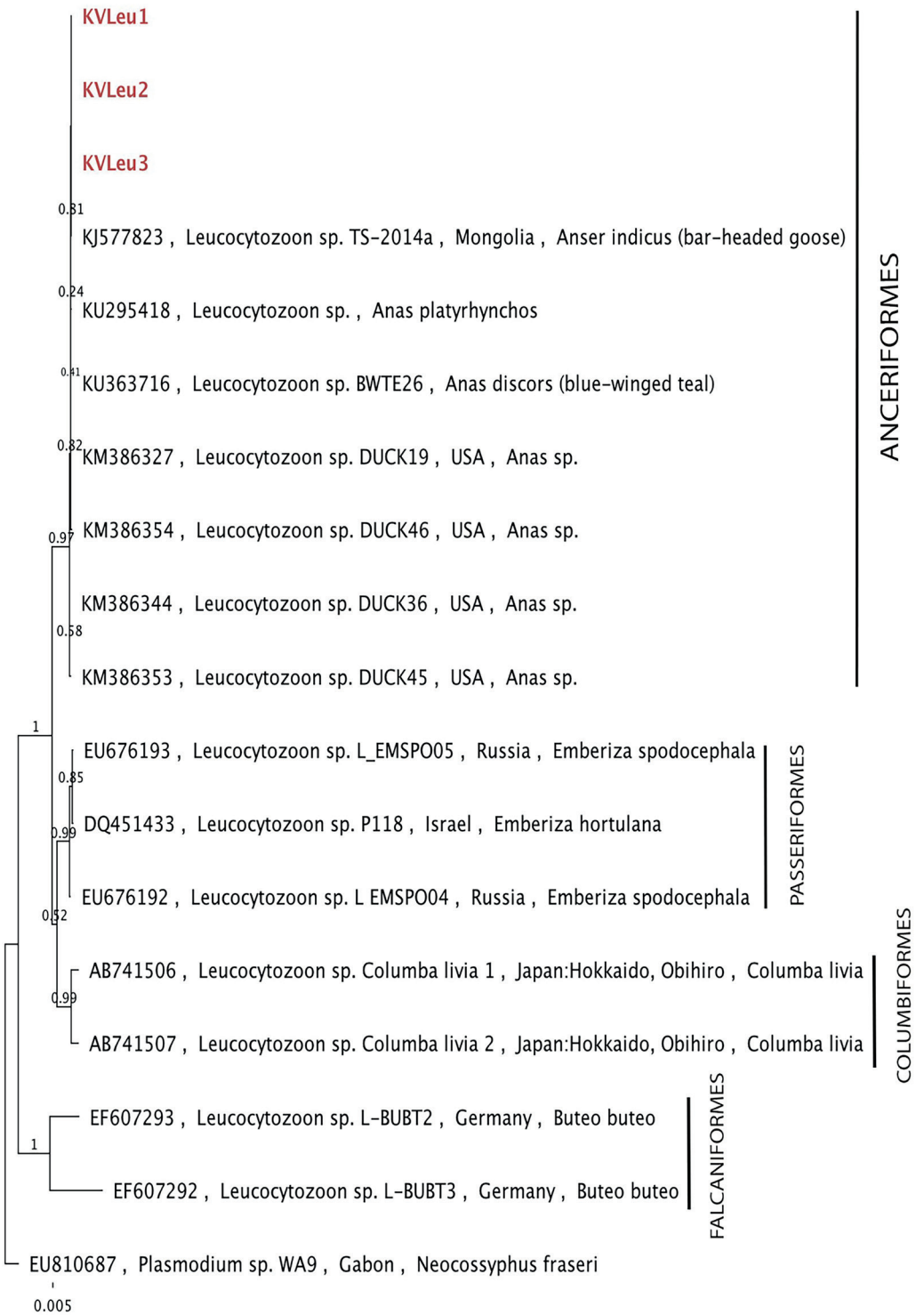

Figure 4. BA tree based on mt-cytb sequences deposited in GenBank and our original data (bold red character) for *Leucocytozoon* lineages. BA posterior probability values are shown before the nodes. *Plasmodium* sp. was used as outgroup taxa. Bars represent 0.1 substitutions per site.

the majority of infections caused by *Leucocytozoon* species. Therefore, we concluded that haemosporidian infections could be one of the sources of disease outbreaks and deaths in domestic geese in the region. Similarly, mortality of birds due to Haemosporidian infections have been reported both due to high parasitaemia and damage caused by phanerozoites (39-40). It has been reported that mixed infections caused by different species and genetic lineages of haemosporidian parasites are common in wildlife, and these infections are significantly more virulent (13). Accordingly, our results indicated a high rates of mix infections among geese populations in the region which might also effect the outcome of the disease.

According to researchers, polymerase chain reaction (PCR)-based methods can be used to detect mix infections caused by *Haemoproteus*/*Plasmodium* and *Leucocytozoon* spp. for wildlife haemosporidian parasites with studies carried out in different regions of world, showing that mixed haemosporidian infection rates ranged between 6-80% in many wild bird species (12, 13, 41-46). However, currently used polymerase chain reaction (PCR)-based detection methods could not fully distinguish *Haemoproteus* and *Plasmodium* from each other and only by utilizing microscopic examinations along with molecular tools can this obstacle be solved (4). In our study, mix infection rate with *Haemoproteus*/*Plasmodium* and *Leucocytozoon* spp. was detected as 21.5%. This prevalence rate was higher than some other studies (12, 47). This might be due to the fact that the domestic geese are maintained extensively outside and therefore exposed to insect vectors.

Currently there are over 2380 unique avian haemosporidian lineages documented in the MalAvi database, (9, 16), and the number of new variants increase regularly with new studies on several kind of bird species from different geographic regions. In our study two new lineages for *Haemoproteus* and *Plasmodium* that infect domestic geese were characterized. The KVHmp lineage exhibited the highest identity (99.6%) to ANSIND01 lineage (GenBank accession: KJ577824) reported from *Anser indicus* (bar-headed goose) which is also in the same avian genus. The avian haemosporidians have a high variance of host specificity. While some lineages are found certain avian genus, family or taxons, some other are localized through all of the Aves class (4). It has been reported that *Haemoproteus* lineages have a more restricted host range based on genus or species level (48, 49). Our phylogenetic analyses on the new *Haemoproteus* linage in geese also support this inference. On the other hand, lineages belonging to *Plasmodium* species have more general characteristics regarding host specificity and infect avian species in different families or taxons (49-51). However, the new lineage KVPlm characterized in our study was highly similar with the lineages reported from *Anas acuta* (northern pintail) (99.4% and 99.2%) which is also in the same avian genus, forming a cluster in the phylogenetic tree. Therefore, we concluded that further studies are required to explore the host range of *Plasmodium* KVPlm lineage.

The *Leucocytozoon* lineage found in domestic geese in the study was identified as the TUSW04 lineage by blast analyses in the MalAvi database (9, 16), and this lineage has been already characterized in *Anser indicus* (bar-headed goose), *Anas discors* (blue-winged teal), *Anas platyrhynchos* (Common Mallard) and *Anas* spp. which are also in the same avian genus. Therefore, this result supports the inference that *Leucocytozoon* species have host specificity generally at the order level and in some cases at the family, subfamily and species levels (52).

In conclusion, this study provides the first molecular epidemiologic data on the haemosporidian parasites of domestic geese. Further studies on the base of host-parasite-vector triangle are needed for exploring the lineage diversity and pathogenicity of haemosporidian parasites infecting domestic geese.

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