Detection of haemosporidian parasites in wild and domestic birds in northern and central provinces of Iran: Introduction of new lineages and hosts

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ABSTRACT

Haemosporidian parasites characterize multi-host and multi-parasite structures which are prevalent among wild bird populations. Here, determination of host records, estimation of the prevalence and diversity of haemosporidian lineages were performed in wild and domestic birds in 11 provinces in Iran. To our knowledge, for the first time in this region, molecular characterization of haemosporidians in migratory water birds, raptors, and domestic birds was carried out: blood or tissue samples were collected from 246 birds belonging to 36 species, 12 families, and 11 orders. The prevalence of *Plasmodium*, *Haemoproteus,* and *Leucocytozoon* were documented as 1.21%, 3.65%, and 0.4%, respectively. Of 36 birds' species inspected in this investigation, 13 individuals of 9 species were parasitized by blood parasites. To our knowledge, five lineages including hANACRE03, hAYTFER01, hAYTFER02, hAQUCYR01, and hSTAL06 were found as un-described lineages, while six known lineages of hLK03, pLK05, lTUSW04, pSW5, hMILANS02, and hHAECOL1 were recorded in hosts within novel geographical regions. Such results are required to fill the gaps in understanding the geographical distribution patterns of wildlife related vector-borne parasites in migratory birds as potential carriers, raptors with high vulnerability, and domestic birds as pet or with economic value.

1. Introduction

Haemosporidian parasites (Haemosporida, Apicomplexa) with a major importance in birds have multi-host and multi-parasite structures which are prevalent among wild bird populations (Valkiūnas, 2005). Many dipteran species are known to be vectors of *Plasmodium* (Culicidae), *Haemoproteus* (Hippoboscidae, Ceratopogonidae), and *Leucocytozoon* (Simuliidae) (Kim et al., 2009; Murdock et al., 2015; Santiago-Alarcon et al., 2012). Avian haemosporidian parasites have the potential to infect a wide range of species in many bird families (Davidar

and Morton, 1993; Krone et al., 2008; Marzal et al., 2005, 2011; Ventim et al., 2012), which may result in significant ecological and evolutionary burdens to the wild bird populations, with great influence on their community structure, existence, conservation, body condition, and reproductive fitness (Atkinson et al., 2009; Dinhopl et al., 2015; Marzal et al., 2005; Ruiz et al., 1995; Ziman et al., 2004). Birds with high movements are often more exposed to various pathogens than sedentary hosts (Hubálek, 2004). It has been demonstrated that wild migratory avian hosts are able to exchange some pathogens, including avian blood parasites between continents. Inter-continental and trans-continental

migration of birds can enhance the potency of disease transmission, while novel avian hosts and vectors may increase the prevalence of diseases in these locations (Aghayan, 2012; Gutierrez-Lopez et al., 2015; Hahn and Bauer, 2018; Hubálek, 2004; Ramey et al., 2015, 2016).

Although several studies have been carried out on the incidence of blood parasites in wild birds in different localities using microscopic examinations in Iran (Nourani et al., 2017a, 2018a, 2018b; Rassouli et al., 2017; Shirazi et al., 2012), few have used molecular methods for detection of the haemosporidian parasites in this region (Nourani et al., 2018b, Nourani et al., 2020b). Previous morphological and molecular studies have focused on detection of haemosporidian parasites in Columbidae, especially *Columba livia* and passerine species (Doosti et al., 2014; Fakhar et al., 2013; Gorji et al., 2012; Nourani et al., 2017a, 2018a, Nourani et al., 2020, Nourani et al., 2020b; Tavassoli et al., 2018). Recent studies have shown the presence of parasitic genera in wild passerine birds in Iran: the prevalence of 22.09% was recorded for *Haemoproteus* infection in north-west of Iran. *Petronia petronia, Sitta tephronota* and *Acrocephalus melanopogon* were new hosts' records captured in this region (Nourani et al., 2017a). In addition, of 136 wild birds collected in three provinces of Iran, 11 species including *Hirundo rustica, Fringilla coelebs, Passer montanus, P. domesticus, Granativora bruniceps, Carduelis* carduelis*, Acrocephalus stentoreus, A. dumetorum, Iduna pallida, Oenanthe pleschanka* and *Motacilla alba* were identified as harboring *Haemoproteus* infection (37.5%) (Nourani et al., 2018a). Molecular analysis discovered 43 lineages related to *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* which infected 35% of 330 individuals seized from eight provinces of Iran (Nourani et al., 2018b). Other morphological (Nourani et al., 2020) have been carried out on blood parasites in raptor samples collected in Iran, resulting in detection of parasite gametocytes (*Haemoproteus* and *Leucocytozoon*) in blood smears.

Nevertheless, the distribution patterns of these diverse groups of avian parasites are poorly understood in many countries including Iran (Valkiūnas, 2005). Mitochondrial DNA sequencing based on nested PCR amplifications and subsequent parasite barcoding using available data in MalAvi have revealed more than 250 morphospecies and over 3900 haemosporidian lineages (Bensch et al., 2000, 2009; Hernández-Lara et al., 2018; Martinsen et al., 2006; Pérez-Tris and Bensch, 2005). Highly sensitive molecular methods are employed to confirm the presence of various lineages of parasites, even with lower parasitemia, to supply more taxonomic data. In the current investigation, we used a 478 base pair fragment of mt-DNA cytochrome *b* gene (*cytb*) to assess the occurrence of haemoparasites in wild and domestic birds collected in different geographical locations of Iran. In the current investigation, we tried to find new haemosporidian parasite diversity and host records, and to define whether previously reported parasite lineages are harbored by different groups of birds sampled in Iran. Furthermore, we studied the phylogenetic relationships of newly discovered parasite lineages and known reported lineages.

2. Materials and methods

2.1. Ethics statement

This study was achieved in accordance with the guidelines and protocols permitted by the ethics committee for the care and use of animals for scientific purposes of the Pasteur Institute of Iran (No: IR. PII.REC.1395.96). During the blood collection, all efforts were made to reduce birds stress and suffering.

2.2. Sampling sites

Blood samples were collected from wild and domestic birds from October 2017 to July 2019. Migratory water birds were trapped using canon nets in Mazandaran province located in north of the country and the rest of wild birds were gathered by live captures in Mazandaran,

Golestan, North Khorasan, Razavi Khorasan provinces (north of Iran) and Kurdistan province (west of the country). The blood samples of captive raptors were obtained from resident birds at three rehabilitation centers, under the supervision of the Department of Environment in North Khorasan, Razavi Khorasan, and Golestan provinces. Moreover, blood samples of poultries and domestic pigeons were gathered from the hosts inhabited in a breeding center in Kurdistan province. Five tissue samples were also collected from dead specimens, provided by the Department of Environment in Mazandaran province. Moreover, Iranian Provincial Veterinary Organization provided 19 tissue specimens collected in Markazi, Hamadan, Kermanshah, Kohgiluyeh and Boyer-Ahmad, Isfahan, and Qom provinces.

2.3. Collection of blood and tissue samples

Peripheral blood was collected from the brachial vein using needles approximately 50–200 μl, preserved in anticoagulant buffer tubes and stored at −20 °C until molecular analysis. The collected tissues from birds (their spleen) were kept in absolute ethanol. Blood and tissue samples were collected from 246 birds belonging to 36 species, 12 families, and 11 orders. Concerning the number of samples from various avian families, Acciptridae members were the most captured hosts (~35%). Common teal, *Anas crecca* was the species yielding the highest number of captures (23.1%). The highest percentage of captured birds belonged to Mazandaran province. The number of examined avian hosts are shown in Table 1.

2.4. Genomic DNA extraction, PCR, and sequencing

Blood and tissue genomic DNA were extracted using PrimePrep Genomic DNA Isolation Kit (GENETBIO Inc. Daejeon, South Korea) according to the instructions provided by manufacturer. DNA extraction was carried out using 50–100 μL of blood and 10 mg of tissue samples. The quality of the extracted DNA was checked by spectrophotometer (DeNovix Inc. USA). For detection of avian haemosporidian parasites, the ~480 base pair fragments of mt-DNA *cytb* were amplified. Nested PCR targeting was conducted using the primers of HaemNFl and HaemNR3 in the first step, and the primer pairs of HaemF, HaemR2 and HaemFL and HaemR2L in the second cycle to amplify *Haemoproteus*, *Plasmodium* and *Leucocytozoon* lineages (Bensch et al., 2000; Hellgren et al., 2004). The final volume of PCR reactions was 25 μl, comprising 50–100 ng/μl of total genomic DNA, 0.6 mM of each primer, 12.5 μl AMPLIQON red PCR master mix (AMPLIQON, Denmark), ddH$_2$O (free nuclease, up to 25 μl). Besides, the ultrapure water and positive PCR product of previous experiments were used as negative and positive controls, respectively. All PCR products were visualized on 1% agarose gels, and the final positive amplicons were purified and sequenced using oligonucleotides of HaemF and HaemR2 (*Haemoproteus* and *Plasmodium*), and HaemFL and HaemR2L (*Leucocytozoon*) by BIONEER Inc. (Seoul, South Korea).

2.5. Bioinformatics, lineage identification and phylogenetic analysis

The resultant sequences were cleaned up, edited, and aligned using ClustalW implemented in BioEdit v. 7.1.7 (Hall, 1999) and MAFFT on-line version (Katoh and Standley, 2013). Parasite lineages were considered unique if they had at least one mutation in comparison with other known sequences deposited in GenBank and MalAvi databases (Bensch et al., 2009; Pérez-Tris and Bensch, 2005; Waldenstrom et al., 2002). Subsequently, amplified sequences were deposited in GenBank (accession numbers: MK929542-MK929552 & MN224223- MN224224), and more information about hosts, parasites, and sequences were made available in MalAvi data center (http://mbioserv4.mbioekol.lu.se/avianmalaria).

In order to compare the genetic diversity of the previously known lineages in the same hosts captured in this study and novel detected

Table 1

Diversity of haemosporidian lineages and hosts. Host's species, family, number of birds sampled, common name, sample types, provinces of sampling, and detected lineages, GenBank accession number, closely related lineage/Reference (s), and known reported host (s)/detection locality are summarized. New lineages are highlighted in bold.

Host family	Host species	Common name	Sample type	Provinces of sampling (n)	Detected lineages (n)	GenBank Accession No.	Similarity percentage to closely related lineage - Closely related lineage (s)/ Reference (s)	known reported host (s)/ detection locality
Acciptridae	*Accipiter nisus*	Eurasian sparrow hawk	B	M (1) R (2)	–	–		
	Aquila chrysaetos	Golden eagle	B	M (4) S (5)	¥ **Haemoproteus sp. AQUCYR01** (1)–	MK929552-	99%- ATN02 (Mata et al., 2015)–	*Athene noctua* (Morocco)
	Aquila heliaca	Eastern imperial eagle	B	M (2)	–	–		
	Aquila clanga	Greater spotted eagle	B	G (13) R (3)	*Plasmodium* sp. LK05 (1)–	MN224224–	100% LK05 (Spurgin et al., 2012; Synek et al., 2013)	*Falco naumanni Anthus berthelotii* (Spain), *Carpodacus erythrinus* (Czech Republic)
	Aquila pomarina	lesser Spotted eagle	B	G (1)	–	–		
	Aquila rapax	Tawny eagle	B	G (1)	–	–		
	Aquila sp.	Unspecified eagle	B	G (1)	–	–		
	Buteo buteo	Eurasian buzzard	B	M (2)S (3) R (15)	* *Haemoproteus* sp. MILANS02 (1)–	MK929542–	100%- MILANS02 (Pérez-Rodríguez et al., 2013)–	*Milvus migrans* (Spain)
	Buteo rufinus	Long-legged buzzard	B	M (1) K (1) G (12) R (9)	–	–		
	Circus cyaneus	Hen harrier	B	G (3)	–	–		
	Milvus migrans	Black kite	B	M (1) G (2)	–	–		
	Neophron percnopterus	Egyptian vulture	B	G (2)	–	–		
	Aegypius monachus	Cinereous vulture	B	G (1)	–	–		
	Haliaeetus albicilla	white-tailed eagle	B	G (1)	–	–		
Anatidae	*Anas acuta*	Northern pintail	B	M (4)	–	–		
	Anas crecca	Common teal	B	M (57)	¥ **Haemoproteus sp. ANACRE03** (1) *P. circumflexum* SW5 (1) * *Leucocytozoon* sp. TUSW04 (1)	MK929548 MK929544 MK929543	99%-CYGNUS01 (Ramey et al., 2016) 100%- SW5 (Bensch et al., 2007; Biedrzycka et al., 2015; Fourcade et al., 2014; Inumaru et al., 2017; Ramey et al., 2013, 2016; Svoboda et al., 2009; Tanigawa et al., 2012; Valkiūnas et al., 2014; Ventim et al., 2012; Waldenstrom et al., 2002; Yohannes et al., 2009) 100%- TUSW04 (Inumaru et al., 2017; Ramey et al., 2012; Seimon et al., 2016; Tasci et al., 2018)	CYGNUS01: *Cygnus columbianus, Anas platyrhynchos, Anas acuta, Anas discors* (USA) *Anas discors* (Canada) SW5: *Acrocephalus arundinaceus* (Sweden) *A. schoenobaenus* (Nigeria, Romania, Poland) *A. scirpaceus* (Portugal, Russia) *Anas acuta, Calidris melanotos* (USA) *Anas discors* (Canada, the United States) *Anas platyrhynchos, Calonectris leucomelas, Fulica atra, Grus japonensis, Ixobrychus eurhythmus, Podiceps cristatus* (Japan) *Crex crex* (Russia) TUSW04: *Cygnus columbianus* (USA) *Anser indicus, Phalacrocorax carbo* (Mongolia) *Anas acuta, Anas crecca, Aythya fuligula, Aythya marila* (Japan) *Anser anser* (Turkey)
	Anas platyrhynchos	Mallard	B	M (13)	* *P. circumflexum* SW5 (1)	MK929545	100%- SW5 (Bensch et al., 2007; Biedrzycka et al., 2015; Fourcade et al., 2014; Inumaru et al., 2017; Ramey et al., 2013; Ramey et al., 2016; Svoboda et al., 2009; Tanigawa et al., 2012; Valkiūnas et al.,	*Acrocephalus arundinaceus* (Sweden) *A. schoenobaenus* (Nigeria, Romania, Poland) *A. scirpaceus* (Portugal, Russia) *Anas acuta, Calidris melanotos* (USA) *Anas discors* (Canada, the United States) *Anas platyrhynchos,*

Table 1 (*continued*)

Host family	Host species	Common name	Sample type	Provinces of sampling (n)	Detected lineages (n)	GenBank Accession No.	Similarity percentage to closely related lineage - Closely related lineage (s)/ Reference (s)	known reported host (s)/ detection locality
							2014; Ventim et al., 2012; Waldenstrom et al., 2002; Yohannes et al., 2009)	*Calonectris leucomelas, Fulica atra, Grus japonensis, Ixobrychus eurhythmus, Podiceps cristatus* (Japan) *Crex crex* (Russia)
	Aythya ferina	Common pochard	B	M (4)	¥ *Haemoproteus* sp. AYTFER01 (1) ¥ *Haemoproteus* sp. AYTFER02 (1)	MK929546 MK929547	99%- CYGNUS01 (Ramey et al., 2016)	*Cygnus columbianus, Anas platyrhynchos, Anas acuta, Anas discors* (USA) *Anas discors* (Canada)
	Spatula clypeata	Northern shoveler	B	M (1)	–	–		
Laridae	*Hydrocoloeus minutus*	Little gull	B	M (1)	–	–		
Falconidae	*Falco tinnunculus*	Common kestrel	B T B	M (2) M (1) G (2) R (2)	*Haemoproteus* sp. LK03 (1)	MN224223	100% LK03 (Krone et al., 2008; Ortego et al., 2007).	*Falco tinnunculus* (Germany), *F. naumanni* (Spain)
	Falco naumanni	Lesser kestrel	B	G (2)	–	–		
Rallidae	*Fulica atra*	Common coot	B	M (19)	–	–		
Gruidae	*Grus grus*	Common crane	T	C (2)	–	–		
Strigidae	*Asio flammeus*	Short-eared owl	B	M (1)	–	–		
	Strix aluco	Tawny owl	B T	M (3) M (1)	– ¥ *Haemoproteus* sp. STAL06 (1)	– MK929551	99%- PLAMIN01(Inumaru et al., 2017) CULKIB01 (Bukauskaitė et al., 2015)	PLAMIN01: *Lanius bucephalus, Passer montanus, Platalea minor, Strix uralensis, Strix uralensis* (Japan) CULKIB01: *Strix aluco* (Russia)
	Bubo bubo	Eurasian eagle-owl	B	G (1) S (1)	–	–		
Ardeidae	*Ixobrychus minutus*	Little bittern	T	M (1)	–	–		
Phasianidae	*Gallus gallus*	Domestic fowl	B T T T T	K (3)H (2) KB (2) Q (3) C (1)	–	–		
	Alectoris chukar	Chukar	B	K (5)	–	–		
	Coturnix coturnix	Common quail	B	K (11)	–	–		
	Meleagris gallopavo	Turkey	B T T	K (2) Q (2) I (3)	–	–		
Columbidae	*Columba livia*	Domestic pigeon	B T	K (2) Ke (1)	*H. columbae* HAECOL1 (2)	MK929549 MK929550–	100%- HAECOL1 (Chagas et al., 2016; González et al., 2015; Scaglione et al., 2015a; Waldenstrom et al., 2002) –	*Columba livia* (Botswana, Nigeria, Colombia, Italy, Brazil)
	Spilopelia senegalensis	Laughing dove	T	M (1)	–	–		
Corvidae	*Corvus monedula*	Western jackdaw	B	S (1)	–	–		
Alcedinidae	*Alcedo atthis*	Common kingfisher	T	I (3)	–	–		
Total				**246**	**13**			

Samples types are B: blood and T: tissue.

The sampling sites are from Provinces including C: Markazi, G: Golestan, H: Hamadan, I: Isfahan, K: Kurdistan, KB: Kohgiluyeh and Boyer-Ahmad, Ke: Kermanshah, M: Mazandaran, Q: Qom, R: Razavi Khorasan, and S: North Khorasan.

New identified lineage of the world (¥), and new host record for Iran (*) are specified.

lineages, aforementioned sequences were retrieved from MalAvi and GenBank. The final dataset included 110 taxa and 478 characters. The genetic differences of blood parasite lineages, based on the pairwise comparison of total sequences, were calculated with Kimura 2-parameter distance matrix, implemented in MEGA6.0 software (Tamura et al., 2013). Bayesian inference analysis were accomplished with two simultaneous Markov Chain Monte Carlo searches of 10 million generations, each with sampling 1 in 1000 trees, using MrBayes v3.2 (Ronquist and Huelsenbeck, 2003). using the sequence evolution model (GTR + I + G) obtained from MODELTEST v3.7 (Posada and Crandall,

1998). To calculate the posterior probabilities at the end of the analyses, the burn-in period of 50% was arranged where the chains reached stationary status. FigTree v1.4 was used to visualize the phylogenetic consensus tree (Rambaut, 2012). As most of newly discovered lineages in this study belonged to *Haemoproteus*, median joining haplotype network was constructed using PopArt 1.7 (Bandelt et al., 1999) to be comparable with other detected lineages in the same hosts, introduced elsewhere in the world.

3. Results

3.1. Prevalence of haemosporidian parasites

Of 246 examined avian hosts, 13 individuals were found to be positive for *Haemoproteus*, *Plasmodium,* and *Leucocytozoon* (5.28%). The infection prevalence for the aforementioned genera was 3.65%, 1.21%, and 0.4%, respectively. Of 36 avian species, 9 (five raptors, three water birds, and one domestic species) were parasitized by haemosporidian parasites. Infected birds pertained to the families Acciptridae, Anatidae, Columbidae, Falconidae, and Strigidae. Most of the parasitized birds belonged to Anatidae (~46%). We did not find any haemosporidian blood parasite in other species (Table 1).

3.2. Lineages diversity and novel parasite lineages

Molecular comparison of the amplified sequences with MalAvi retrieved data, demonstrated that lineages were assigned to *Haemoproteus* (n = 8), *Plasmodium* (n = 2), and *Leucocytozoon* (n = 1) genera. To our knowledge, five lineages including hANACRE03, hAYTFER01, hAYTFER02, hAQUCYR01, and hSTAL06 were found to be novel. In addition, new host records were recorded for six known lineages of lTUSW04, pLK05, pSW5, hMILANS02, hLK03, and hHAECOL1

(*H. columbae*). Common teals were infected with hANACRE03, pSW5 (*P. circumflexum*), and lTUSW04. Moreover, common pochards were parasitized by two different *Haemoproteus* lineages of hAYTFER01 and hAYTFER02, and one mallard was infected with pSW5. Golden eagle and Eurasian buzzard (Family: Accipitridae) were infected with the distinct lineages of hAQUCYR01 and hMILANS02. Greater spotted eagle was parasitized by pLK05 and common kestrel was by hLK03. Strigid tawny owl harbored a novel lineage, hSTAL06, and two domestic pigeons were infected with hHAECOL1. No reason for co-infection by *Haemoproteus* or *Plasmodium,* and *Leucocytozoon* was found in examined hosts. Of all discovered lineages, hHAECOL1 was detected in Kurdistan, and the rest were found in Mazandaran, Golestan, and Razavi Khorasan provinces (Scientific name of birds are given in Table 1). Blood and tissue samples from other provinces were found not to be infected with haemosporidian parasites. Comparison of 110 known lineages demonstrated that the highest frequency belonged to pSGS1, distributed in Asia, Europe, Africa, North America, South America, and Oceania and the highest diversity of lineages was related to *Leucocytozoon* (~38%). The most frequent lineages in each genus were hMW1, pSGS1, and lCIAE02 and the most discovered lineages were from Europe and Asia (Fig. 1).
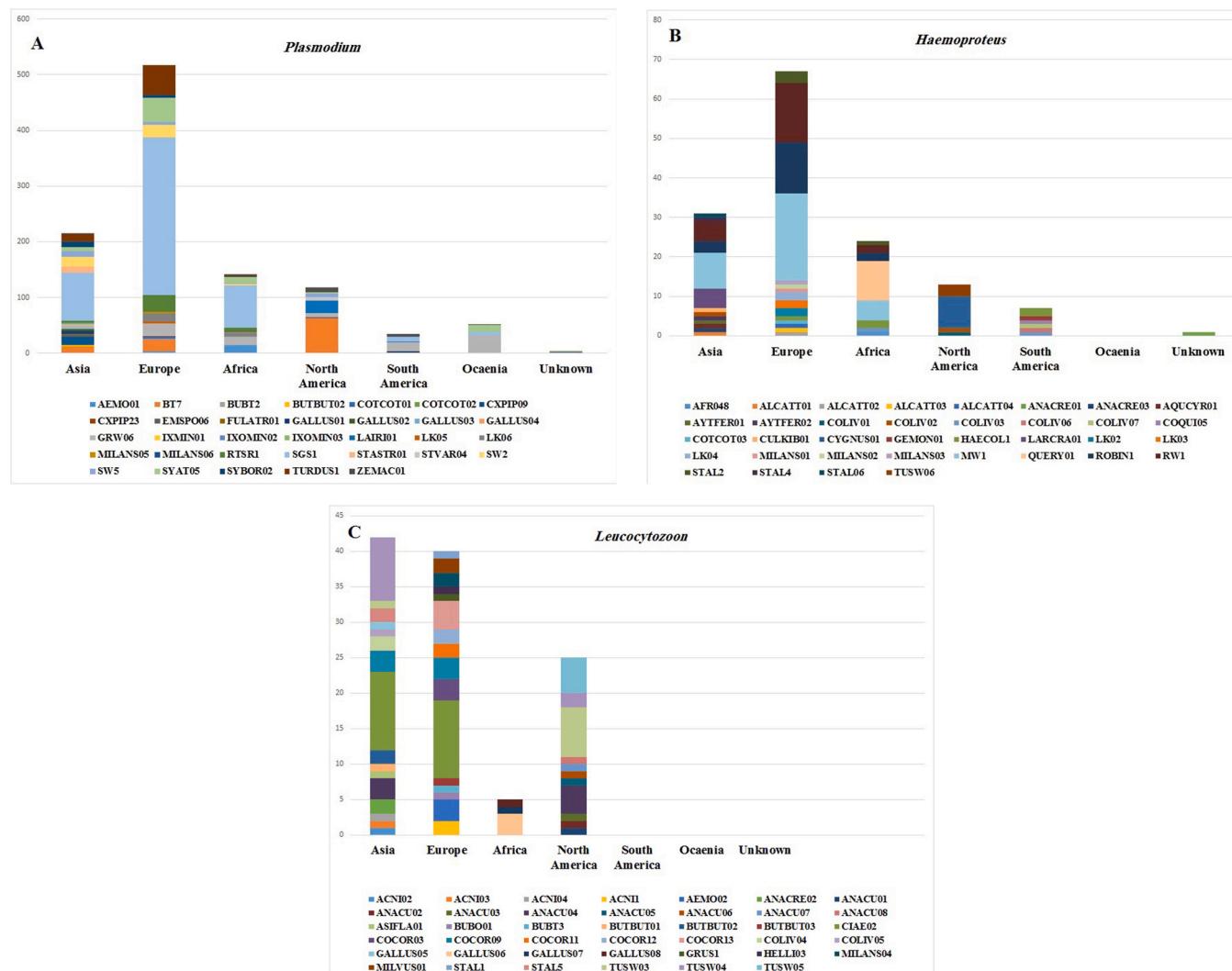

Fig. 1. Diversity of reported lineages in the same hosts in the current study. Frequency and diversity of available lineages of each continent are given from A to C. *Plasmodium* (A)*, Haemoproteus* (B) and *Leucocytozoon* (C). The Y axis is the geographic locations (continents) and the X axis is the frequency of known lineages around the world.

3.3. Phylogeny and median joining haplotype network of haemosporidian lineages

The achieved Bayesian tree of detected lineages in the current study ($n = 13$) and the lineages available for the same hosts in MalAvi ($n = 97$), reconstructed three main clades of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Fig. 2). The positioning of the lineages in the network sections (H) is comparable to the Bayesian phylogenetic tree sub-clades (Fig. 3). All *Plasmodium* lineages clustered in clade C (posterior probability = 100) in which pCOTCOT02 (*Plasmodium* sp.) originated from *Coturnix*, was placed in the basal section. *Plasmodium* lineages are positioned paraphyletic with *Haemoproteus* sub-clades (B and B1) and contain parasites discovered from different families of birds.

Haemoproteus lineages in sub-clade B (pp = 92) were assembled two paraphyletic sub-clades (B2 and B3, pp = 100). Most detected lineages of present study are placed in these sub-clades. STAL06 is clustered with CULKIB01 (*H. syrnii*) and LARCRA01, while AQUCYR01 is clustered with STAL02 (*H. syrnii*) and STAL04. The other paraphyletic branch is suggested to be Columbidae-specific (sub-clade B2) and some of detected lineages are described as *H. columbae*. This sub-clade is in accordance with H2 section in haplotype network (Fig. 3). MILANS02 is placed in sub-clade B10, closely related to previously identified lineages in Acciptridae members (MILANS01 and MILANS03). Within *Haemoproteus* clades, parasites consisted of some family-specific sub-clades; B9 (Phasianidae), B11 (Falconidae), B10 (Acciptridae), B2 (Columbidae), and B5 (Anatidae, excluding some lineages). Sub-clade B9 including lineages found in Phasianidae is consistent with H4 part in the drawn haplotype network. Sub-clade B10 consists of *Haemoproteus* lineages recognized in Acciptridae from Asia and Europe. Detected lineages in Anatidae are placed in H1 section. Sub-clade B11 detected in Falconidae is similar to H3 section (Fig. 3).

Moreover, *Leucocytozoon* lineages were placed in a monophyletic clade (A, pp = 100) with two sub-clades (A1 = 99, A2 = 100). The new TUSW04 lineage is placed in sub-clades A7 which are clustered with other parasitic taxa detected in Anatidae. *Leucocytozoon* sequences are relatively grouped within family-specific sub-clades. Within A2 and A7, all parasites fall into Acciptridae-specific and Anatidae-specific sub-clades.

4. Discussion

The novel finding of this study was the detection of 11 haemosporidian lineages, parasitizing nine species of non-passerine migratory water birds, raptors, and domestic birds in West Asia, where few studies have been carried out. To our knowledge, five lineages including hANACRE03, hAYTFER01, hAYTFER02, hAQUCYR01, and hSTAL06 were found as un-described lineages, while six known lineages of hLK03, pLK05, lTUSW04, pSW5, hMILANS02, and hHAECOL1 were recorded in hosts within novel geographical regions. Our results revealed that most of the novel lineages were found in Mazandaran and Golestan provinces located in northern Iran with a humid climate and aquatic resources including wetlands which are favorable places for breeding of dipteran vectors.

In the present study, a relatively low level of haemosporidian infection was found among 36 species which are strongly related to habitat characteristics influence parasite transmission. These factors comprised scarcity of insect vectors, resistance to parasite, sampling time, host and immune system, habitat characterization such as temperature, elevation, and rain-fall and host specifity of parasite (Bensch et al., 2012; Garcia-Longoria et al., 2019; Gutierrez-Lopez et al., 2015; Rivero de Aguilar et al., 2018; Soares et al., 2016). Blood collection of migratory birds and some raptors are performed in fall and winter, and low prevalence of haemosporidian parasites may be influenced by seasonal and sampling parameters when suitable vectors are absent. In an investigation carried out by Garvin et al., significant prevalence variation was considered as a season-related factor that showed highest and lowest levels in June and August. This temporal pattern was followed in newly infected hosts and chronic relapsing associated with hormonal alteration during breeding (Garvin et al., 2003). Absence or low prevalence of *Plasmodium* in diurnal raptors are indicated by (Ishak et al., 2010) which was reported in our samples. Another study highlighted the variation in prevalence of the same populations, suggesting the influence of sampling period (Dunn et al., 2014). Aquatic habitats of water birds such as coastal and marine are considered as inappropriate places for insect vectors to accomplish their life-cycles (Soares et al., 2016). Besides, geographical variation of competent vectors may be responsible for the prevalence variations. Low abundance of competent vectors is considered for low prevalence of haemosporidian parasites (Bensch et al., 2012; Gutierrez-Lopez et al., 2015).

Due to the high risk of exposure to infectious diseases, captive birds should be given more attentions (Chagas et al., 2017; Sehgal et al., 2006). Stress-inducing situation, such as captivity may decrease the birds' immunity either stimulate relapse of parasites or failure in clearance of parasites (Chagas et al., 2017; Sehgal et al., 2006). We found higher infection rate in raptors which may be related to their susceptibility in captivity and high numbers of examined hosts. Of 102 examined raptor (19 species) from Golestan, North Khorasan, Razavi Khorasan, Mazandaran, and Kurdistan provinces, 5 individuals (5 sp.) were infected by haemosporidians. Previously reported lineage hMILANS02 in *Milvus migrans* in Mazandaran province is suggested to be a new host record in Iran. Moreover, examined raptors were parasitized by new lineages hSTAL06 and hAQUCYR01. Among all lineages detected, the hSTAL06 was acquired through the amplification of owl spleen sample, and the rest of them were found on blood. In this study, we did not test the haemosporidians infection in both tissue and blood samples of same hosts but a comparative investigation for detecting blood parasites in *Anas acuta* in California has revealed little differences between the tissue type and infection rate of parasite genera (Ramey et al., 2013). Moreover, pLK05 was found in *A. clanga* in Golestan province whereas this lineage has been detected in Spain, and Czech Republic (Spurgin et al., 2012; Synek et al., 2013). The novel lineage hSTAL06 in *Strix aluco* in Mazandaran province has previously been detected in the same host in Russia (Bukauskaitė et al., 2015), and Japan (Inumaru et al., 2017). In other part of Iran, blood screening of Eurasian buzzards demonstrated the presence of *Leucocytozoon* spp. Infection in Northwestern (Shirazi et al., 2012) and *Circus aeruginosus* and *Aquila rapax* were recognized as hosts, parasitized by *Leucocytozoon* spp. (Rassouli et al., 2017).

Migratory birds transport parasites from high-latitude landmasses during breeding seasons and in the reversal direction back to the lower latitudes annually (Alerstam, 2003; Alerstam and Lindström, 1990). They are responsible for intercontinental dispersal of avian diseases among spatially remote locations and are principally susceptible to be infected with haemosporidian (Ilgūnas et al., 2016; Ramey et al., 2016; Valkiūnas, 2005; Waldenstrom et al., 2002). Anatidae members with characteristics like communal living in nature have extensive opportunities to be infected with dipteran vectors (Matta et al., 2014; Reisen et al., 2010). 9 species of water birds (105 individuals) from Mazandaran, Markazi, and Isfahan provinces were examined for the presence of haemosporidians and 3 species were infected (7 individuals). Our sequence analysis showed that the pSW5 lineage infected two closely related species of *Anas crecca* and *Anas platyrhynchos,* which are presented as a new host record sampled in Mazandaran province, respectively. Lineage pSW5 (*P. circumflexum*) has been reported in passerine and non-passerine species from different countries (Bensch et al., 2007; Biedrzycka et al., 2015; Fourcade et al., 2014; Inumaru et al., 2017; Krone et al., 2008; Ramey et al., 2013, 2016; Seimon et al., 2016; Svoboda et al., 2009; Tanigawa et al., 2012; Valkiūnas et al., 2014; Ventim et al., 2012; Waldenstrom et al., 2002; Yohannes et al., 2009) which are summarized in Table 1. Our results revealed that *Anas crecca* and *Aythya ferina* were parasitized by three new *Haemoproteus* lineages hANACRE03, hAYTFER01, and hAYTFER02 discovered in Mazandaran province (genetic distances of 0.2%–0.6%). Moreover, *Anas crecca* is
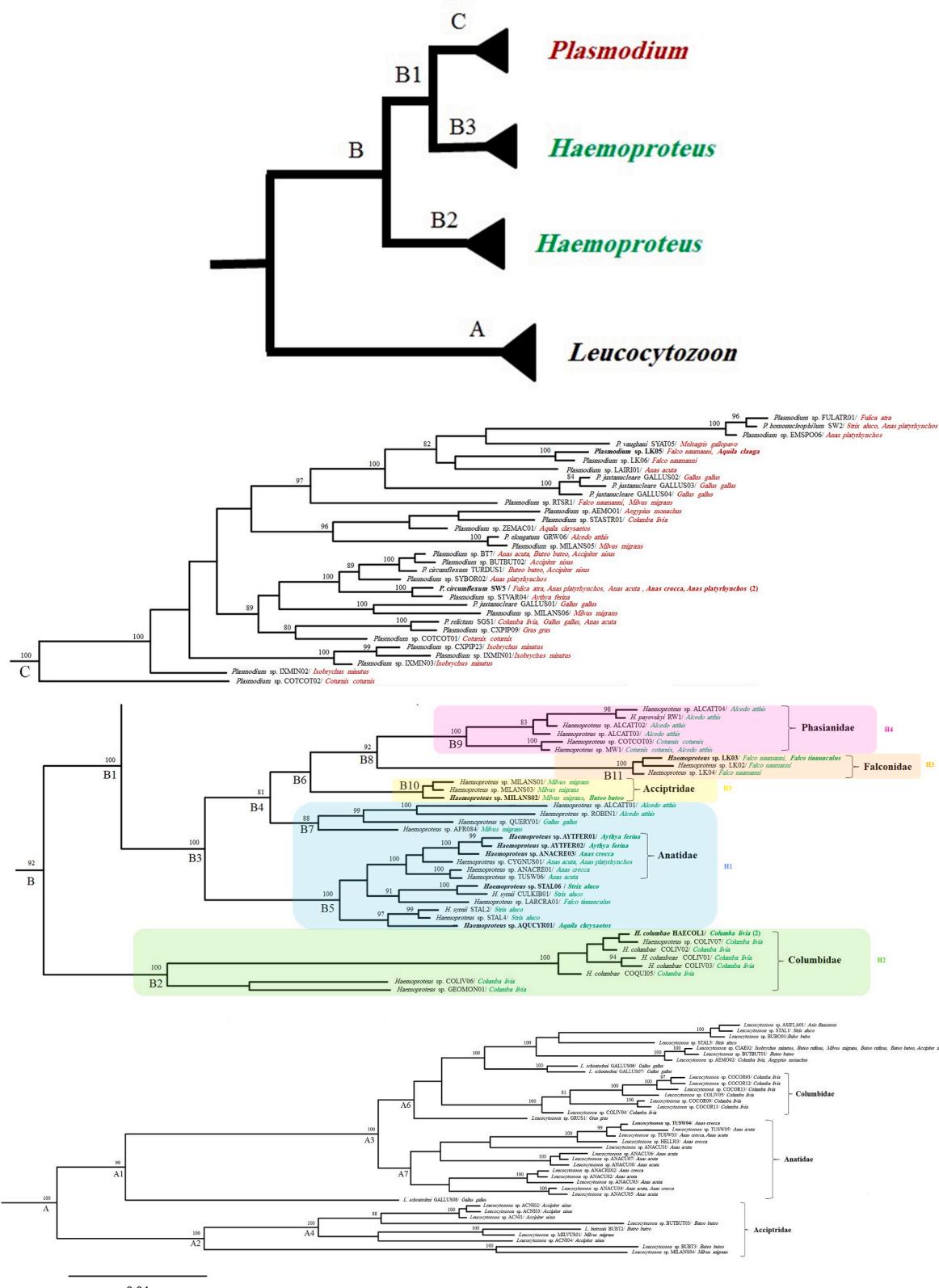
Fig. 2. Bayesian tree reconstructed using 478-bp mitochondrial *cytb* gene for avian blood parasites lineages. The amplified sequences in the current study are highlighted in bold. Posterior probability support of >0.8 is displayed for each branch. Schematic tree is summarized in section A and separated clade for each genus is given in sections of B (*Plasmodium*), C (*Haemoproteus*), and D (*Leucocytozoon*).
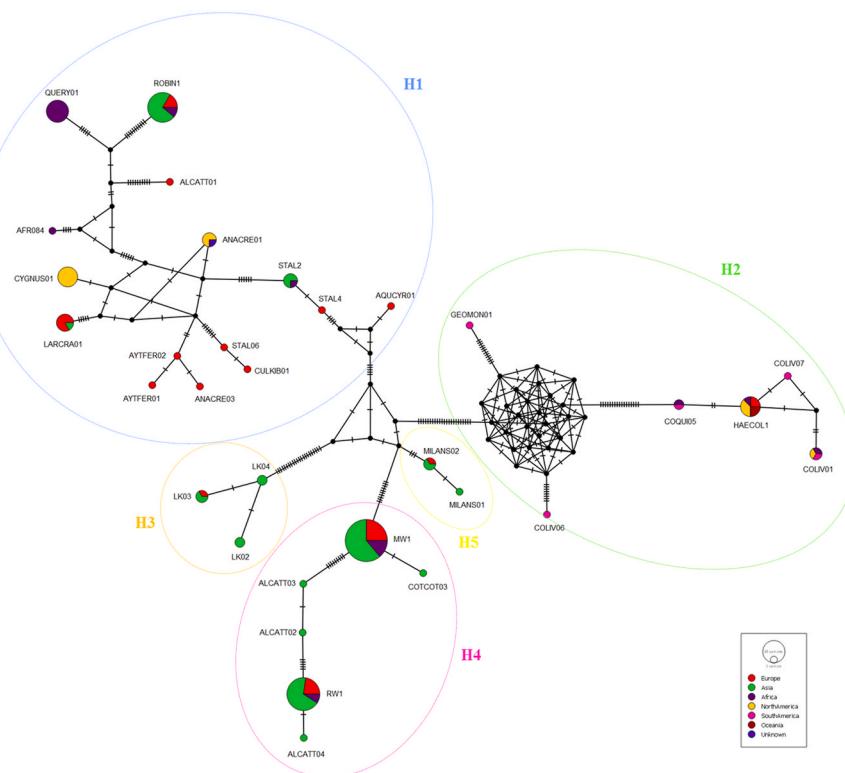
Fig. 3. Median joining haplotype network of *Haemoproteus* lineages. Detected sequences in this study are in bold.

identified as a host harbored by previously known *Leucocytozoon* lineage lTUSW04, as a new infected host captured in Iran.

Haemosporidian infections in domestic birds may lead to quality decline in meat and egg production (Opara et al., 2014). Besides, the disease caused by avian haemosporidians with a significant loss in economic issues will increase the mortality percentage up to 80% (Permin and Juhl, 2002). Hence, detection of the infection agents is required for preventive and control procedures. Although, domestic species of Phasianidae in this study were not found to be infected with avian haemosporidian, some other investigations have reported *Plasmodium* and/or *Haemoproteus* spp. in Galliformes (Atkinson et al., 1988; Greiner and Forrester, 1980; Hasson, 2015; Huchzermeyer, 1993). Of 6 examined domestic species (38 individuals) from Markazi, Hamadan, Isfahan, Kurdistan, Kohgiluyeh and Boyer-Ahmad, Kermanshah, Qom, and Mazandaran provinces, one species was infected by haemosporidians. The domestic species of *Columba livia* were infected by hHAECOL1 (*H. columbae*) which has been reported in same host in different geographic regions such as Botswana, Nigeria, Colombia, Italy, and Brazil (Chagas et al., 2016; González et al., 2015; Kawo, 2012; Scaglione et al., 2015; Waldenstrom et al., 2002). This species has been reported as a common blood parasite in previous morphological studies conducted in Iran, using light microscopy (Nematollahi et al., 2012; Samani et al., 2013) and molecular studies using PCR-RFLP method (Doosti et al., 2014; Tavassoli et al., 2018). The current study is the first report of hHAECOL1 lineage using molecular methods of blood parasites and DNA sequencing in Iran.

Although numerous studies have reported haemosporidians in avian hosts, there is no molecular investigation on migratory water birds, raptorial birds, and domestic hosts within the selected study areas. In a recent study, 20 novel lineages were recorded in birds inspected in Iran. The reported lineages in our study identified in raptors, migratory water birds and domestic birds were not similar to discovered lineages in previous investigation in passerines collected in Iran (Nourani et al., 2018b). According to previous investigations, Iranian birds in northern provinces are more infected by *Haemoproteus* than *Plasmodium* or

Leucocytozoon (Nourani et al., 2020; Nourani et al., 2017a; Nourani et al., 2018a; Nourani et al., 2017b, 2018b; Nourani and Dinparast Djadid, 2019) which is probably due to the presence of Hippoboscidae and Ceratopogonidae vectors rather than Culicidae and Simuliidae members in these sampling sites.

Despite using molecular tool for the detection of blood parasites, molecular methods for estimation of prevalence of haemosporidian parasites have some limitations: Molecular and morphological methods may fail to detect some infections and false positives should be considered (Valkiūnas et al., 2008). Biased amplification of PCR, underestimate the number of species present in the case of mixed infections which is very common in wildlife (Pérez-Tris et al., 2005; Valkiūnas et al., 2006; Valkiūnas et al., 2008). Although, PCR techniques are enabled to detect very light parasitemia of haemosporidian parasites, but they cannot distinguish DNA from circulating sporozoites of parasites that are developed into hosts or complete life cycle of parasite (Valkiūnas et al., 2009). Thus, investigations need to be continued with the utilization of morphological examination as a complementary method.

5. Conclusion

In the current investigation, new records of hosts in different groups of wild and domestic birds, as well as geographical locations are presented for haemosporidian parasites: Eight *Haemoproteus*, two *Plasmodium*, and one *Leucocytozoon* lineages were detected in blood or tissue of specimens. Such results are required to fill significant gaps in characterization of parasite lineages, discovery of novel hosts, studying host-parasite interactions, and understanding the geographical distribution patterns of wildlife vector-borne parasites.

Declarations of competing interest

The authors declare that they have no competing interests.

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