

Genetic diversity of avian blood parasites in SE Europe: Cytochrome *b* lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria

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Abstract

We used a nested PCR protocol to examine the genetic diversity of cytochrome *b* (cyt *b*) lineages from blood parasites of the genera *Plasmodium* and *Haemoproteus* in birds in Bulgaria. In total, 460 birds of 43 species and 14 families (mostly passerines) were examined for the presence of infections. Of them, 267 were recognised as infected with haemosporidian parasites. Mixed infections were recorded in 24 individuals (9%). Besides the 24 individuals with mix infections, 114 (43%) were positive for *Plasmodium* spp. and 129 (48%) for *Haemoproteus* spp. We identified 52 genetic lineages of haemosporidian parasites: 38 of *Haemoproteus* and 14 of *Plasmodium*. Twelve new cyt *b* lineages of *Haemoproteus* were recorded; they occurred in the following hosts: grey-faced woodpecker (*Picus canus*), golden oriole (*Oriolus oriolus*), jay (*Garrulus glandarius*), barred warbler (*Sylvia nisoria*), song thrush (*Turdus philomelos*), spotted flycatcher (*Muscicapa striata*), spanish sparrow (*Passer hispaniolensis*), hawfinch (*Coccothraustes coccothraustes*), and ciril bunting (*Emberiza cirrus*). We also detected 22 new host records for previously known lineages. The most common lineage was SGS1 (*Plasmodium relictum*), which had a total prevalence of 14% and occurred in 8 host species belonging to 5 families. Three of the cyt *b* lineages of genus *Haemoproteus* (DURB1, DURB2 and SYNIS2) showed more than 5% divergence from all described morphologically lineages. These lineages probably represent at least 2 different morphospecies which remains to be identified.

Keywords

Cytochrome *b* lineages, *Plasmodium*, *Haemoproteus*, specificity

Introduction

The number of the species of the order Haemosporida exceeds 400 species in all vertebrates (Valkiūnas 2005). They are parasitic in mammals, amphibians, reptiles and birds, with more than 50% of them occurring in avian hosts (Valkiūnas 2005). Blood parasites of the families Haemoproteidae and Plasmodiidae from wild birds in Bulgaria were studied by Valkiūnas *et al.* (1999), Shurulinkov and Golemansky (2002, 2003) and Shurulinkov and Chakarov (2005). However, the methodological approach of those studies has been limited to light microscopy observations. The parasitemia in birds captured in the wild is often found at low chronic levels, which make it difficult to detect parasites and very often impossible to identify species of haemosporidians in blood films (Sehgal *et al.* 2005, Valkiūnas *et al.* 2005). During the last ten years, the amplification of a specific part of the mitochondrial cytochrome *b*

(cyt *b*) gene (Bensch *et al.* 2000, Perkins and Schall 2002, Hellgren *et al.* 2004, Waldenström *et al.* 2004) has provided new opportunities in the studies of specificity, diversity, distribution, ecology and various aspects of phylogeny and evolution of avian haemosporidian parasites (Bensch *et al.* 2000, Waldenström *et al.* 2002, Beadell *et al.* 2004, Hellgren *et al.* 2004, Fallon *et al.* 2005, Križanauskienė *et al.* 2006). Recent papers have demonstrated that the diversity of cyt *b* lineages is much greater than that of the described morphospecies (Perkins and Schall 2002; Ricklefs and Fallon 2002; Waldenström *et al.* 2002; Beadell *et al.* 2004, 2006; Bensch *et al.* 2004; Pérez-Tris and Bensch 2005; Szymanski and Lovette 2005; Valkiūnas 2005). These studies suggest that many of the genetic lineages may represent distinct evolutionary entities (Bensch *et al.* 2004). The lineages of *Haemoproteus* spp. with a genetic divergence more than 5% are expected to be morphologically differentiated in most cases (Hellgren *et al.* 2007a) which

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does not mean that lineages with divergence less than 5% could not differ in morphology. There are examples of morphologically different species with *cyt b* divergences of less than 2% (Valkiūnas *et al.* 2008a, Križanauskienė *et al.* 2010). A remarkable increase of studies on *Haemoproteus* and *Plasmodium* parasites followed the first publication of the PCR protocol for identification of their lineages (for a survey, see Bensch *et al.* 2009). More than 900 parasite *cyt b* lineages have been identified; however, further information is needed about their geographical distribution and host range.

The present study is a part of a research programme for the examination of haemosporidian parasites in Bulgaria combining an experimental approach with the screening of wild populations of birds (Valkiūnas *et al.* 2007b, 2008b; Zehtindjiev *et al.* 2008, 2009). In the present article, we report results of the molecular screening of bird populations for the presence of haemosporidian parasites, mainly from wetland territories along Bulgarian parts of Danube valley and Black Sea coast. The results presented are relevant to the biodiversity and conservation of these protected areas. We also use the same data to analyse the level of host specificity and the phylogenetic relationships among the species.

Materials and methods

We screened 460 birds of 43 species and 14 families (mostly passerines) for the presence of *Plasmodium* spp. and *Haemoproteus* spp. infections (Table I). The birds were caught by mist nets. All birds were identified, ringed and measured by standard protocol applied at ringing (Svensson 1992, Bairlein

1995). The nomenclature of the birds follows Howard and Moore, 3rd edition (corrigenda 8) (2008). A small amount of blood (c. 20 µl) was taken for DNA analysis in 500 µl SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) by puncturing the brachial vein. The majority of blood samples (>90%) were collected at Kalimok Biological Station (44°00'N, 26°26'E, Silistra District) throughout the whole year in the period 2005–2008. The remaining samples (c. 10%) were collected at 8 localities: Durankulak Lake (43°41'N, 28°33'E), Shablenska Tuzla Lake (43°33'N, 28°35'E), the village of Tyulenovo (43°29'N, 28°35'E, Dobrich District), vicinities of the town of Kavarna (43°26'N, 28°20'E), the village of Kalimantsi (41°27'N, 23°28'E, Blagoevgrad District), the village of Krastatitsa (41°38'N, 24°57'E, Smolyan District), the village of Brodilovo (42°05'N, 27°51'E, Burgas District) and the village of Lekhovo (41°24'N, 23°29'E, Blagoevgrad District).

The DNA was extracted by a standard phenol-chloroform protocol (Sambrook *et al.* 1989) and quantified by spectrophotometer, (Spectromer Genesys 10 Bio) measures the absorption at 280 and 260 nm wavelengths, from which it calculates the protein and DNA concentration. Later the DNA was diluted to standard concentration of 25 ng/µl for standard PCR. Diluted genomic DNA was used as a template for amplification of a 479-bp fragment of the mitochondrial *cyt b* gene of parasites by nested PCR. The latter included two phases: initial PCR with the primers HaemFNI/HaemR3N, which amplified all three genera of haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) and subsequent PCR with primers HaemF/HaemR2 for *Haemoproteus* and *Plasmodium* (Waldenström *et al.* 2004). Positive (1–3 on

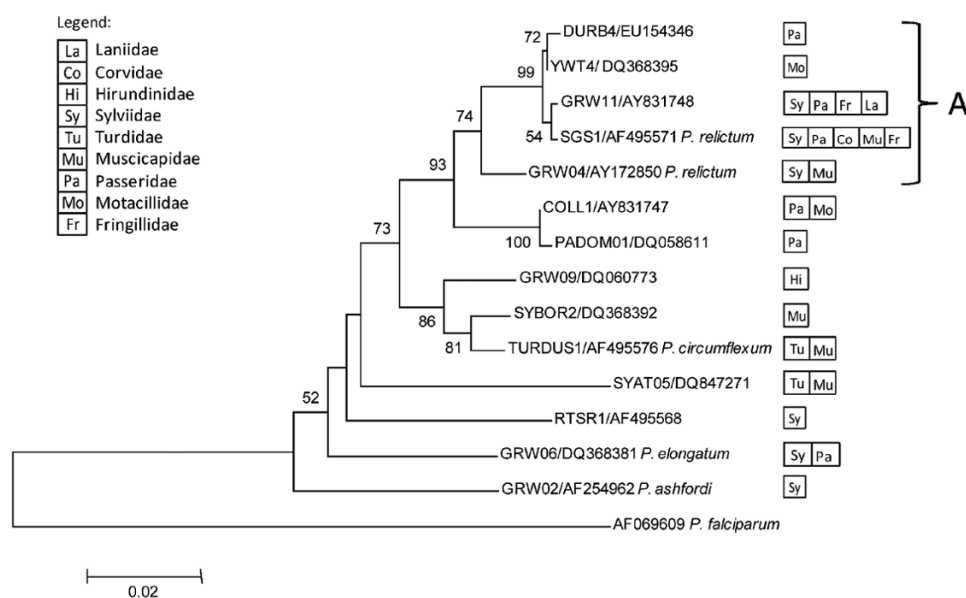


Fig. 1. A neighbour-joining (NJ) phylogenetic tree of cytochrome *b* gene lineages (479 nucleotides) of *Plasmodium* spp. The haplotype abbreviations, Gen Bank accession numbers, scientific names and host families are indicated. The tree was constructed with Kimura 2 parameter distance. Bootstrap values (>50%) are shown. Sequence of human malaria parasite, *Plasmodium falciparum*, was used as an outgroup

every PCR run) and negative (1 on every 8 samples) controls were used to verify the results of every PCR run. The samples were not examined for the presence of *Leucocytozoon* infections. The presence or absence of haemosporidian infection in the samples was evaluated by running 1.5 µl of the final PCR products on 2% agarose gel. All positive samples were sequenced by dye terminator cycling sequencing (big dye) and loaded on an ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, USA). Identified new lineages were sequenced from both directions. The sequences were edited and aligned in BioEdit (Hall 1999). They were compared with

lineages in MalAvi Public Database (Bensch *et al.* 2009). Sequences with double peaks were identified as mixed infections.

Phylogenetic analysis was conducted by MEGA, Version 4 (Tamura *et al.* 2007). Neighbour-joining trees were generated with Kimura 2-parameter distance matrix (Figs 1 and 2). The sequence divergence between and within the different lineages was calculated with the Jukes-Cantor model of substitution, with all substitutions weighted equally. All gaps and missing data in the sequences were excluded from phylogenetic analysis.

The new *cyt b* lineages were submitted to Gen Bank (for accession numbers, see Fig. 2).

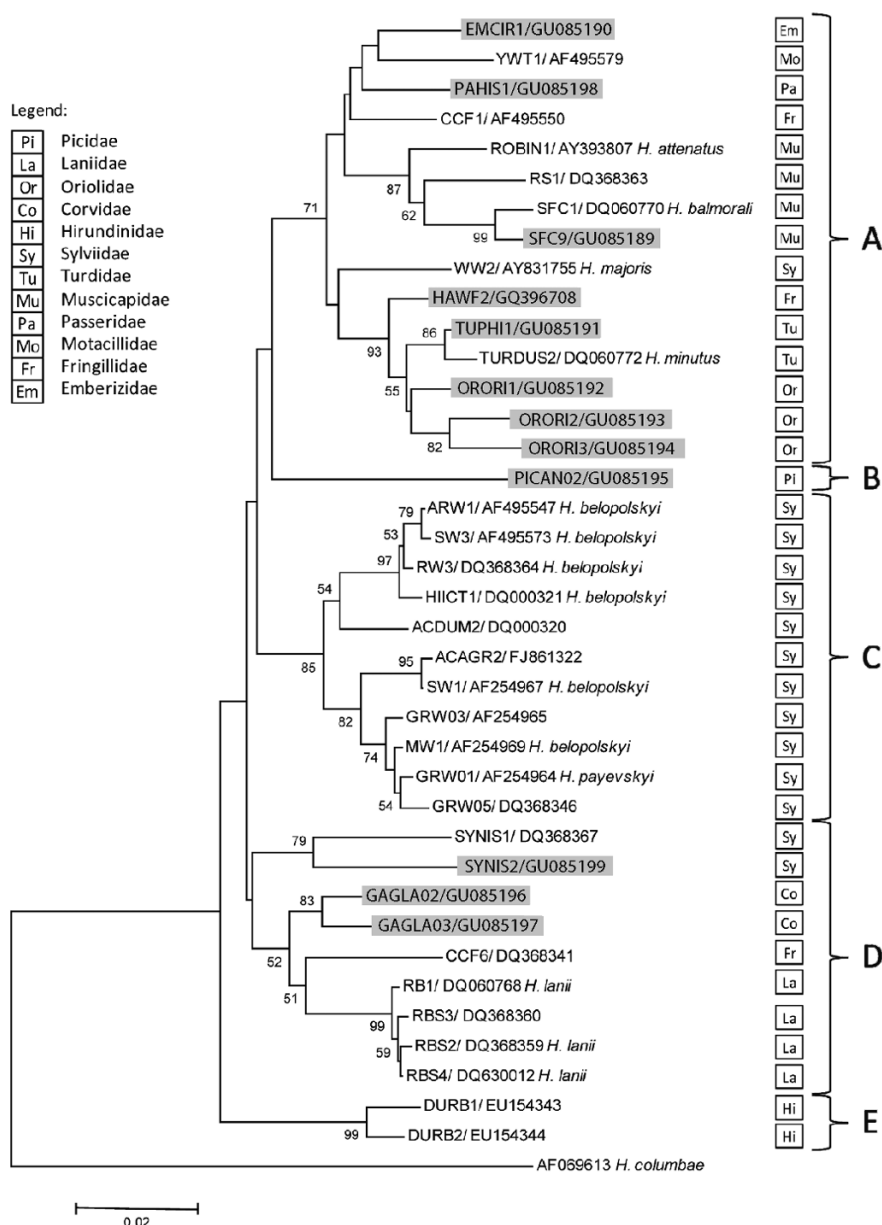


Fig. 2. A neighbour-joining (NJ) phylogenetic tree of cytochrome *b* gene lineages (479 nucleotides) of *Haemoproteus* spp. The haplotype abbreviations, Gen Bank accession numbers, scientific names and host families are indicated. The new lineages found are mark in grey. The tree was constructed with Kimura 2 parameter distance. Bootstrap values (>50%) are shown. Sequence of *Haemoproteus columbae* was used as an outgroup

Table I. List of sampled host species and number of individuals infected by single lineage of *Haemoproteus* spp. (*Haem*) and *Plasmodium* spp. (*Plasm*), or by more than one cyt *b* lineage (Mixed). The prevalence (P%) is given in percentages when 10 or more individuals have been examined. The new cyt *b* lineages are highlighted in grey. Lineages, registered for the first time for the host, are given in bold

Host species	No. of birds studied	No. of birds infected (P%)			No. of lineages	Cytochrome b lineages (infected/examined)
		<i>Haem</i>	<i>Plasm</i>	Mixed		
Meropidae						
<i>Merops apiaster</i>	1	0	0	0	0	
Picidae						
<i>Picus canus</i>	1	1	0	0	1	PICAN02 (1/1)
Laniidae						
<i>Lanius collurio</i>	10	6 (60)	1 (10)	1 (10)	4	GRW11 (1/10); RB1 (3/10); RBS2 (2/10); RBS4 (1/10)
<i>Lanius minor</i>	1	1	0	0	1	RBS3 (1/1)
Oriolidae						
<i>Oriolus oriolus</i>	7	5	0	1	3	ORORI1 (2/7); ORORI2 (2/7); ORORI3 (1/7)
Corvidae						
<i>Garrulus glandarius</i>	4	2	1	0	3	GAGLA02 (1/4); GAGLA03 (1/4); SGS1 (1/4)
<i>Pica pica</i>	1	–	–	1	–	–
Remizidae						
<i>Remiz pendulinus</i>	1	0	0	0	0	
Hirundinidae						
<i>Hirundo rustica</i>	10	0	1 (10)	0	1	GRW09 (1/10)
<i>Delichon urbicum</i>	16	0	14 (87)	0	2	DURB1 (10/16); DURB2 (4/16)
Sylviidae						
<i>Locustella luscinioides</i>	1	0	0	0	0	
<i>Acrocephalus arundinaceus</i>	114	35 (31)	27 (23)	3 (3)	9	GRW01 (27/114); GRW02 (3/114); GRW03 (1/114); GRW04 (13/114); GRW05 (7/114); GRW06 (2/114); GRW11 (1/114); RTSR1 (1/114); SGS1 (7/114)
<i>Acrocephalus schoenobaenus</i>	4	4	0	0	2	SW1 (1/4); SW3 (3/4)
<i>Acrocephalus agricola</i>	57	12 (21)	4 (7)	6 (10)	5	ACAGR2 (7/57); ACDUM2 (4/57); HIICT1 (1/57); GRW11 (1/57); SGS1 (3/57)
<i>Acrocephalus scirpaceus</i>	11	8 (73)	0	0	3	ARW1 (2/11); MW1 (4/11); SW1 (2/11)
<i>Acrocephalus palustris</i>	6	2	0	2	2	MW1 (1/6); RW3 (1/6)
<i>Hippolais icterina</i>	5	4	0	1	1	HIICT1 (4/5)
<i>Sylvia atricapilla</i>	3	1	0	0	1	WW2 (1/3)
<i>Sylvia borin</i>	2	–	–	2	–	–
<i>Sylvia nisoria</i>	6	3	2	1	3	SGS1 (2/6); SYNIS1 (2/6); SYNIS2 (1/6)
<i>Sylvia curruca</i>	1	0	0	0	0	
<i>Sylvia communis</i>	2	0	0	0	0	
<i>Sylvia cantillans</i>	2	1	1	0	2	GRW01 (1/2); GRW11 (1/2)
Regulidae						
<i>Regulus regulus</i>	1	0	0	0	0	
Turdidae						
<i>Turdus torquatus</i>	1	1	0	0	1	TURDUS2 (1/1)
<i>Turdus philomelos</i>	5	4	1	0	2	TUPHI1 (4/5); TURDUS1 (1/5)
<i>Turdus merula</i>	4	0	4	0	1	SYAT05 (4/4)
Muscicapidae						
<i>Erithacus rubecula</i>	1	1	0	0	1	ROBIN1 (1/1)
<i>Luscinia megarhynchos</i>	2	2	0	0	1	ROBIN1 (2/2)
<i>Phoenicurus phoenicurus</i>	4	1	3	0	4	GRW04 (1/4); RS1 (1/4); SGS1 (1/4); SYBOR2 (1/4)
<i>Muscicapa striata</i>	3	2	0	0	2	SFC1 (1/3); SFC9 (1/3)
<i>Ficedula parva</i>	6	1	5	0	2	SYAT05 (1/6); TURDUS1 (4/6)
Passeridae						
<i>Passer domesticus</i>	59	3 (5)	21 (36)	3 (5)	6	COLL1 (1/59); GRW06 (1/59); GRW11 (2/59); PADOM01 (1/59); PAHIS1 (3/59); SGS1 (16/59)
<i>Passer hispaniolensis</i>	15	1 (7)	3 (20)	1 (7)	3	COLL1 (1/15); GRW11 (2/15); PAHIS1 (1/15)
<i>Passer montanus</i>	56	0	36 (64)	1 (2)	3	DURB4 (1/56); GRW11 (8/56); SGS1 (27/56)
Motacillidae						
<i>Motacilla flava</i>	15	6 (40)	2 (12)	1 (7)	3	COLL1 (1/15); YWT1 (6/15); YWT4 (1/15)
<i>Motacilla alba</i>	1	0	0	0	0	

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Fringillidae

<i>Fringilla coelebs</i>	11	3 (27)	1 (9)	1 (9)	3	CCF1 (1/11); CCF6 (2/11); GRW11 (1/11)
<i>Carduelis chloris</i>	1	0	0	0	0	
<i>Carduelis carduelis</i>	2	0	1	0	1	SGS1 (1/2)
<i>Coccothraustes coccothraustes</i>	5	5	0	0	1	HAWF2 (5/5)

Emberizidae

<i>Emberiza calandra</i>	1	0	0	0	0	
<i>Emberiza cirrus</i>	1	1	0	0	1	EMCIR1 (1/1)

Results

Out of the 460 birds from 43 species examined for presence of *Plasmodium* or *Haemoproteus* infections, 267 individuals were defined as positive for these haemosporidian parasites. Birds recognised as carrying mixed infections (24 individuals, 9%) were excluded from further analyses (Table I). In the remaining 243 individuals, we identified 52 genetic lineages of haemosporidian parasites (Table II). Of the infected birds, 114 individuals (43%) were positive for *Plasmodium* spp. and 129 individuals (48%) were infected with *Haemoproteus* spp. The genetic lineages identified belonged to both genera: 38 lineages (73%) of *Haemoproteus* and 14 lineages (27%) of *Plasmodium*. Twelve of the *cyt b* lineages of *Haemoproteus* spp. were recorded for the first time: EMCIR1, GAGLA02, GAGLA03, HAWF2, ORORI1, ORORI2, ORORI3, PAHIS1, PICAN02, SFC9, SYNIS2 and TUPHI1 (marked with grey in Figure 2 and Table I). In 22 cases, we found a new host record for a particular parasite lineage (Table I).

Two *cyt b* lineages with wide host ranges (*Plasmodium* spp.) were found in birds of diverse species and families. The lineage SGS1 of *Plasmodium relictum* was identified in 5 avian families and 8 host species (Table I, Fig. 1). This lineage was recorded in juvenile birds, thus indicating local transmission in the region of Kalimok Station. The second generalist lineage is GRW11 (*Plasmodium* sp.), which was recorded in 4 bird families and 8 species (Table I, Fig. 1). The lineage GRW04 (*P. relictum*), belonging to the same morphospecies as SGS1, was found in 2 bird species of 2 families. The registered lineages from the genus *Haemoproteus* are strictly specific and no one was found to infect hosts from two different families (Fig. 2). However, there are 6 lineages recorded in two host

species (Table II). Four of these cases are among hosts from Sylviidae family, one in Muscicapidae and one in family Passeridae.

The diversity of parasite genetic lineages in each host species varied between 1 and 9 (Table I). The host with the greatest number of recorded lineages was the great reed warbler (*Acrocephalus arundinaceus*) with 9 lineages which probably reflects the sample size of this species (Table I). The prevalence of *Plasmodium* and *Haemoproteus* parasites for this species was 23% and 31%, respectively. In house sparrows (*Passer domesticus*), we recorded 5 lineages of *Plasmodium* and only 1 lineage of *Haemoproteus* (with prevalence 36% for *Plasmodium* and 5% for *Haemoproteus*). The paddyfield warblers (*Acrocephalus agricola*) harbored 5 lineages, 3 of *Haemoproteus* and 2 of *Plasmodium*, with total prevalence of 21% for genus of *Haemoproteus* and 7% for *Plasmodium*.

We constructed NJ phylogenetic trees for the lineages of *Plasmodium* (Fig. 1) and *Haemoproteus* (Fig. 2). In the constructed tree for the *Plasmodium* lineages, the cluster A (Fig. 1) formed a monophyletic group with rather high bootstrap value (74%). This cluster involved 5 lineages, i.e. DURB4, GRW04, GRW11, SGS1 and YWT4. In the phylogenetic tree of the haemoproteid lineages (Fig. 2), we defined 5 clusters (A–E). Among the sequences included in this analysis, the highest divergence was recorded within the clusters A (15 parasite lineages) and D (9 parasite lineages), with 4.4% (SE: $\pm 0.6\%$) and 3.8% (SE: $\pm 0.5\%$) mean divergence, respectively. Group C formed a more homogenous clustering, with a mean divergence of 2.3% (SE: $\pm 0.5\%$). All the lineages of this cluster were recorded in species of the family Sylviidae. Clusters B and E included only one and two lineages, respectively.

Table II. Mitochondrial *cyt b* lineages recorded in the course of the present study, number of individuals infected, number of host species infected and corresponding parasite morphospecies

Cytochrome b lineages	No. of birds infected	No. of hosts species	Morphospecies	Linked by:
ACAGR2	7	1	<i>Haemoproteus</i> sp.	
ACDUM2	4	1	<i>Haemoproteus</i> sp.	
ARW1	2	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
CCF1	1	1	<i>Haemoproteus</i> sp.	
CCF6	2	1	<i>Haemoproteus</i> sp.	
COLL1	3	3	<i>Plasmodium</i> sp.	
DURB1	10	1	<i>Haemoproteus</i> sp.	
DURB2	4	1	<i>Haemoproteus</i> sp.	

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DURB4	1	1	<i>Plasmodium</i> sp.	
EMCIR1	1	1	<i>Haemoproteus</i> sp.	
GAGLA02	1	1	<i>Haemoproteus</i> sp.	
GAGLA03	1	1	<i>Haemoproteus</i> sp.	
GRW01	28	2	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>payevskyi</i> Valkiūnas, Iezhova et Chernetsov, 1994	Bensch <i>et al.</i> (2007)
GRW02	3	1	<i>Plasmodium</i> (<i>Novyella</i>) <i>ashfordi</i> Valkiūnas, Zehtindjiev, Hellgren, Ilieva, Iezhova et Bensch, 2007	Valkiūnas <i>et al.</i> (2007b)
GRW03	1	1	<i>Haemoproteus</i> sp.	
GRW04	14	2	<i>Plasmodium</i> (<i>Haemamoeba</i>) <i>relictum</i> Grassi et Feletti, 1891	Valkiūnas <i>et al.</i> (2007b)
GRW05	7	1	<i>Haemoproteus</i> sp.	
GRW06	3	2	<i>Plasmodium</i> (<i>Huffia</i>) <i>elongatum</i> Huff, 1930	Valkiūnas <i>et al.</i> (2008b)
GRW09	1	1	<i>Plasmodium</i> sp.	
GRW11	17	8	<i>Plasmodium</i> sp.	
HAWF2	5	1	<i>Haemoproteus</i> sp.	
HIICT1	5	2	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
MW1	5	2	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
ORORI1	2	1	<i>Haemoproteus</i> sp.	
ORORI2	2	1	<i>Haemoproteus</i> sp.	
ORORI3	1	1	<i>Haemoproteus</i> sp.	
PADOM01	1	1	<i>Plasmodium</i> sp.	
PAHIS1	4	2	<i>Haemoproteus</i> sp.	
PICAN02	1	1	<i>Haemoproteus</i> sp.	
RB1	3	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>lanii</i> Mello, 1936	Hellgren <i>et al.</i> (2007a)
RBS3	1	1	<i>Haemoproteus</i> sp.	
RBS2	2	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>lanii</i> Mello, 1936	Hellgren <i>et al.</i> (2007a)
RBS4	1	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>lanii</i> Mello, 1936	Hellgren <i>et al.</i> (2007a)
ROBIN1	3	2	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>attenatus</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2006)
RS1	1	1	<i>Haemoproteus</i> sp.	
RTSR1	1	1	<i>Plasmodium</i> sp.	
RW3	1	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
SFC1	1	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>balmorali</i> Peirce, 1984	Hellgren <i>et al.</i> (2007a)
SFC9	1	1	<i>Haemoproteus</i> sp.	
SGS1	59	8	<i>Plasmodium</i> (<i>Haemamoeba</i>) <i>relictum</i> Grassi et Feletti, 1891	Palinauskas <i>et al.</i> (2007)
SW1	3	2	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
SW3	3	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>belopolskyi</i> Valkiūnas, 1989	Valkiūnas <i>et al.</i> (2007a)
SYAT05	5	2	<i>Plasmodium</i> sp.	
SYBOR2	1	1	<i>Plasmodium</i> sp.	
SYNIS1	2	1	<i>Haemoproteus</i> sp.	
SYNIS2	1	1	<i>Haemoproteus</i> sp.	
TUPHI1	4	1	<i>Haemoproteus</i> sp.	
TURDUS1	5	2	<i>Plasmodium</i> (<i>Giovannolaia</i>) <i>circumflexum</i> Kikuth, 1931	Palinauskas <i>et al.</i> (2007)
TURDUS2	1	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>minutus</i> Valkiūnas et Iezhova, 1992	Hellgren <i>et al.</i> (2007a)
WW2	1	1	<i>Haemoproteus</i> (<i>Parahaemoproteus</i>) <i>majoris</i> Laveran, 1902	Križanauskienė <i>et al.</i> (2006)
YWT1	6	1	<i>Haemoproteus</i> sp.	
YWT4	1	1	<i>Plasmodium</i> sp.	

Discussion

We present the first extensive molecular screening of avian malaria parasites in SE Europe. There are two previous publications providing data on cyt *b* lineages of haemosporidian parasites in the region. In a large-scale study of the degree of geographical shifts of transmission areas of vector-borne avian blood parasites, Hellgren *et al.* (2007b) found 4 cyt *b* lineages in great reed warblers and 1 lineage in sardinian warblers (*Sylvia melanocephala*) in samples from Bulgaria. The second study provided data on lineage diversity in a single host species, paddyfield warbler, including from the breeding population in Bulgaria (Zehtindjiev *et al.* 2009).

The relatively high number of new genetic lineages (12 lineages, representing 23% of all the recorded lineages of the genus *Haemoproteus*) suggests that more studies from this region are needed in order to cover the rich avifauna of south-east Europe (BWPi 2006). The host species with registered new cyt *b* lineages are mainly sedentary or species with south and east European distribution (BWPi 2006). The avian host in which we found the highest number of new lineages was the golden oriole (Table I). The present study is the first registration of haemosporidian cyt *b* lineages in this host and our results reflect the scarce investigations. Only two haemosporidian parasites *Haemoproteus orioli* and *Plasmodium rouxi* have been described in golden oriole before (Valkiūnas 2005).

The PCR approach for the identification of haemosporidian parasites provides a unique possibility to check various hypotheses for their specificity, phylogeny and evolution (Bensch *et al.* 2000, Waldenström *et al.* 2002, Beadell *et al.* 2004, Križanauskienė *et al.* 2006, Hellgren *et al.* 2007b). Results of the present study are in accordance with current knowledge of the host specificity of the parasite lineages. *Plasmodium* spp. are predominantly host generalists (Fig. 1) while *Haemoproteus* spp. (Fig. 2) exhibit narrower host ranges (Waldenström *et al.* 2002, Beadell *et al.* 2004). In this study there was no case of haemoproteid lineage found in more than one host family (Fig. 2). At the species level, there are 6 lineages invading two host species (GRW01, HIIC1, MW1, PAHIS1, ROBIN1 and SW1). The transmission of GRW01, recorded initially from great reed warblers, takes place in African wintering quarters of the host (Bensch *et al.* 2007). The same lineage GRW01 is recorded in one more avian host in this study (Table I).

In addition, we found seven *Plasmodium* spp. lineages recorded in only one host species (Table II). DURB4 has previously been found in house martins (*Delichon urbicum*) (Marzal *et al.* 2008), which is a long-distance migrant. However, in the present study, we found the same lineage in the tree sparrow, a resident species in the temperate zone indicating a local transmission of this lineage.

The presence of erythrocyte merogony in *Plasmodium* spp. allows investigation of experimental infections in controlled conditions. At the Kalimok Station (NE Bulgaria), in two experimental studies, three *cyt b* lineages recorded in the course of the present study (GRW02, GRW04 and GRW06) have been linked with three morphological species, i.e. *P. ashfordi*, *P. relictum* and *P. elongatum*, respectively (Valkiūnas *et al.* 2007b, 2008b). Another lineage with known morphology is SGS1, which has been described as *Plasmodium* (*Haemamoeba*) *relictum* (Palinauskas *et al.* 2007). The lineage GRW11 also belongs to the same subgenus (Križanauskienė *et al.* 2006).

The lack of erythrocyte merogony in *Haemoproteus* spp. makes it difficult to perform experimental studies of these parasites. Infections can only be done via vectors and it is difficult to maintain them (Diptera, Ceratopogonidae and Hippoboscidae) in laboratory conditions (Valkiūnas *et al.* 2002). Several recent publications presented the genetic diversity of haemoproteids in wild birds in parallel examinations with the morphology of their blood stages (Križanauskienė *et al.* 2006; Hellgren *et al.* 2007a; Valkiūnas *et al.* 2007a, 2008a). Hellgren *et al.* (2007a) stated that *Haemoproteus* spp. *cyt b* lineages with genetic divergence more than 5% could be differentiated morphologically. However, some papers (Valkiūnas *et al.* 2008a, Križanauskienė *et al.* 2010) showed that genetic divergence in *cyt b* gene between distinct morphospecies can be less than 2%. The degree of genetic divergence (>5%) indicates that 3 haplotypes (DURB1, DURB2 and SYNIS2 isolated from house martin and barred warbler) of the genus *Haemoproteus* that remain to be linked to possibly 2 distinct not yet identified morphospecies (Bensch *et al.* 2009).

The tree for haemoproteid haplotypes obtained in the course of the present study (Fig. 2) contains 5 clades. The lineage PICAN02 of *Haemoproteus* sp. forms its own cluster B showing divergences between 5.5% (with TURDUS2 of *H. minutus*) and 8.6% (with CCF6 of *Haemoproteus* sp.) compared with other haplotypes presented in the same phylogenetic tree (Fig. 2). That is expected because the lineage PICAN02 is found only in grey-faced woodpecker (*Picus canus*) which is the only non-passerine species (order Piciformes). Using the 5% criteria of Hellgren *et al.* (2007a), these should be further tested as possibly representing distinct morphospecies.

In contrast, the lineages ACDUM2, ACAGR2 and GRW03 in clade D probably belong to *H. belopolyskyi* or *H. payevskyi*, which is also included in the same group. This seems to be also the case for the lineage RBS3 (*Haemoproteus* sp.) and *H. lanii* (represented by RB1, RBS2 and RBS4), which differs only between 0.2% and 0.4% thus probably representing intraspecific haplotypes.

The present study adds to the knowledge of the diversity, specificity and possible events of host shifting among haemosporidian parasites. Contributing to the information accumulated in the MalAvi Database, this knowledge of parasite distribution will be useful in conservation biology and disease risk assessment for avian populations.

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