Diversity of avian blood parasites in wild passerine birds in Serbia with a special reference to two new lineages

Daliborka Stanković[1], Jane Jönsson[2] and Marko Raković[1]
1 Natural History Museum, Njegoševa 51, 1100 Belgrade, Republic of Serbia
2 Department of Biology, Lund University, Ecology Building, 2262 Lund, Sweden
Daliborka Stanković: daliborka@nhmbeo.rs, +381 11 3442 147

Abstract

Avian haemosporidians are vector-transmitted blood parasites distributed worldwide, abundant in many bird families and well-studied across Europe and North America. Since avian haemosporidians were poorly examined in the Palearctic migratory flyways of the Western Balkans, the goal of this study was to investigate what species of three haemosporidian genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infect both resident and migratory passerine birds in Serbia. The prevalence, distribution and parasitemia of avian haemosporidian infections were screened using both nested PCR method and microscopy observation. Out of 202 sampled birds at seven localities, 66 were infected with haemosporidians. Total prevalence was 32.7%. The great majority of infected birds (29 individuals) had moderate levels of parasitemia. The most abundant haemosporidian genus was *Haemoproteus* with prevalence of 26.1%. All infected birds were adults, whereas none of tested juveniles were infected. Mixed infection was recorded only in one bird. We identified 31 genetic lineages of haemosporidian parasites. Two new cytochrome *b* lineages of *Plasmodium* and *Leucocytozoon* were identified and found in hosts; Common Chaffinch (*Fringilla coelebs*) and Golden Oriole (*Oriolus oriolus*). We identified three new host records for previously known lineages. The lineage GRW06 (*Plasmodium elongatum*) occurred in Common Chaffinch, while the lineages PARUS20 and PARUS25 (*Leucocytozoon* sp.) were recorded in Willow Tit (*Poecile montanus*) and Crested Tit (*Lophophanes cristatus*), respectively. We found statistically significant differences in the prevalence of three haemosporidian genera among resident and partial migratory birds. The difference in mean parasitemia was significant only between residents and partial migrants.

Keywords: haemosporidians, passerines, *Plasmodium*, *Haemoproteus*, *Leucocytozoon*

Introduction

Haemosporidian parasites from the phylum Apicomplexa are distributed worldwide, from tropical and temperate to subpolar climates, except Antarctica where cold weather prevents development and appearance of particular insect vectors (Bennett et al. 1993). Avian haemosporidian parasites are well-studied across Europe (Western and Northern Europe and Eastern Balkans) (Bensch et al. 2000; Waldenström et al. 2002; Bensch and Åkesson 2003; Scheuerlein and Ricklefs 2004; Ventim et al. 2012) and North America (Ricklefs et al. 2005; Fallon et al. 2006). So far, distribution of avian haemosporidians in wild birds of the Balkan Peninsula was examined only in Bulgaria (Valkiūnas et al. 1999; Shurulinkov and Golemansky 2003; Zehtindjiev et al. 2009; Dimitrov et al. 2010). The prevalence and parasitaemia in birds used to be studied by light microscopy observation. Using this methodology, the prevalence for the genera *Haemoproteus* and *Plasmodium* in Bulgaria was estimated to be 6.3% and 9.5%, respectively (Valkiūnas et al. 1999; Shurulinkov and Golemansky 2003) and 1.3% for the genus *Leucocytozoon* (Shurulinkov and Golemansky 2003). Recent development of efficient protocols for amplification of a specific part of the mitochondrial cytochrome *b* gene (Bensch et al. 2000; Hellgren et al. 2004) have allowed scientists to conduct more detailed studies on diversity, distribution and evolution of avian haemosporidians (Fallon et al. 2003; Bensch et al. 2007; Zehtindjiev et al. 2009). Using nested PCR protocol, Dimitrov et al. (2010) have shown that, of all examined birds in Bulgaria, 43% were positive for *Plasmodium* and 48% for *Haemoproteus*.

Since there is no information about the distribution of avian haemosporidian parasites in Serbia so far, our aim was to obtain primary information on the occurrence of blood parasites in wild birds. As part of the African-Eurasian Flyway, the Western Balkans and Serbia are important for breeding populations of several species of long distant migrants that winter in tropical Africa. In this study, our objective was to investigate the distribution, prevalence and parasitemia of the parasites of the genera *Haemoproteus, Plasmodium* and *Leucocytozoon* among different migratory and resident passerines in Serbia. We consider that the results obtained are valuable for planning further experimental research on avian malaria.

Materials and methods

Using both nested PCR method and light microscopy observation, we screened a total of 202 passerine birds of 43 species and 21 families for the presence of *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. Birds were caught with mist nets during the breeding season (from April to July). All birds were identified, ringed, aged as juveniles or adults and measured using the standard ringing protocol (Svensson 1992). Sampled birds were

divided into three groups based on their migratory status: residents (stay at territory all year round), migrants (annually migrate there-and-back) and partial migrants (some individuals are migratory, some are not) (Berthold 1996). The bird nomenclature follows del Hoyo and Collar (2014). A small amount of blood, approximately 20 µl, was collected from each bird by puncturing the brachial vein. A small drop of blood was used for preparation of blood smears (one smear per bird). The remaining blood was saved for DNA analysis. We obtained blood smears from 199 individuals (one smear per bird) and 134 blood samples (one blood sample per bird). Both blood smears and blood samples were obtained for 134 birds.

Data were collected from seven localities in Serbia, out of which four are wetlands: Ludaš Lake (46º06'N 19º49'E), Ponjavica (44º44'N 20º45'E), Mala Vrbica Fishpond (44º36'N 22º40'E) and Gruža Reservoir (43º57'N 20º41'E); one Pannonic semidesert steppe, Deliblatska Sands (44º51'N 21º06'E) and two mountains: Tara (43º54'N 19º30'E) and Rtanj (43º48'N 21º50'E, foothill). Samples were collected in 2007 (only at Gruža Reservoir) and from 2011 to 2016 from the other localities. The majority of blood samples (48.5%) were collected at Tara Mountain.

The blood slides were air dried, fixed in 96% ethanol for 3 minutes in the field and stained with Giemsa in the laboratory. Blood films were examined by LEICA DMLS light microscope for about 10 minutes at low magnification (x400), and then at least 100 fields were studied at high magnification (x1000), as described by Valkiūnas et al. (2008a). Intensity of infection was estimated as a percentage of counted number of parasites per 1,000 or per 10,000 erythrocytes, as recommended by Godfrey et al. (1987).

The DNA was extracted by a standard ammonium-acetate method (Richardson et al. 2001) and quantified by NanoDrop (IMPLEN Nano photometer P330). The DNA was diluted to a standard concentration of approximately 25 ng/µl and used as a template for amplification of a 479 bp fragment of the mitochondrial cyt *b* gene of parasites by nested PCR assay (Hellgren et al. 2004). The initial PCR was carried out with the primers HaemNFI/HaemNR3, which amplified all three genera of the haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*). PCR reaction was performed in 25 µl total volume containing, per reaction: 1.5 µl $MgCl_2$ (25 mM, Applied Biosystems), 2.5 µl GeneAmp 10X PCR Buffer II (Applied Biosystems), 2.5 µl dNTP (1.25 mM, Thermo Scientific), 0.1µl AmpliTaq DNA polymerase (5 U/µl, Applied Biosystems), 1 µl of each primer (10 µM concentration), 15.4 µl of ddH_2O and 1 µl of diluted total genomic DNA template (25 ng/µl). One negative and two positive controls were used in every PCR run of sixteen samples. Nested PCR was carried out with the primers HaemF/HaemR2 for *Haemoproteus* and *Plasmodium* and HaemFL/HaemR2L only for *Leucocytozoon*. Nested PCR reaction was also performed in 25 µl total volume containing 2 µl of first PCR product as the template and the same reagents. The presence of haemosporidian infection in the samples was evaluated by running 2.5 µl of the final PCR products on 2% agarose gel. All positive samples were sequenced by Sanger sequencing, reaction with BigDye™ Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems (prod. no. 4336776). Samples were loaded on an ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, USA). The sequences were edited in BioEdit (Hall 1999) and aligned using ClustalW algorithm in MEGA version6 (Tamura et al. 2013). After aligning, the sequences were identified by using BLAST algorithm in MalAvi database (Bensch et al. 2009) and GenBank sequences (Benson et al. 2014). Two unique lineages were recorded for the first time and deposited in GenBank (accession numbers MF543057 and MF374497).

Chi-Square test with Bonferroni correction was used for pairwise comparisons to examine the association between the birds' migratory status and the presence of parasites. Kruskal-Wallis test, followed by *post-hoc* Mann-Whitney U signed-rank test with Bonferroni adjustment for pairwise comparisons, was used to assess the differences in parasitemia according to the migratory status. All statistical analyses were carried out using R v3.3.2. (R Core Team, 2016).

Results

According to both independent microscopic examination and PCR analysis, out of the 202 passerine birds of 43 species examined, 66 individuals were found to be infected with haemosporidian parasites (Table 1). All infected individual were adults. None of the juveniles were found to be infected.

Total prevalence for the three haemosporidian genera was 32.7%. It was scored by combining positive results from independent microscopic examination and screening samples with both blood smears and PCR. However, the prevalence differed between the methods. The prevalence showed higher values when PCR was used in combination with blood smears (41.8%) than use of blood smears only (21.6%) (Pearson Chi-Square $\chi^2_{(1)}=7.8724$, $p<0.01$). Considering that blood smears were good enough only for estimation of prevalence and parasitemia, for further analyses we used only data obtained by PCR. Out of 134 analysed birds, *Haemoproteus* spp. infected birds with the prevalence of 26.1%, *Plasmodium* spp. with the prevalence of 9.7% and *Leucocytozoon* spp. with the prevalence of 6.7%. In addition, we identified 31 genetic lineages: 15 lineages of *Haemoproteus* and 8 lineages of both *Plasmodium* and *Leucocytozoon*.

Two unique lineages, one of *Plasmodium* sp. and one of *Leucocytozoon* sp. were recorded for the first time and deposited in GenBank. Lineage CCF25 (MF543057) of the genus *Plasmodium* was recorded in the resident

species, Common Chaffinch (*Fringilla coelebs*), from Deliblatska sands. Lineage ORIORI04 (MF374497) from the genus *Leucocytozoon* was found in the migratory species, Golden Oriole (*Oriolus oriolus*), from Mala Vrbica Fishpond.

We identified three new host records for previously known lineages. In one case, we found a new host record for the cosmopolitan parasite lineage cyt *b* GRW06 (*Plasmodium elongatum*) that occurred in Common Chaffinch at Deliblatska Sands. Lineages PARUS20 and PARUS25 (*Leucocytozoon* spp.) were recorded for the first time in Willow Tit (*Poecile montanus*) and Crested Tit (*Lophophanes cristatus*), respectively, at Tara Mountain. Only one naturally infected Bullfinch (*Pyrrhula pyrrhula*) was positive for mixed infection with *Haemoproteus fringillae* and *Leucocytozoon majoris*. Mixed infection was recorded by using nested PCR approach.

Among the birds examined, 78 were residents, 42 were migratory and 14 were partially migratory. Resident (28 birds) and migratory birds (21 individuals) were infected with all three examined haemosporidian genera, while seven partial migrants had infections only with *Haemoproteus* spp. The majority of resident (15 birds) and migratory (12) hosts were infected with *Haemoproteus* spp., while *Plasmodium* spp. infection was present in seven residents and six migrants. *Leucocytozoon* spp. infection was established in seven residents and two migrants. Resident birds were the most infected group among the birds examined. We found a statistically significant difference between the prevalence of resident and partial migrants ($\chi^2_{(1)}$=12.145, p=0.00049), but there were no significant differences between the prevalence of residents and migrants ($\chi^2_{(1)}$=1.239, p=0.79), nor between the prevalence in migrants and partial migrants ($\chi^2_{(1)}$=4.988, p=0.07).

Lineages MW1 and ARW1 linked with the species *Haemoproteus belopolskyi* were recorded in two different host genera, while two lineages (ACAGR1 and SGS1) of the genus *Plasmodium* were found in more than one host family (Supplementary material, Table 2).

Most hosts from different localities share parasite lineages, but some of the parasites were recorded only at particular sites. Among 17 lineages recorded in total at Tara Mountain, we found 9 lineages of the genus *Haemoproteus*, 6 of *Leucocytozoon* and 2 of *Plasmodium* specific just for this area. Two different haplotypes (RBS4 and RBS2) of *Haemoproteus lanii* were recorded at two distant localities: Tara and Mala Vrbica Fishpond, respectively. Moreover, Kruskal-Wallis Chi-Square test showed absence of overall statistical difference in prevalence of haemosporidian genera among all examined localities ($\chi^2_{(1)}$=6, p=0.42). However, a Binominal test for proportions showed significant difference only between infected birds on Tara Mountain and Rtanj ($\chi^2_{(1)}$=11.829, p=0.01).

The most infected host species was Eurasian Blackbird (*Turdus merula*) with 80% of the examined birds infected only with *Haemoproteus* spp. Common Chaffinch and Eurasian Blackcaps (*Sylvia atricapilla*) also had very high infection levels, with 70% and 58.3% of examined individuals, respectively. Common Chaffinches were infected with *Haemoproteus* spp. and *Plasmodium* spp., while Eurasian Blackcaps were infected with *Haemoproteus* spp.

Calculated parasitemia varied between 0.01% (low and chronic parasitemia) and 4.8% (high parasitemia). Low parasitemia of 0.01% was found in 23 birds. The great majority of hosts (29 birds) had moderate parasitemia between 0.01% - 1%, while high parasitemia above 1.1% was found in 14 birds. The highest parasitemia of 4.8% was found in one Red-backed Shrike (*Lanius collurio*) sampled at Mala Vrbica Fishpond in 2016. Birds with chronic parasitemia were infected with all three haemosporidian genera, as were hosts with moderate parasitemia, while those with high parasitemia had infection with only *Haemoproteus* spp. and one *Plasmodium* spp. Of resident birds, most had either low or moderate level of parasitemia, while high parasitemia was predominantly found in partial migrants.

Mean parasitemia differs significantly between the migratory groups (Supplementary material, figure 1). The highest mean parasitemia of 1.9% was found in partial migrants such as European Robin (*Erithacus rubecula*) and Song Thrush (*Turdus philomelos*) while it was lower in residents and migrants, ranging from 0.3% to 0.6%, respectively. However, Kruskal-Wallis test with *post-hoc* Mann-Whitney U test and Bonferroni correction showed that a statistically significant difference in average parasitemia exists only between resident and partial migrants (U =269, p=0.02); the differences were not statistically significant between residents and migrants (U=426, p=0.939), and migrants and partial migrants (U=72.5, p=0.137).

We detected significant differences in average parasitemia between the examined migratory groups. For resident birds, there was a significant difference in parasitemia only between hosts infected with *Haemoproteus* and *Leucocytozoon* (U=116.5, p=0.0032), while there were no differences between birds infected with *Haemoproteus* and *Plasmodium* (U=90.5, p=0.168) nor between birds infected with *Plasmodium* and *Leucocytozoon* (U=17, p=0.262). On the other hand migrants showed a different pattern: a significant difference was observed among birds infected with *Haemoproteus* and *Plasmodium* (U=82.5, p=0.0034), while there were no difference among birds infected with *Haemoproteus* and *Leucocytozoon* (U=18.5, p=0.653) and *Plasmodium* and *Leucocytozoon* (U=8.5, p=0.382).

Table 1. List of sampled host species (according to their migratory status), places and number of infected individuals by single lineages of *Haemoproteus* spp. *Plasmodium* spp. and *Leucocytozoon* spp.

Family	Prevalence per family (%)	Host species (migratory status)	Sampling site	No. of sampled birds per site	*Haemoproteus* cyt *b* lineages (no. of infected birds)	*Plasmodium* cyt *b* lineages (no. of infected birds)	*Leucocytozoon* cyt *b* lineages (no. of infected birds)
Oriolidae		*Oriolus oriolus* (M)	Mala Vrbica fishpond	1			**ORIORI04** (1)
Laniidae		*Lanius collurio* (M)	Mt Tara	1	RBS4 *H. lanii* (1)		
			Mala Vrbica fishpond	2	RBS2 *H. lanii* (1)		
Corvidae		*Garrulus glandarius* (R)	Mt Tara	1	GAGLA02 (1)		
Paridae	33.3	*Periparus ater* (R)	Mt Tara	8		ACAGR1 (1)	PARUS7 (2)
		Lophophanes cristatus (R)	Mt Tara	3	*Haemoproteus* spp. (1)		PARUS25 (1)
		Poecile montanus (R)	Mt Tara	2			PARUS20 (1), PARUS78 (1)
		Cyanistes caeruleus (R)	Mala Vrbica fishpond	1			
		Parus major (R)	Gruža reservoir	1			
			Mt Rtanj	1			
			Ponjavica	5			
Remizidae		*Remiz pendulinus* (pM)	Ludaš lake	1			
			Mt Rtanj	1			
Alaudidae		*Alauda arvensis* (M)	Mala Vrbica fishpond	1			
Acrocephalidae	47	*Iduna pallida* (M)	Mala Vrbica fishpond	2	HIP2 (1), MW1 *H. belopolskyi* (1)		
		Acrocephalus melanopogon (M)	Ludaš lake	1			
		Acrocephalus palustris (M)	Ponjavica	3		SGS1[b] (1), SYBOR21 (1)	
		Acrocephalus scirpaceus (M)	Ponjavica	2	MW1 *H. belopolskyi* (1)		
			Ludaš lake	2	ARW1*H. belopolskyi* (2)		
			Mala Vrbica fishpond	1			
		Acrocephalus arundinaceus (M)	Mala Vrbica fishpond	5		SGS1[b](1)	
			Ludaš lake	1			
Phylloscopidae		*Phylloscopus collybita* (M)	Deliblatska Sands	2			
			Mt Tara	3			
Aegithalidae		*Aegithalos caudatus* (R)	Mt Rtanj	6			
Sylviidae	64.7	*Sylvia atricapilla* (M)	Gruža reservoir	1			
			Deliblatska Sands	2	*Haemoproteus* spp. (1)		

			Ponjavica	3	ARW1 *H. belopolskyi* (1), SYAT02 *H. parabelopolskyi* (1)		
			Mala Vrbica fishpond	1			
			Mt Rtanj	1	*Haemoproteus* spp. (1)		
			Mt Tara	4	SYAT01 *H. parabelopolskyi* (2), SYAT10 (1)		
		Sylvia borin (M)	Deliblatska peščara sands	1	*Haemoproteus* spp. (1)		
		Sylvia nisoria (M)	Mala Vrbica fishpond	1		GRW11 *P. relictum* (1)	
		Sylvia communis (M)	Mala Vrbica fishpond	3		SGS1[b] (1)	RS4 (1)
Certhiidae		*Certhia familiaris* (R)	Mt Tara	2			PARUS22 (1)
Sittidae		*Sitta europea* (R)	Deliblatska Sands	1			
			Mt Rtanj	1			
Sturnidae		*Sturnus vulgaris* (R)	Mala Vrbica fishpond	1			
			Ponjavica	1			
Turdidae	78.9	*Turdus philomelos* (pM)	Mt Tara	4	TUPHI01 *H. minutus* (3)		
		Turdus merula (R)	Gruža reservoir	3	*Haemoproteus* spp. (1)		
			Mt Tara	9	TURDUS2 *H. minutus* (7), *Haemoproteus* spp. (2)		
			Mt Rtanj	3	*Haemoproteus* sp.		
Muscicapidae	17	*Erithacus rubecula* (pM)	Reservoir Gruža	1	ROBIN1 *H. attenuatus* (1)		
			Rtanj	1	*Haemoproteus* spp. (1)		
			Mt Tara	35	ROBIN1 *H. attenuatus* (3), *Haemoproteus* spp. (3)		
		Luscinia megarhynchos (M)	Gruža reservoir	2			
			Ponjavica	4			
			Fishpond Mala Vrbica	1			
		Phoenicurus ochruros (pM)	MtTara	2			
		Phoenicurus phoenicurus (M)	Gruža reservoir	1			
Regulidae		*Regulus regulus* (R)	Mt Tara	7			

		Regulus ignicapillus (R)	Mt Tara	4			
Prunellidae		*Prunella modularis* (R)	Mt Tara	5		SYAT05 *P. vaughani* (1)	
Passeridae	11.1	*Passer domesticus* (R)	Mt Rtanj	7		SGS1[b] (1)	
			Ponjavica	5			
		Passer hispaniolensis (R)	Mala Vrbica fishpond	1		SGS1[b] (1)	
		Passer montanus (R)	Ludaš lake	1			
			Mala Vrbica fishpond	1			
			Mt Rtanj	1			
			Ponjavica	2			
Motacillidae		*Motacilla cinerea* (M)	Mala Vrbica fishpond	1			
Fringillidae	53.3	*Fringilla coelebs* (R)	Deliblatska Sands	4	CCF2 (1)	ACAGR1 (1); CCF25[a] (1); GRW06 *P. elongatum* (1)	
			Mt Rtanj	2	*Haemoproteus* spp. (1)		
			Mt Tara	4	CCF6 (2)		
		Pyrrhula pyrrhula (R)	Mt Tara	4	CCF3 *H. fringillae* (1) [c]		CB1 *L. majoris*[c]
		Carduelis carduelis (R)	Gruža reservoir	1			
Emberizidae		*Emberiza melanocephala* (M)	Mala Vrbica fishpond	1		AEDVEX01 (1)	
		Emberiza citrinella (R)	Deliblatska Sands	4			
		Emberiza schoeniclus (R)	Ludaš lake	1			

The prevalence (%) is given per family when 10 or more individuals have been examined.
cyt b Cytochrome b, *R* residents, *M* migrants, *pM* partial migrants.
[a] Lineages registered for the first time given in bold
[b] Possibly SGS1 (*Plasmodium relictum*) due to an undetermined nucleotide at position 9 (5')
c Mixed infection

In our study, the females had higher average parasitemia (0.86%) than males (0.65 %). However, statistical analysis showed that there were no significant differences in average parasitemia between the sexes (U=416.5, p=0.0652).

Discussion

Many studies of avian haemosporidian parasites using traditional microscopy (Valkiūnas 1999; Shurulinkov and Golemansky 2003) or molecular approaches (Zehtindjiev et al. 2009; Dimitrov 2010) have been published for Southeaster Europe, but not for the Western Balkans. We present the first results of a molecular and microscopic overview of avian malaria parasites in Serbia, in the Western Balkans.

Overall prevalence, obtained by independently scoring blood slides with results from molecular methods (PCR), in combination with only microscopic examination where both samples were available, was 32.7%. However, the prevalence obtained by PCR (combined with microscopic examination) was significantly higher. Results were not consistent with the study of Valkiūnas et al. (2008a) who showed that overall prevalence of infection was similar after the PCR and microscopic examination, probably due to the good quality of the slides.

Several studies on blood parasites in wild passerines across Europe aimed at amplifying the cyt *b* gene and showed prevalence for *Haemoproteus* of 5.1%, 48% and 17.7%, and for *Plasmodium* of 15.4%, 43% and 82.3% in Germany, Bulgaria and Spain, respectively (Wiersch et al. 2007; Dimitrov et al. 2010; Ventim et al. 2012). As Valkiūnas et al. (2008a) noted, the detection of the genus *Leucocytozoon* was usually omitted due to unskilled

microscopic observation with low prevalence. However, using PCR based methods for *Leucocytozoon*, studies in European passerines recorded prevalence for *Leucocytozoon* of 30%, 2.4% and 85.3% (Valkiūnas et al. 2008a; Rönn et al. 2015; Schmid et al. 2017). Our results are consistent with the literature (Zehtindjiev et al. 2009; Dimitrov 2010) as the most common haemosporidian genus was *Haemoproteus*, ahead of *Plasmodium* and *Leucocytozoon*, respectively.

We recorded two new lineages from the genera *Plasmodium* and *Leucocytozoon*. Lineage CCF25 from the genus *Plasmodium* was found in Common Chaffinch and lineage ORIORI04 from the genus *Leucocytozoon* was isolated from Golden Oriole. To date, only the *Haemoproteus* lineages (ORIORI01, ORIORI02 and ORIORI03) (Dimitrov et al. 2010) and *Plasmodium rouxi* (Valkiūnas 2005) were isolated from Golden Oriole, but no *Leucocytozoon.*

The parasite lineage GRW06, linked with morphological species *Plasmodium elongatum*, was so far recorded in 57 bird hosts (MalAvi database), including Great Reed Warbler (*Acrocephalus arundinaceus*) and House Sparrow (*Passer domesticus*) from Bulgaria (Valkiūnas et al. 2008b). For the first time, we isolated the lineage GRW6 from Common Chaffinch at Deliblatska sands in Serbia.

According to the occurrence of separate bands of both infections in the sequence electrophoregrams, only one Bullfinch had mixed infection with *Haemoproteus fringillae* and *Leucocytozoon majoris*. The detection efficiency might vary due to low quality sequences, because of the combination of parasite lineages or the intensity of infection (Pérez-Tris and Bensch 2005; Zehtindjiev et al. 2012). We could not identify with certainty other mixed infections in our samples, probably due to weak peaks of the lineage with lower parasitemia, as explained by Perez-Tris and Bensch (2005).

In an examination of 460 wild passerines (both migrants and residents) in Bulgaria, Dimitrov et al. (2010) found 267 birds infected with haemosporidian parasites and identified 52 lineages. Of those 52 lineages, 38 belonged to the genus *Haemoproteus* and 14 to the genus *Plasmodium*. Hellgren et al. (2009) obtained similar results in Western Europe: 63 lineages in the genus *Haemoproteus* and 35 *Plasmodium* lineages. In our study of 202 wild birds, we found 66 individuals that tested positive for haemosporidian parasites and we identified 31 genetic lineages, 15 of which belonged to the genus *Haemoproteus* and 8 to each of the *Plasmodium* and *Leucocytozoon* genera. This shows that the most common haemosporidian parasites in wild passerine birds in Serbia belong to the genus *Haemoproteus*.

Latta and Ricklefs (2010) have shown that haemosporidian prevalence can differ significantly depending on the migratory status of the birds. According to the authors, the most infected bird species were residents, mostly infected by the *Haemoproteus* species, while the endemic residents had the highest rates of infection and the parasite assemblage was dominated by the *Plasmodium* lineages. In our study, among 78 residents, 42 migrants and 7 partial migrants, residents were the most infected group of birds, with the presence of all three haemosporidian genera in 28 individuals. Migrants (21 infected birds) were also infected by all three haemosporidian genera, whereas partial migrants (7 birds) were infected only with the genus *Haemoproteus*. Unlike Ventim et al. (2012), who found the genus *Haemoproteus* only in migratory birds, we found that the residents were mostly infected by *Haemoproteus*. Moreover, infection with the genus *Plasmodium* was established both in resident birds (young and adults) and young migrants (Ventim et al. 2012). We found *Plasmodium* spp. and *Leucocytozoon* spp. infections in both residents and migrants. None of the juvenile birds in our sample were infected, which lead us to conclude that adults might obtain the haemosporidian infection in the non-breeding part of their range or that young birds were insufficiently exposed to vectors to become infected at the nest sites. However, we did not investigate the diversity of vectors at the breeding sites, which is the next step necessary to obtain sufficient information for better understanding of haemosporidian infection in Serbian birds.

As shown by Waldenström et al. (2002), *Haemoproteus* spp., which infect fewer host species, are considered host specialists, while *Plasmodium* spp. are predominantly host generalists. Accordingly, we detected two *Haemoproteus* and one *Plasmodium* lineage infecting different host species and genera. Using the same bird nomenclature as Waldenström et al. (2002), we observed the same pattern; host species infected with *Plasmodium* lineages were from different families, whereas host species infected with *Haemoproteus* lineages were from the same family.

According to Valkiūnas (2005), who followed the bird nomenclature after Sibley and Monroe (1990), the most infected bird family was Sylviidae. Applying the same systematics to this study, we observed the same pattern as Valkiūnas (2005) and Palinauskas et al. (2005): birds from the family Sylviidae were infected with all three examined haemosporidian genera, while species from the family Turdidae were infected only with *Haemoproteus* spp. Such a difference is probably due to the ecological demands of the family Turdidae during the breeding season; species from this family place their nests on the ground or 1.5 – 2 m above the ground (Cramp 1988) where there is higher activity of biting midges as potential vectors of *Haemoproteus spp*. (Diarra et al. 2014).

The most infected host species was Eurasian Blackbird. It was found that 80% of examined Eurasian Blackbirds were infected, which is in accordance with the studies conducted in Great Britain and the Azores

(Hatchwell et al. 2000; Hellgren et al. 2011), where 80% and 57% of the examined Eurasian Blackbirds were infected respectively. Moreover, Bentz et al. (2006) found only one *Haemoproteus* lineage and two *Plasmodium* lineages in Eurasian Blackbird, while Hatchwell et al. (2000) determined all three haemosporidian genera. Unlike the listed authors, we found only one lineage from the genus *Haemoproteus* (TURDUS2), most likely due to the lack of suitable insect vectors for other haemosporidian genera at sampling habitats and locations in Serbia. Eurasian Blackcaps were also highly infected, with a prevalence of 58.3% but only with the *Haemoproteus* spp. which is in line with the research of Arizaga et al. (2010) from Spain, where 34.1% of Eurasian Blackcaps were infected with both *Haemoproteus* and *Plasmodium* genera. Similarly to Eurasian Blackbird, the absence of other haemosporidian genera in Eurasian Blackcaps could be due to the lack of insect vectors for the *Plasmodium* and *Leucocytozoon* lineages at sampling sites in Serbia.

Parasitemia of less than 0.01% was considered as a chronic infection and more than 80% of infected birds from places in Europe, North America and Africa had parasitemia less than 0.01% (Valkiūnas et al. 2008a). However, in our study, large number of infected birds (14.3%) had moderate infection (mean 0.4%, σ = 32.79%). Since birds with moderate infection were residents, one could say that breeding grounds in Serbia had suitable vectors for haemosporidia and birds were exposed for long enough.

Contrary to the theory that female birds are usually less infected than males, due to better immunity (Zuk, 1990), many studies report that females had higher parasitemia than males (Hatchwell et al. 2000; Bentz et al. 2006; Asghar et al. 2011; Sorensen et al. 2016). In our study, although females had higher medium parasitemia than males, we did not find any statistically significant difference.

The results presented in this paper are the first data about distribution of haemosporidian genera in wild birds in Serbia. We found resident birds to be the most infected group of birds in contrast to migratory and partial migratory birds; however, partial migrants have the highest parasitemia. *Haemoproteus* spp. tend to be the most common haemosporidian genus in wild passerines in Serbia. Future research on both the parasites and the vectors in Serbia will give better insights into their relationships and presence.

Acknowledgement

Laboratory work was supported partially by the Natural History Museum in Belgrade, Serbia and the Lund University, Department of Biology, Sweden. Aleksandra Urošević is acknowledged for her help in the laboratory at the Institute for Medical Research, University of Belgrade, Serbia. We are thankful to Dr. Staffan Bensch and two unknown reviewers for valuable comments on earlier versions of the manuscript. We thank Dr. Bojana Stanić for proof reading the manuscript and Nicola Crockford for correcting the English. The samples involved in this study comply with the current legislation of the Ministry of Environmental Protection of the Republic of Serbia.

Literature

Arizaga J, Esparza X, Barba E. (2010): Haemosporidians in migratory blackcaps (*Sylvia atricapilla*): a comparison between autumn and spring periods of passage. Anales de Biología 32: 87-93.

Asghar M, Hasselquist D, Bensch S. (2011): Are chronic avian haemosporidian infections costly in wild birds. Journal of Avian Biology 42: 530-537.

Bennett G. F, Peirce MA, Ashford RW. (1993): Avian Haematozoa: mortality and pathogenicity. Journal of Natural History 27 (5): 993-1001. [Taylor & Francis Online].

Bensch S, Stjernman M, Hasselquist D, Östman Ö, Hansson B, Westerdahl H, Pinheiro RT. (2000): Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proceedings of the Royal Society of London, Series B 267: 1583-1589.

Bensch S, Åkesson S. (2003): Temporal and spatial variation of Hematozoans in Scandinavian Willow Warblers. Journal of Parasitology, 89: 388–391.

Bensch S, Waldenström J, Jonzén N, Westerdahl H, Hansson B, Sejberg D, Hasselquist D. (2007): Temporal dynamic and diversity of avian malaria parasites in a single host species. Journal of Animal Ecology 76: 112-122.

Bensch S, Hellgren O, Pérez-Tris J. (2009): MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Molecular Ecology Resources 9: 1353-1358.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers E.W. (2014): GenBank. Nucleic Acids Research 41: 36-42.

Bentz S, Rigaud T, Barroca M, Martin-Laurent F, Bru D, Moreau J, Faivre B. (2006): Sensitive measure of prevalence and parasitaemia of haemosporidia from European blackbird (*Turdus merula*) populations: value of PCR-RFLP and quantitative PCR. Parasitology 133: 685-692.

Berthold P. (1996): Control of bird migration. Chapman & Hall, London, pp: 25-26.

Cramp S. (1988): Handbook of Europe the Middle East and North America. The Birds of the Western Palearctic, Volume V. Oxford University Press.
del Hoyo J, Collar N. J. (2014): HBW and BirdLife International Illustrated Checklist of the Birds of the World. Volume 2: Passerines. Lynx Edicions, Barcelona.
Dimitrov D, Zehtindjiev P, Bensch S. (2010): Genetic diversity of avian blood parasites in SE Europe: cytochrome *b* lineages of the genera *Plasmodium* and *Haemoproteus* (Haemosporida) from Bulgaria. Acta Parasitologica 55: 201-209.
Diarra M, Fall M, Fall GA, Diop A, Seck MT, Garros C, Balenghien T, Allène X, Rakotoarivony I, Lancelot R, Mall I, Bakhoum M, Dosum AM, Ndao M, Bouyer J, Guis H. (2014): Seasonal dynamics of *Culicoides* (Diptera: Ceratopogonidae) biting midges, potential vectors of African horse sickness and bluetongue viruses in the Niayes area of Senegal. Parasites and Vectors 7: 147.
Fallon SM, Ricklefs RE, Swanson BL, Bermingham E. (2003): Detecting avian malaria: an improved polymerase chain reaction diagnostic. Journal of Parasitology 89 (5): 1044–1047.
Fallon SM, Fleischer RC, Graves GR. (2006): Malarial parasites as geographical markers in migratory birds? Biology letters 2: 213-216.
Godfrey RD, Fedynich AM, Pence DB. (1987): Quantification of hematozoa in blood smears. Journal of Wildlife Diseases, 23: 558–565.
Hall TA. (1999): BIOEDIT: a user friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. Nucleic Acid Symposium Series 41: 95-98.
Hatchwell BJ, Wood MJ, Anwar M, Perrins CM. (2000): The prevalence and ecology of the haematozoan parasites of European black birds, *Turdus merula*. Canadian Journal of Zoology 78 (4): 684-687.
Hellgren O, Waldenström J, Bensch S. (2004): A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. Journal of Parasitology: 90 (4): 797–802.
Hellgren O, Pérez-Tris J, Bensch S. (2009): A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. Ecology 90 (10): 2840–2849.
Hellgren O, Križanauskienė A, Hasselquist D, Bensch S. (2011): Low haemosporidian diversity and one key-host species in a bird malaria community on a mid-Atlantic island (São Miguel, Azores). Journal of Wildlife Diseases 47 (4): 849-859.
Latta SC, Ricklefs RE. (2010): Prevalence patterns of avian haemosporida on Hispaniola. Journal of Avian Biology 41: 25-33.
MalAvi. A database for avian haemosporidian parasites. www.mbio-serv.mbioekol.lu.se/Malavi. (Accessed on 23 December 2017).
Palinauskas V, Markovets MY, Kosarev VV, Efremov VD, Sokolov LV, Valkiûnas G. (2005): Occurrence of avian haematozoa in Ekaterinburg and Irkutsk districts of Russia. Ekologija (Lithuania) 4: 8-12.
Pérez-Tris J, Bensch S. (2005): Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. Parasitology 131 (1): 15-23.
R Core Team (2016): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org. (Accessed on 14 October 2017).
Ricklefs RE, Swanson BL, Fallon SM, Martínez-Abraín L, Cheuerlein A, Gray J, Latta SC (2005): Community relationships of avian malaria parasites in Southern Missouri. Ecological Monographs 75 (4): 543-559.
Richardson DS, Jury FL, Blaakmeer J, Komdeur J, Burke T. (2001): Parentage assignment and extra – group paternity in a cooperative breeder: The Seychelles Warbler (*Acrocephalus sechellensis*). Molecular Ecology 10: 2263-2273.
Rönn J. A. C, Harrod C, Bensch S, Wolf J. B. W. (2015): Transcontinental migratory connectivity predicts parasite prevalence in breeding populations of the European barn swallow. Journal of Evolutionary Biology 28: 535-546.
Scheuerlein A, Ricklefs R. (2004): Prevalence of blood parasites in European passeriform birds. Proceedings of the Royal Society of London, Series B 271: 1363-1370.
Schmid S, Fachet K, Dinkel A, Mackenstedt U, Woog F. (2017): Carrion crows (*Corvus corone*) of southwest Germany: important hosts for haemosporidian parasites. Malaria Journal 16 (1): 369.
Sorensen MC, Asghar M, Bensch S, Fairhurst GD, Jenni-Eiermann S, Spottiswoode CN. (2016): A rare study from the wintering ground provides insight into the cost of malaria infection for migratory birds. Journal of Avian Biology 47: 575-582.
Shurulinkov P, Golemansky V. (2003): *Plasmodium* and *Leucocytozoon* (Sporozoa: Haemosporida) of wild Birds in Bulgaria. Acta Protozoologica 42 (3): 205-214.
Svensson L. (1992): Identification Guide to European Passerines. British Trust for Ornithology, 4th ed. Stockholm.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729.

Valkiūnas G, Iezhova T, Golemansky G, Pilarska D, Zehtindjiev P. (1999): Blood protozoan parasites (Protozoa: Kinetoplastida and Haemosporida) in wild birds from Bulgaria. Acta Zoologica Bulgarica 51: 127-129.

Valkiūnas G. (2005): Avian Malaria Parasites and other Haemosporidia. CRC press, Boca Raton, FL.

Valkiūnas G, Iezhova T. A. Križanauskienė A, Palinauskas V, Sehgal R. N. M. (2008a): A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology 94 (6): 1395-1401.

Valkiūnas G, Zehtindjiev P, Dimitrov D, Križanauskienė A, Iezhova TA, Bensch S. (2008b): Polymerase chain reaction–based identification of *Plasmodium* (*Huffia*) *elongatum*, with remarks on species identity of haemosporidian lineages deposited in GenBank. Parasitology Research 102: 1185-1193.

Ventim R, Tenreiro P, Grade N, Encarnação P, Araújo M, Mendes L, Pérez-Tris J, Ramos JA. (2012): Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds. Journal of Ornithology 153: 505-512.

Waldenström J, Bensch S, Kiboi S, Hasselquist D, Ottosson U. (2002): Cross-species infection of blood parasites between resident and migratory songbirds in Africa. Molecular Ecology 11: 1545-1554.

Wiersch SC, Lubjuhn T, Maier WA, Kampen H. (2007): Haemosporidian infection in passerine birds from Lower Saxony. Journal of Ornithology 148: 17-24.

Zehtindjiev P, Ilieva M, Križanauskienė, Oparina O, Oparin M, Bensch S. (2009): Occurrence of haemosporidian parasites in the paddyfield warbler, *Acrocephalus agricola* (Passeriformes, Sylviidae). Acta Parasitologica 54 (4): 295–300.

Zehtindjiev P, Križanauskienė A, Bensch S, Palinauskas V, Asghar M, Dimitrov D, Scebba S, Valkiūnas G. (2012): A new morphologically distinct avian malaria parasite that fails detection by established polymerase chain reaction-based protocols for amplification of the cytochrome b gene. Journal of Parasitology 98 (3): 657-665.

Zuk M. (1990): Reproductive strategies and diseases susceptibility, an evolutionary viewpoint. Parasitology Today 6 (7): 231-233.