A comparison of microscopy and PCR diagnostics for low intensity infections of haemosporidian parasites in the Siberian tit *Poecile cinctus*

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This study compares the sensitivity of two methods for diagnosing avian haemosporidian parasite (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) prevalence in Siberian tits *Poecile cinctus* breeding in northern Finland: a polymerase chain reaction (PCR)based method and microscopy examination of blood smears. During molecular analysis, we used PCR screening and RFLP as a post-PCR diagnostic step. PCR screening detected haemosporidian parasites in the blood of 38 out of 40 (95%) breeding Siberian tits. Microscopy examination of blood smears and PCR diagnostics showed the same prevalences for *Leucocytozoon* spp. infections. In contrast, prevalences of *Haemoproteus* spp. and *Plasmodium* spp. determined by molecular methods were significantly higher than the prevalence determined by microscopy screening. However, habitat and sex effects on the total count of parasites, *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. counts appeared to be similar when assessed with either screening method, giving the same interpretation for the ecological hypotheses tested. Finally, this study shows that transmission of avian malaria can occur in the area of the Arctic Circle in northern Europe.

Introduction

Avian malaria, haemoproteosis and leucocytozoonosis are diseases caused by Plasmodium, Haemoproteus and Leucocytozoon parasites of the apicomplexans (Valkiūnas et al. 2005). Recent results by Outlaw and Ricklefs (2011) clearly demonstrated that the genera of Leucocytozoon, Haemoproteus and Plasmodium are genetically closely related to form a sister group referred to as haemosporidian parasites (Perkins & Schall 2002, Ricklefs et al. 2004, Pérez-Tris & Bensch 2005, Szollosi et al. 2011). Haemosporidian parasites have been described in many bird groups and from many geographical areas (Bennett 1993, Bennett et al. 1993, Bennett et al. 1994, Valkiūnas 2005, Garamszegi 2011). With few exceptions, haemosporidian parasites occur worldwide, due to the high mobility and migration patterns of most host species (Spielman & D'Antonio 2001). Although these parasites are most readily seen in peripheral blood, they initially invade other internal organs, such as the liver, where they may remain for many years (Cox 1993). It has been shown that infections of haemotozoa are costly to host reproduction (Merino et al. 2000, Martínez-De la Puente et al. 2010) and haemosporidian parasites may play a crucial role in the evolution of avian life histories (Møller 1997, Ots & Hõrak 1998, Wiehn et al. 1999, Martínez-De la Puente et al. 2011). All haemosporidians have complex life cycles requiring vectors for transmission (Cox 1993, Bush et al. 2001). Avian malaria consists of a diverse group of parasites that are transmitted by dipteran insects (Bennett & Coombs 1975, Atkinson & van Ripper 1991, Cox 1993, Bush et al. 2001) infecting red blood cells in their vertebrate host and also white blood cells in the case of Leucocytozoon (Atkinson & van Ripper 1991, Valkiūnas 1993).

Most of the current data on haemosporidians, their distribution, vectors, parasite-host interactions and seasonality of infections have been collected primarily by microscopy (Atkinson & van Ripper 1991, Valkiūnas 2005). New molecular techniques have been considered to improve the detection of vector-borne blood parasites (Bensch *et al.* 2000, Ricklefs & Fallon 2002, Waldenström *et al.* 2002, Waldenström *et al.* 2004). The prevalence of these parasites has been found to be significantly higher than previously considered. According to some studies (Jarvi et al. 2002, Durrant et al. 2006), PCR tests were many-fold better than microscopy for detecting chronic blood parasite infections. However, the study by (Valkiūnas et al. 2008) obtained comparable infections rates of haemosporidian parasites when comparing microscopy to PCR-based methods. They demonstrated that both methods slightly underestimate the overall prevalence of infections. Although Valkiūnas et al. (2008) verified that microscopy is a reliable tool in detecting haemosporidians in many bird species, Jarvi et al. (2002) suggested that PCR diagnostics may be more sensitive in the case of low intensity infections. It has recently been shown (Garamszegi 2010) that relative to microscopy methods, the PCR-based molecular methods generally provide higher estimates for Plasmodium spp., and comparable estimates for species of Haemoproteus and Leucocytozoon.

There is now a range of highly sensitive PCR-based assays to detect the presence of the haemosporidians Plasmodium spp. and Haemoproteus spp. in samples of bird blood. However, the high degree of sequence conservation between species of Plasmodium and Haemoproteus presents difficulties for designing a PCR test to differentiate between them (Bensch et al. 2004, Martínez et al. 2009). So far, no such assay based purely on PCR has been forthcoming, and existing PCR screening protocols co-amplify both genera of haemosporidian parasites, relying on direct sequencing, or RFLP analysis (Beadell & Fleischer 2005) to separate the genera. There is also significant sequence conservation between species of Plasmodium, Haemoproteus, and a third haemosporidian parasite taxon, Leucocytozoon, which is sister to Plasmodium and Haemoproteus according to the mt cyt b phylogeny (Perkins & Schall 2002, Outlaw & Ricklefs 2011). Therefore, Cosgrove et al. (2006) suggested the use of a post-PCR diagnostic step, such as RFLP analysis or sequencing, to discriminate between Plasmodium, Haemoproteus and Leucocytozoon spp.

The aim of the present study was to compare the sensitivity of microscopy and PCRbased screening in determining the prevalence of haemosporidian parasite infections in the Siberian tit *Poecile cinctus*. We collected blood smears and blood samples for molecular analysis while testing the effects of forest management on reproduction, haematological parameters and blood parasites of Siberian tits breeding in northern Finland. The results obtained by using microscopy screening have been published earlier (Krams *et al.* 2010). Here, we compare the results already published (Krams *et al.* 2010) with those obtained using molecular analysis.

Material and methods

Study site, the birds and habitat

The field study was carried out near Kuusamo (66°N, 29°E), northern Finland in 2007. Scots pine Pinus sylvestris, Norway spruce Picea abies and downy birch Betula pubescens are the dominant tree species in the study area. To attract Siberian tits, wooden nestboxes arranged in lines were used. During the breeding season of Siberian tits, all of the nest boxes were checked twice a week as a part of a long term project on the ecology of Siberian tits carried out by the University of Oulu. The total size of our study area was ca. 100 km². Basic breeding parameters such as clutch size, brood size and the number of fledglings were recorded. We captured breeding adult Siberian tits in their nestboxes (n =23) when their nestlings were at the age of 9-12days. All of the breeding individuals were individually marked, and sex and age of all adult Siberian tits were known (see Krams et al. 2010 for details).

The habitat of breeding Siberian tits differed markedly between two main patches of northern taiga: (i) moderately-managed forests, and (ii) heavily-managed areas. The heavily-managed areas had been extensively thinned, leaving only middle-sized pines. The moderately-managed areas included both areas thinned at least a decade ago and patches of old growth coniferous forests (Virkkala 1990) consisting of pines, spruces and an admixture of birches. The moderately-managed forests also comprised many dead trees which increased the quality of habitat for Siberian tits (Virkkala 1990, Orell *et al.* 1999). We had 12 and 11 nestboxes of Siberian tits in the moderately- and heavily-managed forests, respectively. We estimated distances from the nestboxes to the nearest lakes and streams by using GPS.

Preparation and examination of blood smears

The blood samples were taken from a total of 45 (23 males and 22 females) breeding adult Siberian tits. We punctured the tarsal vein of each bird and prepared three individually marked slides following the recommendations of Bennett (1970) and Valkiūnas (2005). The blood smears were screened with a light microscope under oil immersion at 1000× magnification for Haemoproteus and Plasmodium and at 500× magnification for Leucocytozoon. Parasites were enumerated from 100 fields by moving the slide to areas where blood cells formed a monolayer for Leucocytozoon and from more than 200 fields for Haemoproteus and Plasmodium. For infected birds, parasite intensity was calculated as the number of parasites per 10 000 erythrocytes in randomly chosen fields of the blood smear, as recommended by Godfrey et al. (1987) and Valkiūnas (2005). Although species of Trypanosoma (the overall prevalence of infection was 20%) and Hepatozoon (4.4%) were also found during microscopic examinations of blood smears, they were not considered in the further analysis because we used PCR protocols to detect haemosporidian parasites only. Blood smears were screened by T.K., V.S. and D.C.

Molecular analysis

We also collected blood $(50-60 \ \mu$ l) from the same individuals as above for molecular analysis, and stored the samples in SET buffer (0.015M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) at -20 °C. We used the PCR protocol by Cosgrove *et al.* (2006), representing an improved modification of the nested PCR protocols by (Hellgren *et al.* 2004) and Waldenström *et al.* (2004) targeting mitochondrial cytochrome *b* (mt cyt *b*) genes. Although the PCR protocols by Hellgren *et al.* (2004) and Waldenström et al. (2004) were supposed to use Plasmodium- and Haemoproteusspecific primers in the second round PCR, a significant coamplification of Leucocytozoon spp. may occur (Cosgrove et al. 2006). The protocol by Cosgrove et al. (2006) was used to check for the presence of Plasmodium, Haemoproteus and Leucocytozoon parasites in samples of 40 Siberian tits (10 males and 10 females from both the moderately- and heavily-managed forests). We could not obtain enough blood from 5 Siberian tits (3 males and 2 females), and the molecular analysis was not performed for these birds. They also were not included in the subsequent comparison between molecular and microscopy methods of blood parasite detection.

For extractions of the DNA, the Invisorb[®] Spin Blood Mini Kit was used. The presence and quality of the extracted DNA was assessed by electrophoresis of 2 μ l of the extract in 2% agarose containing ethidium bromide, and visualizing under UV light. In the first round we used primers HaemNF (5'-CATATATTAAGAGAAT-TATGGAG-3') and HaemNR2 (5'-AGAGGT-GTAGCATATCTATCTAC-3'). A PCR was performed in volumes of 25 μ l, which contained 2 μ l of genomic DNA, 0.125 mM each dNTP, 0.2 µM each primer, 3 mM MgCl₂, 0.25 units of Taq DNA polymerase and $1 \times PCR$ buffer. The PCRs including primers HaemNFI and HaemNR3 were run using the following conditions: 120 s at 94 °C of enzyme activation step, followed by 20 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C, ending with an elongation step of 600 s at 72 °C.

We used the second round primers HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCA TTATCTGGATGTGATAAT-GGT-3'). The second PCR was performed in volumes of 25 μ l containing of 0.125 mM each dNTP, 0.4 μ M each primer, 3 mM MgCl2, 0.5 units of *Taq* DNA polymerase, 1× PCR buffer, and 2 μ l of the PCR product from the first round, which was used as template instead of genomic DNA. The PCRs including primers HaemF and HaemR2 were run using the following conditions: 120 s at 94 °C of enzyme activation step, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C. Two to 8 μ l of the

second-round reactions were run on 2% agarose stained with ethidium bromide and visualized under UV light.

Cosgrove et al. (2006) obtained amplicons of 478 bp, which were attributable to species of Plasmodium and Haemoproteus according to the results of direct sequencing. They also obtained larger amplicons of approximately 550 bp, which proved to be species of Leucocytozoon. In the cases when both PCR products were obtained, they distinguished the larger fragment from the smaller amplicons when electrophoresed on high-resolution agarose gels for 2 hr, but in some cases they reported less clear distinction. To be certain that a PCR product is because of Plasmodium and Haemoproteus and not Leucocytozoon spp., Cosgrove at al. (2006) recommended a further screening stage for all PCR products of approximately 450-600 bp. We confirmed the presence of Leucocytozoon spp. in our amplified PCR products, by performing RFLP analysis (Cosgrove et al. 2006), using the enzymes NcoI and EcoRV. We digested 3 µl of a PCR product in a total volume of 20 μ l for 4 hr at 37 °C, using 1 U of each of the restriction enzymes NcoI and EcoRV, 1 mg ml-1 bovine serum albumin, and 1× restriction enzyme buffer D (Thermo Fisher Scientific, Fermentas). For *Plasmodium* spp. sequences, the digest produces bands of 114 bp, 149 bp, and 262 bp, and for Leucocytozoon spp. sequences, bands of 173 bp and 352 bp are obtained (Cosgrove et al. 2006). Haemoproteus spp. sequences are not cut by either enzyme (Cosgrove et al. 2006).

To find the blood parasites, we used positive and negative controls. Positive controls were from birds with known infections evident from microscopy results, and the negative controls used purified water instead of DNA template. The PCR products were run on a 1.8% agarose gel using 1× TBE and visualized by an ethidium bromide stain under ultraviolet light.

Statistical analysis

Parasite counts were normally distributed (onesample Kolmogorov-Smirnov test), hence parametric statistical procedures were used for further analyses. Parasites loads in male and female Siberian tits from the moderately- and heavily-managed forests were analysed with GLM models. Number of nestlings as a measure of reproductive effort in the models did not change the results and, since that covariate itself remained always non-significant, we dropped it from the final models. Most of the adult birds were yearlings (41 out of 45 individuals in the microscopy data set, and 37 out of 40 individuals in the combined microscopy and molecular data set). Since we did not find any age-related effects and their interactions, age was also dropped from the final models. We included all birds (infected and non-infected) in the models.

Results

During microscopic examination of blood smears, haemosporidian parasites were found in the blood of 75.6% of the breeding Siberian tits (34 infected and 11 individuals with undetected infections). Since samples had a mean $(\pm SE)$ of 2 ± 0.23 infected cells per 10 000 (range = 0-4), the level of parasitemia was considered to be low in all of the inspected samples. When considering only individuals (n = 40) that were screened both by microscopy and molecular methods, the blood parasites were detected in 75% of the Siberian tits (30 infected and 10 individuals with undetected infections). Most of the infected Siberian tits (26 out of 40 individuals) had single infections. In 4 cases, the Siberian tits were double-infected (Table 1). There was no significant

difference in total parasite prevalence between sexes (χ^2 with Yates' correction = 0.07, p = 0.80; Table 1).

Using molecular screening, the blood parasites were found in 95% of the breeding Siberian tits (38 infected and 2 individuals with undetected infections). This shows that the prevalence of haemosporidian parasites was significantly higher when the same samples were tested by a combination of PCR and RFLP methods (χ^2 with Yates' correction = 4.80, p = 0.028; Table 1 and Fig. 1). In 4 cases, double infections of Leucocytozoon spp. × Haemoproteus and Leucocytozoon spp. × Plasmodium spp. as detected by microscopy were found to be triple infections consisting of Plasmodium, Haemoproteus and Leucocytozoon spp. according to the results obtained by molecular screening (Table 1 and Fig. 1). Two single infections of *Plasmodium* spp. as detected by microscopy appeared to be double infections of *Plasmodium* spp \times *Haemoproteus* spp.

Prevalence of *Haemoproteus* spp. determined by molecular methods (35%, Table 1) was significantly higher than the prevalence determined by microscopy (2.22%, Table 1) (χ^2 with Yates' correction = 13.48, p = 0.0002). Prevalence of *Plasmodium* spp. was also found to be significantly higher after molecular screening (35%, Table 1) as compared with microscopy examination of blood smears (12.5%, Table 1) (χ^2 with Yates' correction = 4.42, p = 0.036). In contrast, prevalence of *Leucocytozoon* spp. remained the same (70%) irrespective of the method used to detect blood parasites (Table 1). The total para-

 Table 1. Number of single, double and triple infections of blood parasites in Siberian tits regarding to individual's sex.

Type of single/combined infection	Method							
	Micros	сору	RFLP					
	Females	Males	Females	Males				
Plasmodium	1	1	0	0				
Haemoproteus	0	0	0	0				
Leucocytozoon	14	10	14	10				
Plasmodium × Haemoproteus	0	0	4	6				
Leucocytozoon × Plasmodium	1	2	0	0				
Leucocytozoon × Haemoproteus	0	1	0	0				
Leucocytozoon × Plasmodium × Haemoproteus	0	0	1	3				
Total	16	14	19	19				

336



Fig. 1. Diagnostic *Plasmodium* and *Leucocytozoon* species banding patterns resulting from post-PCR digestion with restriction enzymes *Eco*RV and *Ncol*. Lane numbers and genus are assigned. P = Plasmodium spp., L = Leucocytozoon spp., and M = mixed.

site prevalence did not differ between sexes after molecular screening (χ^2 with Yates' correction = 0, p = 1.00; Table 1).

The GLM analysis of the data obtained by molecular screening revealed that prevalences of *Leucocytozoon*, *Plasmodium* and that of all haemosporidians taken together were similar to those obtained by microscopy (Tables 2 and 3). Both methods showed that probability of being infected by *Leucocytozoon*, *Plasmodium* and by any of the blood parasites was not affected by habitat and sex. Molecular and microscopy screening showed that the risk of being infected by Leucocytozoon increased significantly with the decreasing distance to the nearest stream (Table 2). The risk of being infected by Plasmodium increased with the decreasing distance to the nearest lakes and streams (Tables 2 and 3). The main discrepancy between the parasite screening methods was that according to microscopy the risk of being infected by any of the parasites increased in the vicinity of lakes, while it was not supported by the results of molecular screening (Tables 2 and 3). Molecular screening gave us an opportunity to evaluate the prevalence of *Haemoproteus*, which was not possible by using microscopy only. The PCR and RFLP analyses showed that the risk of being infected by Haemoproteus increased significantly near lakes and streams (Table 3).

Discussion

The current and the earlier study by Krams *et al.* (2010) are the first showing that avian malaria transmission can occur in the area of the Arctic Circle. Recently migratory Fennoscandian blue-throats (*Luscinia svecica*) have been found to be infected with a *Plasmodium* lineage (Hellgren 2008). However, these birds most probably have been infected outside northern Sweden, since the samples were collected only from adult individuals. Studies carried out in North America, demonstrated that the transmission of *Plasmodium* parasites do not occur at northern latitudes (Deviche *et al.* 2001, Yohannes *et al.* 2009, Barnard *et al.* 2010).

Table 2. The effects of habitat, sex, distance to the nearest lake and distance to the nearest stream on the blood parasite prevalence (infected *vs.* non-infected) of breeding Siberian tits (GLM analysis) revealed by microscopy.

	Leucocytozoon			Plasmodium			Haemoproteus			All genera of parasites		
	df	F	p	df	F	p	df	F	p	df	F	p
Habitat	1	3.18	0.10	1	3.20	0.10	1	0.0001	1.00	1	0.05	095
Sex	1	1.63	0.23	1	0.54	0.48	1	0.0001	1.00	1	1.05	0.37
Distance to lake	2	0.0001	1.00	2	5.33	0.01	2	0.0001	1.00	2	3.98	0.04
Distance to stream	6	4.19	0.02	6	4.50	0.02	6	0.0001	1.00	6	1.69	0.18
Error	13			13			13			13		

We found that microscopy and PCR diagnostics showed the same prevalences for *Lecocytozoon* spp. infections in the same samples of Siberian tits. This indicates that optical microscopy may be as sensitive as PCR screening to detect *Leucocytozoon* spp., and microscopy does not underestimate the prevalence of infection even in infections of low intensity. We would suggest the use of microscopy only when the research interests are related solely to *Leucocytozoon* species, because preparation of good blood smears is easy, their examination is cheap, and the detection of this relatively large blood parasite is not difficult.

The present study shows that PCR diagnostics appeared to be more sensitive than microscopy in the case of Plasmodium spp. and Haemoproteus spp. It is known that after an initial acute phase of infection by blood parasites, the hosts develop chronic, low intensity parasitemias, that are regulated by host cellular and humoral immunity (Schmid-Hempel 2011). Whereas high intensity acute parasitemias are typically easy to monitor and quantify by inspecting blood smears (Valkiūnas et al. 2008), the use of microscopy for diagnosing chronic, low intensity infections may considerably underestimate parasite prevalence (McCurdy et al. 1998, Jarvi et al. 2002). Our results support conclusions of previous studies about insufficient sensitivity of microscopy when parasitemias are low. On the other hand, we cannot agree with the notion that PCR tests are 3 to 4 or even 10-fold better than microscopy diagnostic for detecting chronic blood parasite infections. Although the difference between prevalences revealed by PCR-based techniques and microscopy was significant, we found that sex- and habitat-related relationships found in our previous study on the blood parasites in breeding Siberian tits were not biased by low sensitivity of microscopy. We found that PCR diagnostics and microscopy showed no sex differences in the total count of parasites, or in counts of Leucocytozoon and Plasmodium. PCR and microscopy also demonstrated similar results with regard to the lack of association between the total count of parasites or counts of Leucocytozoon and Plasmodium, and habitat of their hosts. However, we found that microscopy examination of blood smears significantly underestimated infections by Haemoproteus spp. in the peripheral blood of Siberian tits. It is important to note that identification of Haemoproteus parasites was possible only because of the RFLP step in molecular analysis Cosgrove et al. 2006). Otherwise coamplification of all three sister genera of haemosporidia (Perkins & Schall 2002, Outlaw & Ricklefs 2011) may occur during the PCR protocols suggested by Hellgren et al. (2004) and Waldenström et al. (2004).

The low numbers of *Haemoproteus* spp. from microscopy samples may be explained mainly by difficulties to detect the patent infections of this haemosporidian genus during examining of blood smears of exceptionally light infections, when just a few parasites are present in samples. It is difficult to rule out a possibility that sensitive PCR-based tools amplify the DNA of sporozoites, which are not numerous in the circulation, so usually difficult to detect using microscopy (Valkiūnas 2005). This is a likely explanation because PCR diagnostics revealed also much higher prevalence of *Plasmodium*

Table 3. The effects of habitat, sex, distance to the nearest lake and distance to the nearest stream on the blood parasite prevalence (infected *vs.* non-infected) of breeding Siberian tits (GLM analysis) revealed by molecular screening (PCR and RFLP).

	Leucocytozoon			Plasmodium			Haemoproteus			All genera of parasites		
	df	F	p	df	F	p	df	F	p	df	F	p
Habitat	1	2.93	0.11	1	0.0001	1.00	1	0.09	0.77	1	0.0001	1.00
Sex	1	1.47	0.25	1	0.14	0.72	1	3.38	0.09	1	0.09	0.77
Distance to lake	2	0.0001	1.00	2	5.33	0.01	2	5.33	0.01	2	0.0001	1.00
Distance to stream	6	3.55	0.03	6	3.05	0.04	6	6.04	0.004	6	1.89	0.16
Error		13			13			13			13	

than the results obtained by microscopy. It is also possible but perhaps less likely that gametocytes of *Haemoproteus* spp. were less mature and harder to detect than gametocytes of *Plasmodium* spp.

Either screening method showed that the risk of being infected by Leucocytozoon significantly increased with the decreasing distance to the nearest stream, where simuliid flies (Diptera: Siimulidae) reproduce (Bennett & Fallis 1971, Ashford et al. 1991, Krams et al. 2010), while the risk of infection by *Plasmodium* significantly increased with decreasing distance to streams and lakes. Thus, sex, habitat and parasite-vector related results do not support the idea that the use of microscopy for diagnosing chronic infections could unavoidably underestimate and bias studies that test for the role of sex and habitat-related stress in parasitism. Therefore, we would suggest that microscopy may be a suitable method for detecting blood parasites during times when parasitemias and transmission rates are low, except perhaps in the case of *Haemoproteus* spp. It is important to note that blood smears, which are made from blood containing parasitemias of low intensity, should be of high quality, they need to be properly prepared, and the smears should be examined by highly skilled investigators (Valkiūnas et al. 2008). We also would suggest the use of greater sample sizes to reach life-history related conclusions, though microscopy is time-consuming, which may make it less convenient especially for large-scale screening.

Leucocytozoon, Plasmodium and Haemoproteus rarely occurred together and this fact enables us to assert that they are transmitted by different vectors. It is known that blood-sucking simuliid blackflies (Diptera: Siimulidae) are the vectors of Leucocytozoon (Valkiūnas 2005, Hellgren et al. 2008). According to the PCR results, Plasmodium and Haemoproteus always occurred together, suggesting the same vector for these parasites. However, biting midges (Diptera: Creratopogonidae) and hippoboscid flies (Diptera: Hippoboscidae) are the vectors of bird haemosporidian parasites, while blood-sucking mosquitoes (Diptera: Culicidae) are the vectors of Plasmodiidae species (Valkiūnas 2005). Supposedly, hippoboscid flies and mosquitoes are so abundant in northern Finland (Adler et al. 1999)

that both closely related genera of blood parasites often occur together in prevalences as high as 80%, according to microscopy examination of blood smears, and as high as 95%, according to molecular screening of the blood of breeding Siberian tits.

Finally, our results confirm that the PCR screening protocol and use of RFLP analysis suggested by Cosgrove *et al.* (2006) is sufficient to avoid the risk of co-amplification of *Leucocy-tozoon* while performing PCR to detect *Plasmo-dium* and *Haemoproteus* parasites. This method is sensitive enough despite the high degree of sequence similarity between *Plasmodium* and *Haemoproteus* avian malaria species, and *Leucocytozoon* in target genes. We would suggest the use of this technique as a standard method of molecular screening for blood parasites of birds in a combination with a microscopy approach to identify and detect the intensity of infection.

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