Parasitology Research (2019) 118:191–201
https://doi.org/10.1007/s00436-018-6153-7

GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites

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Received: 25 May 2018 / Accepted: 12 November 2018 / Published online: 7 December 2018

Abstract

Accurate detection and identification are essential components for epidemiological, ecological, and evolutionary surveys of avian haemosporidian parasites. Microscopy has been used for more than 100 years to detect and identify these parasites; however, this technique requires considerable training and high-level expertise. Several PCR methods with highly sensitive and specific detection capabilities have now been developed in addition to microscopic examination. However, recent studies have shown that these molecular protocols are insufficient at detecting mixed infections of different haemosporidian parasite species and genetic lineages. In this study, we developed a simple, sensitive, and specific multiplex PCR assay for simultaneous detection and discrimination of parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in single and mixed infections. Relative quantification of parasite DNA using qPCR showed that the multiplex PCR can amplify parasite DNA ranging in concentration over several orders of magnitude. The detection specificity and sensitivity of this new multiplex PCR assay were also tested in two different laboratories using previously screened natural single and mixed infections. These findings show that the multiplex PCR designed here is highly effective at identifying both single and mixed infections from all three genera of avian haemosporidian parasites. We predict that this one-step multiplex PCR assay, being convenient and inexpensive, will become a widely used method for molecular screening of avian haemosporidian parasites.

Keywords *Plasmodium* · *Haemoproteus* · *Leucocytozoon* · Parasite detection · Mixed infection · Multiplex PCR

Introduction

Since the end of the nineteenth century, avian haemosporidians (Apicomplexa: Haemosporida), which are dipteran-vectored blood parasites belonging to the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, have been documented in birds all over the world (Valkiūnas 2005). Studies of these parasites have addressed ecological and evolutionary questions regarding host-parasite interactions (Bensch et al. 2013; Sehgal 2015) and avian conservation in places where they threaten host populations (e.g., van Riper et al. 1986; Levin et al. 2013). Avian haemosporidians are diverse (Bensch et al. 2009; Clark et al. 2014) and can reach high prevalence in host populations (Valkiūnas et al. 2003; Mata et al. 2015). Infections can result in anemia, weight loss, morbidity, and mortality (Merino et al. 2000; Valkiūnas 2005; Palinauskas et al. 2008, 2015), and severe pathology may also occur in non-competent hosts (i.e., hosts in which the parasite does not complete its development). Both non-competent vertebrate hosts and insect vectors may suffer increased mortality when infected (Cannell et al. 2013; Valkiūnas et al. 2014a, b). Therefore, accurate detection of avian haemosporidians is very important, not only for investigating host-parasite interactions but also for understanding epidemiology of infections and developing control strategies against these diseases and vectors (van Riper et al. 1986; Marzal et al. 2008; Levin et al. 2013; Bernotienė et al. 2016).

Section Editor: Larissa Howe

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-018-6153-7) contains supplementary material, which is available to authorized users.

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Molecular techniques are routinely used to detect and characterize avian haemosporidian parasites (Feldman et al. 1995; Escalante et al. 1998; Bensch et al. 2000; Ricklefs and Fallon 2002; Richard et al. 2002; Fallon et al. 2003; Waldenström et al. 2004; Hellgren et al. 2004; Ricklefs et al. 2005). One widely used PCR protocol was developed by Bensch et al. (2000) and modified with external and internal primers as a nested PCR by Hellgren et al. (2004) and Waldenström et al. (2004). These assays target a fragment of the cytochrome *b* (cyt *b*) gene which is sufficient for lineage and genus identification; genetic avian haemosporidian lineages are recorded in the publically available MalAvi database (Bensch et al. 2009). However, these PCR protocols (Bensch et al. 2000; Hellgren et al. 2004; Waldenström et al. 2004) and other often used PCR-based assays (Richard et al. 2002; Beadell et al. 2004; Martinsen et al. 2008) are ineffective at detecting mixed infections between parasites of the genera *Haemoproteus* and *Plasmodium* (Valkiūnas et al. 2006; Bernotienė et al. 2016). This is because the PCR favors the amplification of the most abundant parasite in the sample (i.e., the parasite with highest parasitemia) or the parasite for which the primers are a better match (Valkiūnas et al. 2006; Martínez et al. 2009; Dimitrov et al. 2013). This is unfortunate because these mixed infections are very common in the wild and have been shown to be particularly virulent in wild birds (Valkiūnas et al. 2003; Marzal et al. 2008; Martínez et al. 2009; Bernotienė et al. 2016).

Recent studies suggest the use of a combination of PCR and microscopic methods for reliably detecting avian haemosporidians in mixed infections (Valkiūnas et al. 2008, 2014b; Bernotienė et al. 2016; Ciloglu et al. 2016). However, microscopy has some disadvantages. First, compared to molecular techniques, microscopy may fail to detect low levels of parasitemia (Garamszegi 2010; Ellis et al. 2014; Lutz et al. 2015; Ishtiaq et al. 2017), except when observers are highly trained (Valkiūnas et al. 2008). Second, successful microscopic detection of parasites requires taxonomic expertise for accurate identification of parasite genera and developmental stages (Valkiūnas et al. 2008). Because of these difficulties, avian haemosporidian research would benefit from sensitive, accurate, cost-effective, and easily implemented detection methods for identification of mixed infections.

In this study, we consider the challenges and limitations of the most commonly used avian haemosporidian detection methods and develop a sensitive and simple multiplex PCR assay for providing simultaneous detection and discrimination of parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in single and mixed infections. We tested the specificity and sensitivity of this new multiplex PCR assay by using it to identify parasites in known single and mixed infections and in natural infections previously screened by other methods. We performed relative quantifications (RQs) of parasite DNA in single and mixed infections by designing genus-specific primers and using them with quantitative PCR (qPCR) to test the amplification sensitivity of the here-designed multiplex PCR method.

Material and methods

Design of multiplex PCR primers

Whole mitochondrial (mtDNA) genome sequences of avian *Plasmodium*, *Haemoproteus* (*Parahaemoproteus*), and *Leucocytozoon* (*Leucocytozoon*) isolates previously reported by Pacheco et al. (2018a) were aligned using Geneious v. R11 (Kearse et al. 2012). To this alignment, we added additional avian haemosporidian sequences from isolates containing mtDNA sequences > 1000 bp (13 *Plasmodium* spp., 21 *Haemoproteus* spp., and 10 *Leucocytozoon* spp. isolates) found on GenBank (Benson et al. 2005). The primers were designed using a modified version of Primer3 v. 2.3.7. (Rozen and Skaletsky 2000) in Geneious according to standard guidelines (Dieffenbach et al. 1993). The 3′ end of each pair of primers was designed to target sequence motifs unique to each genus in order to ensure genus-specific amplification. The primer pairs were checked using the Primer-BLAST program (Ye et al. 2012) in order to confirm their specificity and the absence of non-specific amplification. The primer pairs for *Plasmodium* spp. were located in a non-coding region of the mtDNA, for *Haemoproteus* spp. between the 5′ end of cyt *b* and a non-coding region of mtDNA, and for *Leucocytozoon* spp. in the cytochrome *c* oxidase subunit 1 (COX1) gene of mtDNA. The primer sequences and sizes of amplified regions are listed in Table 1.

Samples

The primary dataset of this study consisted of 180 samples collected at Lake Krankesjön in southern Sweden (55° 41′ N, 13° 26′ E), between June and August 2015 as part of an ongoing community study of avian haemosporidian parasites. These samples were processed at the Molecular Ecology and Evolution Laboratory at Lund University in Lund, Sweden. Briefly, genomic DNA was extracted using an ammonium acetate protocol (Richardson et al. 2001) and diluted to a concentration of 25 ng/μl. The samples were screened using the nested PCR protocols of Bensch et al. (2000) and Hellgren et al. (2004). The amplification products (2.5 μl) were identified by gel electrophoresis in 2% agarose gel. PCR products from positive amplifications were precipitated and sequenced with the forward primer using Big-Dye on an ABI PRISM™ 3100 sequencer (Applied Biosystems, FL, USA). Sequences were edited and aligned in Geneious. Mixed infections were identified, and genus identification of parasites was determined by analyzing double nucleotide peaks on sequence

Table 1 Sequences and amplification sizes of the designed primers

	Parasite genus	Primer name	Primer sequence (5′ to 3′)	Product size (bp)
Multiplex PCR primers	*Plasmodium*	PMF	CCTCACGAGTCGATCAGG	377–379
		PMR	GGAAACCGGCGCTAC	
	Haemoproteus	HMF	ATTGGATGTCAATTACCACAATC	525–533
		HMR	GGGAAGTTTATCCAGGAAGTT	
	Leucocytozoon	LMF	TGGAACAATAATTGSATTATTTACAYT	218
		LMR	AACATATCATATTCCATCCATTTAGATTA	
qPCR primers	*Plasmodium*	PQF	CATGGATTTGTGGTGGATATCTTG	198
		PQR	TATCRAGACTTAAWAGATTTGGATAGAAG	
	Haemoproteus	HQF	GGATTTGTGGWGGATATAYTATWAGTGAT	194
		HQR	ATCTARACATAATAGACTTGGATARAAA	
	Leucocytozoon	LQF	GGWCAAATGAGTTTYTGGGGA	212
		LQR	CCTAAAGGATTAGTGCTACCWTGAAT	

electropherograms. The obtained cyt *b* lineages were named following standard nomenclature (Bensch et al. 2009) and deposited in the MalAvi database (Table 2). These samples were then screened with the multiplex PCR assay, and the results of the two screening methods were compared.

A smaller dataset of 21 naturally infected (both single and mixed infections) individual birds collected in Ventes Ragas (55° 20′ N, 21° 11′ E), Lithuania, in 2015–2017 and previously screened by microscopy was also screened by multiplex PCR in the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania (Table 3).

Multiplex PCR assay

The multiplex PCR was performed using equimolar concentrations of three primer sets (Table 1) in a single reaction tube. The PCRs were set up in total volumes of 10 μl that contained 5 μl of commercial master mix (2× Qiagen Multiplex PCR Master Mix, Qiagen, Hilden, Germany), 0.2 μl of each primer (10 μM concentration), 1.8 μl of ddH_2O, and 2 μl of DNA template at a concentration of 25 ng/μl. The PCR amplification protocol has an initial denaturation step of 95 °C for 15 min (for activation of HotStarTaq DNA Polymerase). This is followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 90 s, and extension at 72 °C for 30 s; the final extension occurs at 72 °C for 10 min. All PCR experiments contained one negative control (ddH_2O) for every 16 samples. Amplification products (2.5 μl) were electrophoretically resolved after 1 h at 90 V in 2% agarose gels containing GelRed™ gel stain (Biotium, Inc., Hayward, CA, USA) and visualized on a Gel Doc™ XR+ imaging system with Image Lab™ software (Bio-Rad, Hercules, CA, USA).

The specificity of the multiplex PCR amplification for the corresponding target fragments was assessed by sequencing amplicons of 12 samples which were single infections of 12 different parasite lineages, with the corresponding forward primers, as described previously. Sequences were edited with Geneious. The processed sequences were aligned to homologues available from GenBank (Benson et al. 2005) using the BLASTn algorithm with default settings.

The multiplex PCR of the smaller dataset previously screened by microscopy was performed using a Platinum™ Multiplex PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The thermal protocols and gel electrophoresis analysis were carried out as described previously.

Relative quantification analyses

The amplification sensitivity of the here-designed multiplex PCR method was tested with RQ analyses using qPCR. First, new genus-specific qPCR primer sets were designed by aligning a total of 46 avian haemosporidian cyt *b* lineages from 115 single and mixed infected individuals obtained from the Krankesjön dataset (Table 2). The primer pairs were checked for specificity as described previously. The qPCR primer sequences with their names and product sizes are listed in Table 1.

The qPCR protocols were performed on 16 single and mixed infection samples that were also tested by multiplex PCR. The qPCR were carried out in a Mx3005P real-time PCR instrument (Agilent Technologies, CA, USA) using a SYBR-green (Platinum™ SYBR™ Green qPCR SuperMix-UDG, Thermo Fisher Scientific, Waltham, MA, USA) detection method. Each reaction of 25 μl included 12.5 μl of SYBR-green SuperMix, 0.1 μl of ROX dye, 1 μl of each primer (10 μM concentration), 4 μl of the DNA template (1 ng/μl concentration), and 6.4 μl of ddH_2O. Thermal cycling conditions were as follows: initial incubation at 50 °C for 2 min followed by 94 °C for 2 min, then 43 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s, and immediately followed by a melting curve analysis to check for correct amplification following the default setting in the instrument (1 cycle of 95 °C for 1 min, 48 °C for 30 s, and 95 °C for 30 s).

Table 2 List of the parasite species, parasite cytochrome *b* (cyt *b*) lineage names, bird species, and number of infections per lineage of the Krankesjön dataset

Parasite genus	Parasite species	Lineage name	Bird species	No. of infections/lineage
Haemoproteus	*Haemoproteus belopolskyi*	HIICT3	*Muscicapa striata*	1
	Haemoproteus fringillae	CCF3	*Carduelis chloris*	2
			Fringilla coelebs	1
	Haemoproteus lanii	RB1	*Lanius collurio*	1
	Haemoproteus magnus	ROFI1	*Fringilla coelebs*	1
	Haemoproteus majoris	CCF5	*Fringilla coelebs*	1
		PARUS1	*Parus ater*	1
			Parus caeruleus	9
			Parus major	6
			Sitta europaea	1
		PHSIB1	*Erithacus rubecula*	1
			Parus caeruleus	3
			Phylloscopus sibilatrix	1
		WW2	*Phoenicurus phoenicurus*	1
			Phylloscopus collybita	4
			Phylloscopus trochilus	5
			Sylvia borin	3
			Sylvia communis	4
	Haemoproteus minutus	TURDUS2	*Turdus merula*	2
	Haemoproteus pallidus	COLL2	*Sylvia borin*	1
		SFC3	*Muscicapa striata*	1
	Haemoproteus parabelopolskyi	SYBOR01	*Sylvia borin*	1
		SYBOR03	*Sylvia communis*	1
	Haemoproteus sp.	CCF1	*Fringilla coelebs*	1
		CCF2	*Fringilla coelebs*	5
		CWT4	*Sylvia communis*	1
		EMCIR01	*Emberiza citrinella*	4
		SYAT14	*Sylvia atricapilla*	1
	Total	*18*	*18*	*64*
Plasmodium	*Plasmodium circumflexum*	TURDUS1	*Fringilla coelebs*	1
			Parus caeruleus	3
			Parus major	5
			Prunella modularis	1
			Troglodytes troglodytes	1
	Plasmodium matutinum	LINN1	*Turdus merula*	1
	Plasmodium relictum	SGS1	*Erithacus rubecula*	1
			Phylloscopus trochilus	1
			Sylvia borin	1
	Plasmodium sp.	ANTTRI01	*Anthus trivialis*	1
		BT7	*Carduelis cannabina*	1
			Parus ater	1
			Parus caeruleus	1
			Parus major	1
		GRW09	*Muscicapa striata*	1
		PARPAL01	*Parus palustris*	1
	Total	7	14	22
Leucocytozoon	*Leucocytozoon* sp.	AFR164	*Sylvia communis*	1
		BT2	*Sylvia curruca*	1
		CIAE02	*Dryocopus martius*	1
		CORMON02	*Corvus monedula*	1
		CWT12	*Sylvia communis*	1
		PARUS4	*Parus caeruleus*	4
			Parus major	2
		PARUS12	*Parus caeruleus*	1
		PARUS14	*Parus caeruleus*	1
		PARUS17	*Parus major*	1
		PARUS18	*Parus caeruleus*	2
		PARUS19	*Parus major*	1
		PARUS21	*Parus caeruleus*	2
			Sitta europaea	1
		PARUS27	*Parus major*	1
		PARUS74	*Parus major*	1

Table 2 (continued)

Parasite genus	Parasite species	Lineage name	Bird species	No. of infections/lineage
		PARUS87	*Parus caeruleus*	1
		REB11	*Sylvia communis*	1
		RECOB3	*Sylvia communis*	1
		RS4	*Sylvia communis*	1
		SYBOR07	*Sylvia borin*	1
		SYBOR33	*Sylvia borin*	1
		TRPIP1	*Anthus trivialis*	1
	Total	21	9	29
Overall		46	26	115[a]

[a] The total is higher than the number of infected birds due to mixed infections

The samples were run in duplicate on each plate together with two no-template controls (ddH_2O in place of template DNA). The samples were scored as positives if both of the duplicates showed evidence of amplification (fluorescence higher than the threshold, i.e., the level of fluorescence deemed above background and used to determine cycle threshold, set by the software) of a single product; the latter was determined by inspecting the melting curves. The cycle threshold (Ct) values were scored as the average of the two samples.

After running the first set of qPCR (for each parasite genus separately), a single sample (a *Haemoproteus* infection, which was part of a mixed infection) was identified with the lowest Ct value, and this sample was considered to be the calibrator. Multiple plates were run and always included the same calibrator sample (the calibrator remained the lowest Ct value across all plates). Then, the Ct values from all other samples were compared to this calibrator to generate RQ values (the threshold values were set to always be the same). RQ values were calculated following the formula, $RQ = 2^{-(Ct\ target - Ct\ calibrator)}$, which is based on the comparative Ct method ($2^{-\Delta Ct}$) defined by Livak and Schmittgen (2001). Using RQ values allowed for the comparison of samples among the different plates and the accurate assessment of the amplification sensitivity of the multiplex PCR.

Statistical analysis

To compare the sensitivity of the multiplex PCR method described here to that of the typical nested PCR protocol of Bensch et al. (2000) and Hellgren et al. (2004), both methods (nested PCR and multiplex PCR) were used to assess the infection status of 180 individual bird blood samples from Krankesjön (described previously). The proportion of individuals found infected (prevalence) by each parasite genus was compared between the two methods using chi-squared tests. This was done for all infections and separately for mixed infections (i.e., individuals infected by more than one parasite genus) only. The proportions of mixed infections found by the two methods were also compared. Statistical tests were conducted in R v.3.4.0 (R Core Team 2017).

Results

A total of 46 avian haemosporidian lineages from single and mixed infected individuals belonging to 26 bird species sampled at Krankesjön were successfully amplified using the multiplex PCR designed here (Table 2 and Table 4). These lineages represent a phylogenetically diverse sample of all known avian haemosporidian lineages (Supplementary Fig. 1). The amplifications yielded products of different sizes for each genus (533 bp for *Haemoproteus*, 378 bp for *Plasmodium*, and 218 bp for *Leucocytozoon*) allowing for rapid and accurate genus identification (Fig. 1). There were no overlapping bands among the genera and no interference between primer pairs; in rare cases, faint non-specific products greater than 600 bp in size were observed.

Two *Plasmodium*, five *Haemoproteus*, and five *Leucocytozoon* lineages were successfully sequenced to confirm the specificity of the amplification for each target. According to alignment analysis, the *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* lineages showed 98.0–100.0%, 89.9–99.1%, and 84.4–100.0% similarities to haemosporidian isolates deposited in GenBank, respectively.

Among all 180 samples screened for infection, the multiplex PCR method found 95 individuals infected with one or more parasite genera (52.7%) while the nested PCR method found 88 infected individuals (48.8%; chi-squared = 0.40, df = 1, $P = 0.527$). The two methods did not differ in terms of the total number of individuals infected (single and mixed infections together) with *Plasmodium* (chi-squared = 1.66, df = 1, $P = 0.197$), *Haemoproteus* (chi-squared = 1.93, df = 1, $P = 0.165$), or *Leucocytozoon* (chi-squared = 0.18, df = 1, $P = 0.675$; Fig. 2) parasites. The multiplex PCR recovered more mixed infections (37/180 individuals found with mixed infections) than the nested PCR (26/180), but the difference was not statistically significant (chi-squared = 1.92, df = 1, $P =$

Table 3 Results of microscopic examination and multiplex PCR screening of samples from Ventes Ragas, Lithuania. Differences between the microscopy and multiplex PCR methods are highlighted in bold

Sample number	Avian host species	Microscopy results			Multiplex PCR results		
		H	P	L	H	P	L
1	*Acrocephalus arundinaceus*	*H. nucleocondensus*	*Plasmodium* sp.	*Leucocytozoon* sp.	+	+	+
2	*Asio otus*	*H. noctuae*	–	*L. danilewskyi*	+	–	+
3	*Coccothraustes coccothraustes*	*H. tartakovskyi*, *H. fringillae*	*Plasmodium* sp.	*Leucocytozoon* sp.	+	+	+
4	*Delichon urbica*	*Haemoproteus* sp.	***Plasmodium* sp.**	–	+	–	–
5	*Delichon urbica*	*H. hirundinis*	*Plasmodium* sp.	–	+	+	–
6	*Erithacus rubecula*	–	–	*Leucocytozoon* sp.	–	–	+
7	*Erithacus rubecula*	–	–	*Leucocytozoon* sp.	–	+	+
8	*Hippolais icterina*	*H. belopolskyi*	*P. homonucleophilum*	–	+	+	–
9	*Luscinia luscinia*	*H. balmorali*	*P. matutinum*	–	+	+	–
10	*Luscinia luscinia*	*H. balmorali*	*Plasmodium* sp.	–	+	+	–
11	*Luscinia luscinia*	*H. attenuatus*	*Plasmodium* sp.	–	+	+	–
12	*Muscicapa striata*	*H. balmorali*	–	*L. fringillinarum*	+	–	+
13	*Strix aluco*	*H. syrnii*	–	*L. danilewskyi*	+	–	+
14	*Sylvia atricapilla*	*H. parabelopolskyi*	–	***Leucocytozoon* sp.**	+	–	–
15	*Sylvia atricapilla*	*H. parabelopolskyi*	***Plasmodium* sp.**	–	+	–	–
16	*Sylvia communis*	*H. belopolskyi*	*Plasmodium* sp.	–	+	+	–
17	*Sylvia communis*	*H. belopolskyi*	*Plasmodium* sp.	–	+	+	–
18	*Sylvia communis*	*H. belopolskyi*	*P. relictum*	–	+	+	–
19	*Turdus merula*	*H. minutus*	*P. vaughani*	***Leucocytozoon* sp.**	+	+	–
20	*Turdus merula*	*H. minutus*	*P. vaughani*	*Leucocytozoon* sp.	+	+	+
21	*Turdus merula*	*H. minutus*	*P. vaughani*	***Leucocytozoon* sp.**	+	+	–

H, *Haemoproteus* spp.; *P*, *Plasmodium* spp.; *L*, *Leucocytozoon* spp.

0.165). Among individuals infected by more than one parasite genus, the multiplex PCR recovered proportionally more *Plasmodium* infections than the nested PCR method (29 individuals infected by *Plasmodium* in mixed infections determined by multiplex PCR vs. 13 individuals infected by *Plasmodium* in mixed infections determined by nested PCR; chi-squared = 6.06, df = 1, $P = 0.014$). For *Haemoproteus* (34/180 vs. 23/180; chi-squared = 2.08, df = 1, $P = 0.149$) and *Leucocytozoon* (28/180 vs. 23/180; chi-squared = 0.37, df = 1, $P = 0.546$), a similar number of samples in mixed infections were found for these genera (Fig. 2, Table 4).

The multiplex PCR also gave results similar to the microscopic evaluation of infected birds in the smaller dataset (Table 3). The multiplex PCR agreed completely with the microscopy results in 15 of the 21 individuals. In five cases, the multiplex PCR method missed one infection that

Table 4 The multiplex PCR and nested PCR screening results of 180 samples collected from Lake Krankesjön in southern Sweden. These results show that some samples that were found to be single infections by nested PCR were revealed to be mixed infections by multiplex PCR. For example, while both nested and multiplex PCR found five samples infected with *Leucocytozoon* only, the nested PCR found one sample to be a single infection of *Leucocytozoon* that the multiplex PCR revealed to be a mixed infection of *Leucocytozoon* and *Plasmodium*

Nested PCR results			Single infected			Mixed infected					
			H	P	L	H, P	H, L	P, L	H, P, L	Negative	Total
Multiplex PCR results	Single infected	H	40	–	–	–	–	–	–	8	48
		P	–	5	–	–	–	–	–	–	5
		L	–	–	5	–	–	–	–	–	5
	Mixed infected	H, P	4	3	–	2	–	–	–	–	9
		H, L	–	–	–	–	8	–	–	–	8
		P, L	–	2	1	–	–	–	–	–	3
		H, P, L	1	–	–	1	5	3	7	–	17
	Negative		–	1	–	–	–	–	–	84	85
	Total		45	11	6	3	13	3	7	92	180

H, *Haemoproteus* spp.; *P*, *Plasmodium* spp.; *L*, *Leucocytozoon* spp.

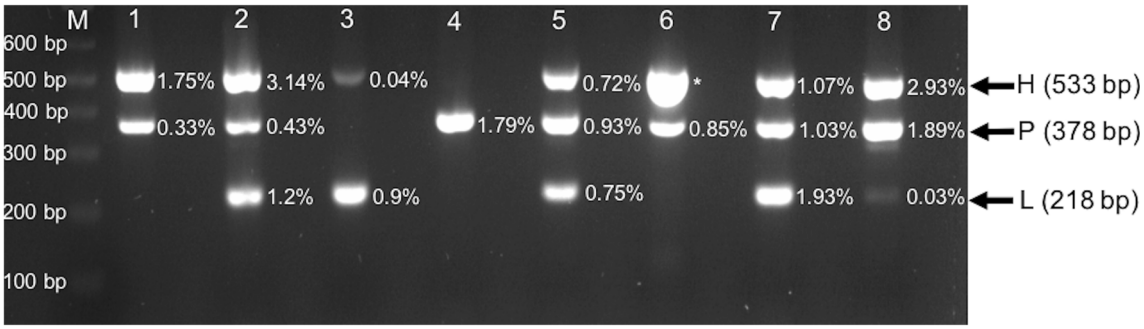

Fig. 1 Agarose gel electrophoresis of multiplex PCR products. The relative quantification (RQ) values, expressed as a percentage of the intensity of the calibrator sample (i.e., the highest intensity sample, indicated by an asterisk symbol), are included to the right of the bands (for more details, see Supplementary Table 1). Lane M, 100-bp molecular marker; lanes 1–8, amplicons from single and mixed infections (H, *Haemoproteus* spp.; P, *Plasmodium* spp.; L, *Leucocytozoon* spp.)

microscopy found. However, one bird found by microscopy to be infected by a *Leucocytozoon* sp. only was shown by multiplex PCR to also have a *Plasmodium* infection. The multiplex PCR also agreed with the microscopy results in two species of owls that carried mixed infections of *Haemoproteus* and *Leucocytozoon* species (Table 3).

The relative amount of parasite DNA for the 16 single and mixed infected samples was determined by qPCR analysis. The Ct value of the calibrator sample was measured as 25.94. The $2^{-\Delta Ct}$ values of the 15 samples (not including the calibrator) ranged from 0.1961 to 0.0003 (Fig. 1, Supplementary Table 1). Thus, the data showed that the multiplex PCR could amplify infections covering a large range of intensities and therefore is highly sensitive.

Discussion

In this study, we report the development of a new multiplex PCR assay for detecting and distinguishing the parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* in a single reaction. The multiplex PCR was tested in two different laboratories with different master mixes and was shown to perform as expected. We compared the results of the multiplex PCR with the most widely used nested PCR protocol. While not statistically significant, the multiplex PCR method found more infected individuals (52.7%) than the nested PCR method (48.8%) in a sample of birds from a community in southern Sweden. The multiplex PCR was also used to test for single and mixed infections in a small set of individuals previously screened and found to be infected by one or more parasite genera using microscopy. The multiplex PCR had similar results compared with the results from microscopy. Finally, we used RQ analyses to investigate the amplification sensitivity of the multiplex PCR. These analyses showed that the multiplex PCR can amplify samples that have very low relative parasite intensities (as low as 0.03% of the highest intensity infection identified in a group of 16 infections). These results show that the multiplex PCR is effective and highly sensitive in identifying both single and mixed infections from all three genera of avian haemosporidian parasites.

Since Bensch et al. (2009) compiled MalAvi as a public database of haemosporidian parasites in avian hosts, the number of molecular based studies has increased remarkably. Although several nuclear (DHFR-TS, SSU-rRNA, etc.) and mitochondrial (cytochrome oxidase 3) markers exist, a nested PCR protocol targeting 479 bp of cyt *b* (Bensch et al. 2000; Hellgren et al. 2004) has become the most widely used protocol (Bensch et al. 2009; Clark et al. 2014). Because of this, we compared the detection sensitivity of our multiplex PCR method with that of the aforementioned nested PCR protocol. We found more infected individuals with the multiplex PCR method (52.7%) than with the nested PCR method (48.8%), although the difference was not statistically significant (Fig. 2, Table 4). These comparative results show that the multiplex PCR method described here is at least as sensitive as the nested PCR for detection of avian haemosporidian infections. Furthermore, the lineages amplified by the multiplex PCR method represent a phylogenetically diverse sample of all known avian haemosporidians (Supplementary Fig. 1). This suggests that the multiplex PCR, like the nested PCR, will be able to amplify infections in birds globally.

Several PCR protocols that are widely used are inefficient at amplifying and insensitive at detecting mixed haemosporidian infections (Valkiūnas et al. 2006; Martínez et al. 2009; Bernotienė et al. 2016). Bernotienė et al. (2016) tested the ability of five PCR assays (Bensch et al. 2000; Richard et al. 2002; Hellgren et al. 2004; Beadell et al. 2004; Martinsen et al. 2008), which are broadly used in wildlife avian haemosporidian research, to detect experimentally created mixed avian haemosporidian infections (double and triple mixed infections of *Plasmodium* and *Haemoproteus* spp.). The tested molecular assays had low to moderate success to detect mixed infections (7–53% of mixed infections detected) and therefore underestimated the rates of mixed infections. We compared detectability of mixed infections (i.e., mixed genus infections)

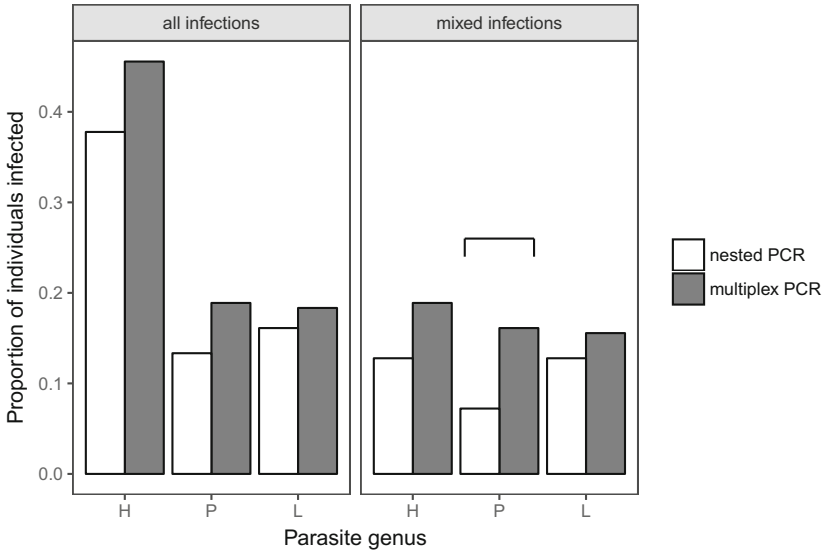

Fig. 2 Proportion of individuals infected by parasites from the three genera investigated (H, *Haemoproteus*; P, *Plasmodium*; L, *Leucocytozoon*) using a standard nested PCR method (Bensch et al. 2000; Hellgren et al. 2004) and the multiplex PCR method developed here. The total number of individuals screened in this analysis was 180. All infected individuals and individuals with mixed infections only are presented separately. The proportion of individuals infected by *Plasmodium* spp. in mixed infections was significantly greater in the multiplex PCR method compared to the nested PCR method (indicated by a bracket on the figure); no significant differences were detected for the other categories (see text for statistical results)

of our multiplex PCR with the nested PCR protocol of Bensch et al. (2000) and Hellgren et al. (2004) which were reported by Bernotienė et al. (2016) as being able to detect 20% of their experimentally created mixed infections. We report that the multiplex PCR detected more mixed infected individuals (20.5%) than the nested PCR protocol (14.4%) in the sample of birds from southern Sweden, although this result was not statistically significant. However, we found that the multiplex PCR detected *Plasmodium* infections in mixed infected individuals significantly more often than the nested PCR. Our study supports the conclusion of Bernotienė et al. (2016) that the nested PCR most often amplifies the DNA of *Haemoproteus* parasites in *Plasmodium*/*Haemoproteus* spp. double mixed infections. This may be due to the primer sequences being more similar to *Haemoproteus* than to *Plasmodium* or because *Haemoproteus* reaches higher parasitemias than *Plasmodium* (Valkiūnas 2005; Fallon and Ricklefs 2008; Asghar et al. 2011; Mukhin et al. 2016).

Several studies have indicated that microscopic examination can better identify mixed genera avian haemosporidian infections than PCR methods (Valkiūnas et al. 2006; Zehtindjiev et al. 2012; Dimitrov et al. 2013; Ivanova et al. 2018). Therefore, we tested and compared the mixed infection detectability of our multiplex PCR method with 21 DNA samples of previously microscopically screened natural single and mixed infections. The multiplex PCR detected the same parasite genera as microscopy in 15 of 21 (71.4%) infected samples (Table 3). Moreover, one microscopically identified single infection was determined to be a mixed infection by the multiplex PCR, highlighting the benefit of combining molecular and microscopic approaches when possible (Valkiūnas et al. 2008). For the remaining five mixed infection samples, the multiplex PCR only missed one of the parasite genera that microscopy found in each sample (i.e., the multiplex PCR found at least one parasite genus infecting in all samples). This discrepancy is expected and may depend on several factors such as DNA quality and quantity, and parasite intensity (Altshuler 2006). Moreover, the multiplex PCR could amplify infections with very low parasite intensities as shown by the RQ analyses. These results show that the multiplex PCR method is quite specific and nearly as sensitive as microscopic examination for the detection of naturally occurring mixed infections. However, it should be emphasized that the multiplex PCR is insufficient for detecting mixed infections of haemosporidians belonging to the same genus. It is difficult to distinguish congeneric mixed infections by microscopy, particularly at early parasite developmental stages (young gametocytes/trophozoites); such analyses require the preparation of high-quality blood films and detailed ability to identify haemosporidian species morphologically (Valkiūnas et al. 2008, 2014b). Alternatively, phasing multiple peaks in a sequence chromatogram can be used to distinguish mixed infections when the parasite lineages have already been identified from single infections (Matthews et al. 2016). For efficient and accurate detection of mixed infections, we recommend the use of both molecular and microscopic techniques.

Thymine adenine (TA) cloning and DNA sequencing (Pérez-Tris and Bensch 2005), laser microdissection of single blood cells or vector stages (Palinauskas et al. 2010; Valkiūnas et al. 2015), and next-generation sequencing (NGS) assays (Karadjian

et al. 2016) can be used in order to characterize mixed infections of haemosporidian parasites. Among these methods, NGS is the most promising for specific detection of mixed infections (even congeneric mixed infections) and for quantification of their relative intensities (Karadjian et al. 2016). However, all these methods are quite expensive and labor-intensive, and therefore of limited use when screening larger datasets from samples collected in the wild. Moreover, for NGS methods to accurately recover mixed infections, one must carefully design unbiased primers (i.e., the primers should not bind preferentially with one parasite genus over another) and determine sufficient sequencing depth in order to obtain reads from parasites with relatively low intensities (Bensch et al. 2016; Karadjian et al. 2016).

Pacheco et al. (2018b) designed genus-specific mitochondrial PCR primers and a nested multiplex PCR protocol for detecting *Haemoproteus*/*Plasmodium* spp. mixed infections. Although this method is useful and very sensitive for detecting mixed infections of *Plasmodium* and *Haemoproteus* parasites, it does not include *Leucocytozoon* spp. infections which are common in wild birds (Lotta et al. 2016; Freund et al. 2016). Therefore, we developed the multiplex PCR method presented here to detect and discriminate parasites belonging to all three avian haemosporidian genera (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) simultaneously in a single reaction. In terms of best practices for detection of infected samples, we recommend that researchers first use our three-genus multiplex PCR protocol or the two genus protocol of Pacheco et al. (2018b). However, Pacheco et al.'s (2018b) protocol will require an additional assay for identification of *Leucocytozoon* infections, such as the protocol of Hellgren et al. (2004). To determine the molecular lineages of the samples identified as infected will require further analyses, the choice of primers, and protocol depending on the specific question. For example, to compare with lineages in the MalAvi database requires that the sequences overlap with the cyt *b* region used to define lineages. However, phylogenetic studies will require longer sequences, such amplifying the whole mtDNA genome (Pacheco et al. 2018a) or phylogenetically informative nuclear genes (Huang et al. 2018). Another important consideration is that the primer set designed for amplification of *Haemoproteus* and *Leucocytozoon* parasites in the multiplex PCR described here targets the subgenera *Parahaemoproteus* and *Leucocytozoon*. Future studies will need to test whether these primers also amplify *Haemoproteus* (*Haemoproteus*) and *Leucocytozoon* (*Akiba*) species which are often found infecting birds belonging to the orders Columbiformes, Pelecaniformes, Charadriiformes, and Galliformes, respectively (Valkiūnas 2005; Levin et al. 2012).

We also tested the primers in an assay with ordinary Taq DNA polymerase (AmpliTaq™ DNA Polymerase, Thermo Fisher Scientific, Waltham, MA, USA) using the same thermal protocol (except for the initial denaturation time that was set at 95 °C for 2 min). This resulted in substantially more non-specific products than when using the master mix designed for multiplexing (2× Qiagen Multiplex PCR Master Mix, Qiagen, Hilden, Germany; data not shown). Although it might be possible to optimize an assay with ordinary Taq DNA polymerase, we recommend using a polymerase enzyme developed for multiplexing with pre-optimized concentrations for simple, fast, and high-yield multiplex PCR.

In conclusion, the presently described one-step multiplex PCR assay fills an important gap in the avian haemosporidian detection toolkit by allowing for the simultaneous detection and genus-level identification of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* parasites. This novel assay has several advantages over the currently available detection methods and represents a significant improvement in detecting mixed infections. The availability of this one-step multiplex PCR assay will reduce labor and handling time, the costs of reagents, and disposable consumables. It will also limit contamination risk during the transfer of first PCR amplicons to second PCR mixtures because the nested PCR will no longer need to be used as a screening protocol, so fewer samples (only known infections) will be subjected to the nested reaction. These advantages make this quick, specific, and highly sensitive multiplex PCR assay suitable and useful for molecular epidemiological surveillance and biodiversity studies.

Acknowledgments We thank Xi Huang for assisting with quantitative PCR analyses in the laboratory and Tatjana A. Iezhova for microscopic identification of parasites.

Funding information This work was supported by the Swedish Research Council (grant 621-2013-4839 to SB) and also partly funded by the Research Council of Lithuania (grant S-MIP-17-27 to RB). VAE was supported by a postdoctoral fellowship from the Carl Tryggers Foundation.

Compliance with ethical standards Permission for taking blood samples from birds in Sweden was approved by the Malmö/Lund Committee for Animal Experiment Ethics (M45-14). Procedures with birds in Lithuania were performed by licensed researchers and were approved by the Ethical Commission of the Baltic Laboratory Animal Science Association, Lithuania; Lithuanian State Food and Veterinary Office and Environmental Protection Agency, Vilnius.

Conflict of interest The authors declare that they have no conflict of interest.

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