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Avian haemosporidian parasites (Haemosporida): A comparative analysis of different polymerase chain reaction assays in detection of mixed infections

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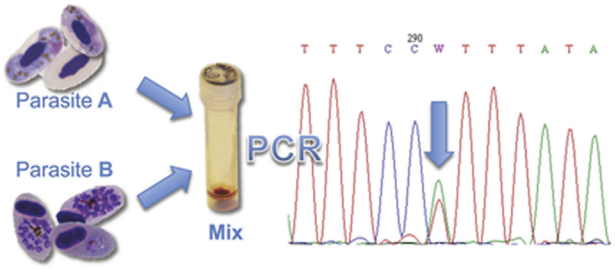
HIGHLIGHTS

- Sensitivity of 5 different PCR assays in detection of mixed haemosporidian infections was studied.
- Each PCR assay remarkably underestimate haemosporidian diversity.
- Application of 3–5 different PCR assays in parallel detect the majority of mixed infections.
- Preferences of different PCR assays were determined in haemosporidian diagnostics.

GRAPHICAL ABSTRACT



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ABSTRACT

Mixed infections of different species and genetic lineages of haemosporidian parasites (Haemosporida) predominate in wildlife, and such infections are particularly virulent. However, currently used polymerase chain reaction (PCR)-based detection methods often do not read mixed infections. Sensitivity of different PCR assays in detection of mixed infections has been insufficiently tested, but this knowledge is essential in studies addressing parasite diversity in wildlife. Here, we applied five different PCR assays, which are broadly used in wildlife avian haemosporidian research, and compared their sensitivity in detection of experimentally designed mixed infections of *Haemoproteus* and *Plasmodium* parasites. Three of these PCR assays use primer sets that amplify fragments of cytochrome *b* gene (cyt *b*), one of cytochrome oxidase subunit I (COI) gene, and one target apicoplast genome. We collected blood from wild-caught birds and, using microscopic and PCR-based methods applied in parallel, identified single infections of ten haemosporidian species with similar parasitemia. Then, we prepared 15 experimental mixes of different haemosporidian parasites, which often are present simultaneously in wild birds. Similar concentration of total DNA was used in each parasite lineage during preparation of mixes. Positive amplifications were sequenced, and the presence of mixed infections was reported by visualising double-base calling in sequence electropherograms. This study shows that the use of each single PCR assay markedly underestimates biodiversity of haemosporidian parasites. The application of at least 3 PCR assays in parallel detected the majority, but still not all lineages present in mixed infections. We determined preferences of different primers in detection of parasites belonging to different genera of haemosporidians during mixed infections.

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1. Introduction

Haemosporidian parasites (Haemosporida) are widespread in wildlife and are cosmopolitan in birds (Garnham, 1966; Valkiūnas, 2005; Perkins, 2014). Some species cause severe diseases both in avian hosts and blood sucking insects (Atkinson et al., 2008; Valkiūnas et al., 2014). Mixed haemosporidian infections i. e., the infections of different species or genetic lineages occurring in same individual hosts, predominate in wildlife, and such infections are often particularly virulent (Pérez-Tris and Bensch, 2005; Palinauskas et al., 2011; Jarvi et al., 2013; Dimitrov et al., 2013, 2015). Different studies reported prevalence of mixed haemosporidian infections between 6% and over 80% in many wild bird species all over the world (Valkiūnas et al., 2003, 2006; Beadell et al., 2004; Pérez-Tris and Bensch, 2005; Loiseau et al., 2010; Silva-Iturriza et al., 2012; Dimitrov et al., 2014).

Polymerase chain reaction (PCR)-based detection methods are widely used in wildlife haemosporidian studies (Bensch et al., 2000; Clark et al., 2014; Perkins, 2014). Application of these diagnostic tools have revealed remarkable genetic diversity of haemosporidian parasites (Ricklefs and Fallon, 2002; Bensch et al., 2004; Hellgren et al., 2004; Ricklefs et al., 2004; Schaer et al., 2015). However, it has been recognised that these methods often are insensitive in reading mixed haemosporidian infections, which can be overlooked if solely PCR-based diagnostic tools are used (Pérez-Tris and Bensch, 2005; Valkiūnas et al., 2006; Martínez et al., 2009; Zehtindjiev et al., 2012). Resolving this methodological problem is important from ecological and evolutionary perspectives (Merino et al., 2000; Marzal et al., 2008); that is also essential in haemosporidian biodiversity studies aiming better understanding host–parasite interactions in wildlife (Sorci et al., 1997; Pérez-Tris and Bensch, 2005; Braga et al., 2011; Clark et al., 2014). Determination of mixed infections is important in conservation projects because such infections often are associated with high virulence (Petney and Andrews, 1998; Marzal et al., 2008; Palinauskas et al., 2011).

PCR-based methods particularly often underestimate haemosporidian mixed infections during co-existence of genetically similar parasite lineages (Pérez-Tris and Bensch, 2005; Zehtindjiev et al., 2012). Dimitrov et al. (2014, 2015) used both PCR-based and microscopic tools in parallel and showed that this methodology markedly increases detectability of haemosporidian infections in wild bird populations. The PCR-based methods were proved to be less sensitive than microscopic examination of blood films in determining mixed infections of different haemosporidian morphospecies, particularly of pigment-forming parasites belonging to *Plasmodium* and *Haemoproteus* spp. (Valkiūnas et al., 2006; Martínez et al., 2009). However, microscopic examination of the same blood samples might be less sensitive in detection of light parasitemia, abortive infections and is not applicable for detecting cryptic haemosporidian species (Bensch et al., 2004; Hellgren et al., 2004; Jarvi et al., 2013; Palinauskas et al., 2015). A sensitive problem of PCR-based detection of mixed infections using general primers is the different affinity of primers shown for different parasite lineages or light intensity of infection by one of these lineages, resulting in low DNA concentrations and poor amplification (Marzal et al., 2008). Interestingly, Zehtindjiev et al. (2012) and Schaer et al. (2015) reported that the success of different primers in detection of haemosporidian infections varies, and PCR does not always amplify DNA of a clearly visible or even predominant parasite in blood samples. Valkiūnas et al. (2006) noted that the sensitivity of the PCR diagnostics was not always directly related to intensity of parasitemia. The usage of different primers in parallel was recommended during initial screening of samples in studies aiming to estimate diversity of malarial infections in wildlife (Zehtindjiev et al., 2012). However, the sensitivity of different PCR assays in detection of mixed infections remains insufficiently tested, but is important for better understanding parasite diversity in wildlife populations.

Here, we tested experimentally the sensitivity of different PCR-based assays in detection of mixed *Haemoproteus* and *Plasmodium* infections, which are widespread and often occur naturally. Five primer sets, which are often used in molecular diagnostics and detection of avian haemosporidians were used (Table 1). This experimental study 1) provides information about preferences and shortcomings of different PCR assays in diagnostics of certain mixed haemosporidian infections and 2) suggests ways how to improve the detectability of mixed haemosporidian infections in wildlife.

2. Materials and methods

2.1. Sample collection

All *Haemoproteus* infections used in this study were obtained from naturally infected birds, and all *Plasmodium* infections were experimental ones (Table 2). To obtain single *Haemoproteus* infections, birds were captured with mist nets at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°09′ N, 20°51′ E) in 2011. A drop of blood was taken from each bird by puncturing the brachial vein to make two or three blood films. The smears were air-dried, fixed in absolute methanol and stained with Giemsa, as described by Valkiūnas et al. (2008). Blood films were examined microscopically in order to detect blood samples with single *Haemoproteus* infection and to determine intensity of parasitemia. About 30 μl of blood was collected in heparinized microcapillaries and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) at ambient temperature while in the field, and then preserved at −20 °C in the laboratory. Samples were examined using PCR to identify the cytochrome *b* (cyt *b*) genetic lineages of *Haemoproteus* parasites in the laboratory at the Nature Research Centre, Vilnius.

At the same time, birds were captured and processed in order to obtain single *Plasmodium* infections, as described for *Haemoproteus* species. Naturally infected birds with single *Plasmodium* infections were used as donors to multiply the parasite strains and to freeze the infected blood in liquid nitrogen, as described by Palinauskas et al. (2015). We used the *Plasmodium* lineages pGRW2, pGRW11, pSGS1, and pTURDUS1, which were isolated from naturally infected common cuckoo *Cuculus canorus*, house sparrow *Passer domesticus*, common crossbill *Loxia curvirostra* and Eurasian wren *Troglodytes troglodytes*, respectively. Siskins *Carduelis spinus* and crossbills were used as recipients for multiplication of these lineages, and blood samples from the recipient birds were used in present experiment (Table 2). Blood samples with single parasite infection, as determined both by microscopic examination of blood films and PCR-based testing (see below), were used to prepare experimental mixed infections. To minimize opportunities of preferable amplification, the blood samples with similar intensity of parasitemia (ranging between 1% and 5%) were chosen (Table 2). Eight different *Haemoproteus* and four *Plasmodium* genetic lineages were used in this study. All these parasites are widespread in wild birds (Valkiūnas et al., 2006; Bensch et al., 2009; Clark et al., 2014).

2.2. Experimental design

Total DNA was extracted from samples with single parasite infections using ammonium acetate extraction method (Richardson et al., 2001). Quantification of the DNA was performed by using nanodrop spectrophotometer (IMPLEN Nanophotometer P330).

Table 1
Five PCR assays used in detection of experimental mixed haemosporidian infections.

Gene fragment	Primer code	Primer sequence	Reference
Cytochrom *b* [a]	Outer HaemNFI	5′-CATATATTAAGAGAAITATGGAG-3′;	Bensch et al., 2000; Hellgren et al., 2004
	HaemNR3	5′-ATAGAAAGATAAGAAATACCATTC-3′	
	Inner HAEMF	5′-ATGGTGCTTTCGATATATGCATG-3′	
	HAEMR2	5′-GCATTATCTGGATGTGATAATGGT-3′	
Cytochrom *b*	621F	5′-AAAAATACCCTTCTATCCAAATCT-3′	Richard et al., 2002
	983R	5′-CATCCAATCCATAATAAAGCAT-3′	
Cytochrom *b*	3760F	5′-GAGTGGATGGTGTTTTAGAT-3′	Beadell et al., 2004
	4292Rw2	5′-TGGAACAATATGTARAGGAGT-3′	
Cytochrome oxidase subunit I [a]	Outer COIF	5′-CTATTTATGGTTTTCATTTTTATTTGGTA-3′	Martinsen et al., 2008
	COIR	5′-AGGAATACGTCTAGGCATTACATTAAATCC-3′	
	Inner COIF2	5′-ATGATATTTACARTTCAYGGWATTATTATG-3′	
	COIR2	5′-GTATTTTCTCGTAATGTTTTACCAAAGAA-3′	
Apicoplast [a]	Outer ClpcF	5′-AAACTGAATTAGCAAAAATATTA-3′	Martinsen et al., 2008
	ClpcR	5′-CGWGCWCCATATAAAGGAT-3′	
	Inner ClpcF2	5′-GATTTGATATGAGTGAATATATGG-3′	
	ClpcR2	5′-CCATATAAAGGATTATAWG-3′	

[a] Nested PCR.

Table 2
Samples of single haemosporidian infections used in this study.

Parasite species and cytochrome *b* lineage code	Avian host	Parasitemia, %
Haemoproteus belopolskyi hHIICT1	*Hippolais icterina*	4
H. lanii hRBS1	*Lanius collurio*	4
H. minutus hTURDUS2	*Turdus merula*	3
H. motacillae hYWT1	*Motacilla flava*	3
H. pallidus hPFC1	*Ficedula hypoleuca*	1
H. parabelopolskyi hSYBOR1	*Sylvia borin*	5
H. tartakovskyi hHAWF1	*Coccothraustes coccothraustes*	2
H. tartakovskyi hSISKIN1	*Carduelis spinus*	4
Plasmodium ashfordi pGRW2	*Loxia curvirostra*	1
P. circumflexum pTURDUS1	*Carduelis spinus*	3
P. relictum pGRW11	*Loxia curvirostra*	1
P. relictum pSGS1	*Carduelis spinus*	2

Table 3
Genbank accession numbers of haemosporidian parasite lineages used in this study.

Parasite name and cytochrome *b* lineage code	Genome and primers used in PCR assays				
	Mitochondrial cytochrome *b*			Mitochondrial cytochrome oxidase	Apicoplast
	HaemNFI/NR3 HAEMF/R2	621F/983R	3760F/4292Rw2	COIF/R COIF2/R2	ClpcF/R ClpcF2/R2
Haemoproteus belopolskyi hHIICT1	JX026904	**KM361498**[a]	KM361487	**KM434209**	**KM361469**
H. lanii hRB1	JX026907	**KM361500**	KM361489	**KM434211**	**KM361471**
H. minutus hTURDUS2	JX026900	KM361496	KM361485	**KM434207**	**KM361466**
H. motacillae hYWT1	KM361477	KM361495	KM361484	–	**KM361465**
H. pallidus hPFC1	KM361479	KM361499	KM361488	**KM434210**	**KM361470**
H. parabelopolskyi hSYBOR1	KM361478	-[b]	KR080370	**KM434216**	**KM361467**
H. tartakovskyi hHAWF1	JX026903	**KM361497**	KM361486	**KM434208**	**KM361468**
H. tartakovskyi hSISKIN1	JX026908	KM361501	KM361490	**KM434212**	**KM361472**
Plasmodium ashfordi pGRW2	KM361483	KM361505	KM361494	**KM434215**	**KM361476**
P. circumflexum pTURDUS1	KM361481	KM361503	KM361492	KM434214	**KM361474**
P. relictum pGRW11	KM361482	KM361504	KM361493	KR080371	KM361475
P. relictum pSGS1	KM361480	KM361502	KM361491	KM434213	KM361473

[a] New sequences are given in Bold font.
[b] Unsuccessful amplification.

The samples, in which total DNA concentration exceeded 100 ng/μl, were resolved with TE buffer, so that the final DNA concentration in all samples was 41 ± 5.3 ng/μl. Five PCR assays were used for DNA amplification from 12 samples with single infection (Tables 1–3).

Using the genomic DNA from single infections (Table 3), we prepared 15 experimental mixes of different haemosporidian combinations, which often occur naturally in wild birds (Valkiūnas et al., 2006; Dimitrov et al., 2014, 2015) The same amount (15 μl) of similar concentration DNA was used. The following experimental mixes containing different combinations of parasites were prepared (Table 4): 1) two different *Haemoproteus* lineages (two samples); 2) one *Haemoproteus* and one *Plasmodium* lineages (eight samples); 3) two different *Plasmodium* lineages (three samples), and 4) one *Haemoproteus* and two different *Plasmodium* lineages (two samples).

Five PCR assays, which are broadly used in wildlife avian haemosporidian research, were compared in regard of their sensitivity in detection of experimental mixed infections of *Haemoproteus* and

Table 4
Results of five PCR assays used in detection of experimental mixed haemosporidian infections. Correctly determined mixed infections are given in bold font.

Experimental mixed infections of different parasite lineages	Detected lineages of the corresponding genome using different primers				
	HaemNFI/NR3 HAEMF/R2	621F/983R	3760F/4292Rw2	COIF/R COIF2/R2	ClpcF/R ClpcF2/R2
hSISKIN1/hHAWF1	**hSISKIN1/ hHAWF1**	**hSISKIN1/ hHAWF1**	**hSISKIN1/hHAWF1**	**hSISKIN1/ hHAWF1**	hHAWF1
hSYBOR1/hHIICT1	**hSYBOR1/hHIICT1**	-[a]	hSYBOR1	–	**hSYBOR1/ hHIICT1**
hSISKIN1/pSGS1	hSISKIN1	pSGS1	pSGS1	hSISKIN1	pSGS1
hHAWF1/pSGS1	hHAWF1	pSGS1	**hHAWF1/pSGS1**	pSGS1	pSGS1
hTURDUS2/pSGS1	hTURDUS2	pSGS1	**hTURDUS2/pSGS1**	hTURDUS2	pSGS1
hRB1/pSGS1	pSGS1	pSGS1	hRB1	–	hRB1
hYWT1/pSGS1	hYWT1	pSGS1	hYWT1	pSGS1	pSGS1
hSYBOR1/pSGS1	pSGS1	pSGS1	**hSYBOR1/pSGS1**	hSYBOR1	pSGS1
hPFC1/pSGS1	hPFC1	pSGS1	**hPFC1/pSGS1**	hPFC1	**hPFC1/pSGS1**
hHIICT1/pSGS1	pSGS1	pSGS1	hHIICT1	pSGS1	pSGS1
pTURDUS1/pSGS1	pTURDUS1	**pTURDUS1/pSGS1**	**pTURDUS1/pSGS1**	pTURDUS1	pSGS1
pGRW11/pSGS1	**pGRW11/pSGS1**	pGRW11 = pSGS1[b]	**pGRW11/pSGS1**	pGRW11 = pSGS1[b]	**pGRW11/pSGS1**
pGRW2/pSGS1	pGRW2	pGRW2	pSGS1	pGRW2	pSGS1
hHAWF1/pTURDUS1/pSGS1	pTURDUS1	pTURDUS1/pSGS1	**hHAWF1/pTURDUS1/ pSGS1**	pTURDUS1	pSGS1
hSISKIN1/pTURDUS1/pSGS1	pTURDUS1	pTURDUS1/pSGS1	pTURDUS1/pSGS1	pSGS1	pSGS1
Total number of determined mixed infections (%)	3 (20)[c]	2 (14)	8 (53)	1 (7)	3 (20)

[a] Unsuccessful amplification.
[b] Cytochrome *b* lineages, which are indistinguishable in gene region amplified using certain set of primers.
[c] Number of correctly detected mixed infections is given, followed in parenthesis by their percentage.

Plasmodium species (Table 4). Four of these PCR assays used primer sets, which amplify fragments of the mitochondrial genome: three of cyt *b* gene, one of cytochrome oxidase subunit I (COI) gene, and one target apicoplast genome (see Table 1).

The detectability of mixed infections using different PCR assays was compared by Fisher's exact test. A *P*-value of 0.05 or less was considered significant. The analysis was carried out using 'Statistica 7' package.

2.3. PCR protocols

All reactions were performed in 25 μl total volumes, including 50 ng of a total genomic DNA template (2 μL), 12.5 μL of DreamTag Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 μL nuclease-free water and 1 μL of each primer. One negative control (nuclease-free water) and one positive control (an infected sample, which was positive by microscopic examination of blood films) were used per every 14 samples to control for false amplifications. No cases of false positive samples were found. All samples classified as false negatives were tested at least twice to ensure that the negative results were not caused by PCR failure.

The 479 bp length segments of parasite mitochondrial cyt *b* gene were amplified using nested PCR protocol (Bensch et al., 2000; Hellgren et al., 2004) and two pairs of primers: HaemNFI/HaemNR3 (Table 1), which amplifies gene fragment of haemosporidians belonging to genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, and the primers HAEMF/HAEMR2, which are specific to *Haemoproteus* and *Plasmodium* spp. The "Basic Local Alignment Search Tool" (National Centre for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST) was used to determine cyt *b* lineages of detected DNA sequences.

The segments of 338 bp of cyt *b* were amplified using the primers 621F/983R (Richard et al., 2002) and a larger fragment of cyt *b* gene (533 bp) was amplified using primers 3760F/4292Rw2 (Beadell et al., 2004) (Table 1). The mitochondrial COI gene fragment (918 bp) was amplified using COIF/R and COIF2/R2 primers and nested PCR protocol presented by Martinsen et al. (2008). The primers ClpcF/R and ClpcF2/R2 and nested PCR assay were used for the amplification of apicoplast gene fragments of 536 bp length (Martinsen et al., 2008).

Temperature profiles in all PCRs' were the same as in the original protocol descriptions (see references in Table 1). All amplifications were evaluated by running 2 μl of the final PCR products on a 2% agarose gels. Fragments of DNA from all positive amplifications were sequenced with corresponding primers twice for both strands. The obtained sequences were aligned and analysed using Bioedit program (Hall, 1999).

Mixed infections were determined by visualising double-base calling in sequence electropherograms (Pèrez –Tris and Bensch, 2005). All sequences obtained from experimental mixes were compared with corresponding sequences from initial single parasite infections. We considered reports of mixed infections to be positive if 1) double-base calling in sequence electropherogram was visualised and 2) sequences of initial lineages, which were used to prepare mixed samples, were identified.

2.4. Ethical statement

The experiments described herein comply with the current laws of Lithuania and Russia. Experimental procedures of this study were approved by the International Research Co-operation Agreement between the Biological Station "Rybachy" of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25-05-2010).

3. Results

3.1. Detection of lineages in single infection

All samples containing single infections were successfully amplified, and all sequences were obtained using apicoplast primers (Martinsen et al., 2008) and cyt *b* primers using two PCR protocols published by Bensch et al. (2000) and by Beadell et al. (2004). The assay with cyt *b* primers 621F/983R (Richard et al., 2002) did not detect the lineage hSYBOR1, and the assay with COI primers (Martinsen et al., 2008) did not detect the lineage hYWT1 (Table 3); both these PCR assays were repeated twice with the same result. All sequences obtained in this study were submitted to

GenBank; 21 of them were new (Table 3).

3.2. Detection of double mixed infections

The PCR assay described by Bensch et al. (2000) (primers HaemNFI/NR3 and HAEMF/R2) was the most sensitive in detection of mixed infections of two *Haemoproteus* spp. (Table 4). This primer set detected mixed infections of *Haemoproteus* parasites in both experimentally designed samples. All other primer sets detected *Haemoproteus* mixed infections only in one of two samples (Table 4).

The most sensitive primer set for detection of *Plasmodium* mixed infections were the cyt *b* primers described by Beadell et al. (2004) (3760F/4292Rw2). These primers detected two of three *Plasmodium* mixed infections. All other primer sets detected only one of three experimentally designed *Plasmodium* mixed infections. It should be mentioned that detection of some closely related lineages pSGS1 and pGRW11 of *Plasmodium relictum* was impossible using the COI primers and the cyt *b* primers 621F/983R. Obtained DNA fragments of these parasites are identical using mentioned primers, which thus cannot be used for detection of mixed infections of these two closely related *Plasmodium* lineages (Table 4).

Experimental mixed infections of *Haemoproteus* and *Plasmodium* species were the most difficult to detect using all primer sets. The assay using the cyt *b* primers 3760F/4292Rw2 detected 50% (four of eight) of such mixed infections, while the COI primer sets, the cyt *b* primers designed by Bensch et al. (2000) (HaemNFI/FR3 and HAEMF/R2) and Richard et al. (2002) (621F/983R) detected only one genetic lineage in all investigated mixes (Table 4). Only one of eight experimental mixes of *Haemoproteus* and *Plasmodium* species was detected using the apicoplast primer set. The latter primer set and the cyt *b* primers 621F/983R showed preference to detect *Plasmodium* infections, and they markedly underestimated *Haemoproteus* spp. All other investigated PCR protocols amplified *Plasmodium* and *Haemoproteus* approximately equally (Table 4).

3.3. Detection of triple mixed infections

The PCR assay using the cyt *b* primers 3760F/4292Rw2 detected one of two experimentally designed triple mixed infections, while other primer sets were insensitive to read such infections: they detected only two genetic lineages (primers 621F/983R) or one genetic lineage of three lineages that were present in the samples (Table 4).

3.4. Summary of experiment

The detectability of mixed infections was between 7% and 20% in all PCR assays, except for the cyt *b* primers 3760F/4292Rw2, which detected 53% of all experimental mixed infections. The ability of the latter primers to detect mixed infections was significantly greater in comparison to the COI primer sets and cyt *b* primers 621F/983R ($P < 0.05$ for both tests), which indicates the low sensitivity of the later two primers to read mixed haemosporidian infections.

None of the PCR assays showed true composition of mixes in all samples tested however, the application of two PCR assays in parallel (the cyt *b* primers 3760F/4292Rw2 combined with the apicoplast primers set or the cyt *b* primers 3760F/4292Rw2 combined with the cyt *b* primer set HaemNFI/NR3 and HAEMF/R2) detected 60% of lineages present in experimental mixes. We show that the application of three primer sets (apicoplast, cyt *b* 3760F/4292Rw2 and cyt *b* HaemNFI/NR3, HAEMF/R2) in parallel can detect up to 90% of mixed haemosporidian infections. The application of all tested primer sets in parallel detect the great majority, but still not all mixed infections.

4. Discussion

This is the first experimental study, which tested the sensitivity of different primer sets in detection of mixed avian haemosporidian infections (Table 4). Twenty-one new lineages of haemosporidian parasites were obtained and deposited in Genbank (Table 3). We support conclusions of former investigations about insufficient sensitivity of the currently used PCR-based protocols in reading haemosporidian mixed infections (Valkiūnas et al., 2006; Martínez et al., 2009; Loiseau et al., 2010; Dimitrov et al., 2013, 2015). The key result of this experiment is that the use of each single PCR assay remarkably underestimate diversity of haemosporidians, but the application of 3–5 different PCR assays in parallel markedly increase detectability of mixed infections, thus can be recommended for haemosporidian biodiversity studies in wildlife. We identified such PCR assays and determined their preferences in detection of parasites of genera *Haemoproteus* and *Plasmodium* (Table 4).

PCR-based detection of mixed infections depends on the parasite lineages present in a sample and DNA quantity (Beadell and Fleischer, 2005; Pérez-Tris and Bensch, 2005; Martínez et al., 2009). Failure in detection of mixed infections of *Plasmodium* and *Haemoproteus* spp. have been often observed (Pérez-Tris and Bensch, 2005; Valkiūnas et al., 2006; Martínez et al., 2009; Zehtindjiev et al., 2012). That is expected because many PCR assays were developed using conserved *Plasmodium* spp. sequences, and hence often preferentially amplify DNA of parasites of this genus (Richard et al., 2002). It is important to note that preferential amplification of *Plasmodium* spp. is often reported without relation to the DNA concentration of *Haemoproteus* parasites present in the same sample. For example, Pérez-Tris and Bensch (2005) did not detect DNA sequence signals of *Haemoproteus* parasites using cyt *b* primers HAEMF/R2, despite significant initial DNA concentration of the latter parasites in mixed infection with *Plasmodium* sp. Similar results were recorded in this study using apicoplast primers and cyt *b* primers 621F/983R (Table 4).

It worth mentioning that PCR does not always amplify DNA of a clearly visible and even predominant parasite in blood samples (Zehtindjiev et al., 2012; Schaer et al., 2015). Even more, PCR-based tools were reported to detect different lineages in two blood samples collected from the same individual host during a day. For example, Palinauskas et al. (2015) tested blood of the same individual bird co-infected with 2 different malaria parasites; the sample collected in the morning showed presence of single *Plasmodium homocircumflexum* (lineage pCOLL4) infection, and the blood sample collected in the afternoon showed a single infection of *P. relictum* (lineage pLZFUS01). These parasites are readily distinguishable based on morphology of their blood stages. We thus point out the need of careful analyses of haemosporidian prevalence and biodiversity data, which are based solely on PCR-based testing, particularly in wildlife where natural combinations of mixed infections are difficult to predict.

The cyt *b* primers 621F/983R were designed using *Plasmodium* sp. cyt *b* sequences of mammalian parasites deposited in GenBank (Richard et al., 2002). According to our study (Table 4), this primer set can be successfully used in detection of *Plasmodium* spp. in mixed infections with *Haemoproteus* parasites. However, the detection of mixed infections of different *Plasmodium* spp. remains problematic because numerous mixed malarial infections remained undetected using this primer set (Table 4).

Bensch et al. (2000) developed primers using conserved regions of the cyt *b* gene of parasites belonging to *Haemoproteus* and *Plasmodium*. DNA of parasites of these two genera was used to locate conserved regions of cyt *b* gene to create the primer sets HAEMF/HAEMR2 (Bensch et al., 2000; Hellgren et al., 2004) and 3760F/4292Rw2 (Beadell et al., 2004). In this study (Table 4), both

primer sets amplified DNA of *Plasmodium* and *Haemoproteus* parasites approximately equally, but the primer set created by Bensch et al. (2000) and Hellgren et al. (2004) was less sensitive in determining mixed infections belonging to different genera of haemosporidians.

Apicoplast primers (Martinsen et al., 2008) cannot be recommended in haemosporidian diversity studies due to the low detectability of *Haemoproteus* spp. in various mixed infections (Table 4). This study also points out (Table 4) that some primers cannot be used for detection of closely related genetic lineages (for example, pSGS1 and pGRW11) because some DNA fragments of these lineages are identical.

Pérez-Tris and Bensch (2005) applied Thymine adenine (TA) cloning and sequencing for detection of mixed avian haemosporidian infections. This method reads some mixed infections, but is time and funding consuming. It is expected that each PCR product containing mixed haemosporidian infection can be separated by cloning (Waldenstrom et al., 2004). This method is suffering of PCR errors such as single nucleotide mutations and jumping PCR artefacts (Pérez-Tris and Bensch, 2005), and it remains rarely used in practical work in wildlife haemosporidian research. Recently, multiplex PCR and real-time PCR techniques have been applied to screen for mixed parasite infections (Cheesman et al., 2003; Perandin et al., 2004; Yamasaki et al., 2004; Bell et al., 2015). However, these assays remain relatively expensive and hardly applicable for diagnostics of mixed infections when undescribed new parasite lineages are present in samples (Pérez-Tris and Bensch, 2005), which is an often case in current wildlife haemosporidian research (Zehtindjiev et al., 2012).

Laser microdissection of single blood cells or vector stages of avian *Haemoproteus* and *Plasmodium* spp. can be used in reading haemosporidian mixed infections (Palinauskas et al., 2010; Valkiūnas et al., 2015). The dissected single cells, meronts or oocysts can be used for DNA isolation, DNA amplification, and sequencing, providing information about lineages of certain parasites present in samples. This method is readily applicable in experimental research, but is difficult to use during initial screening of blood samples obtained in wildlife, particularly in remote study sites. Development of primers specific to certain parasite lineages can be helpful in detection of targeting infections. However, this methodology is difficult to apply in wildlife biodiversity research due to hardly predictable parasite lineage combinations, which often are unique at each study site and bird population. This is particularly true for tropical areas where diversity of haemosporidians is high and remains insufficiently described (Durrant et al., 2006).

In conclusion, we call for more careful research and data analysis of mixed haemosporidian infections in wildlife by application of microscopic and several PCR-based assays, which ideally should be applied in parallel. This study identifies a combination of currently widely used PCR-based assays, which can be sensitive in reading many mixed infections and can be used in studies of diversity of haemosporidian parasites in wildlife populations.

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