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

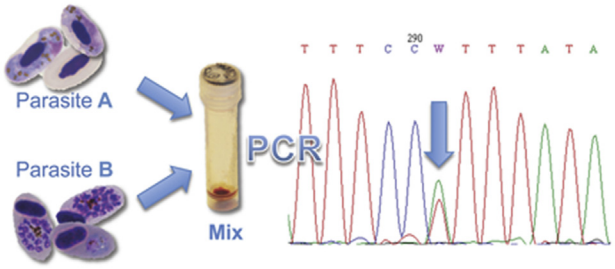
Rasa Bernotienė*, Vaidas Palinauskas, Tatjana Iezhova, Dovilė Murauskaitė, Gediminas Valkiūnas

Nature Research Centre, Akademijos 2, Vilnius 2100, LT-08412, Lithuania

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.

* Corresponding author.
E-mail addresses: rasab@ekoi.lt (R. Bernotienė), vaidas@ekoi.lt (V. Palinauskas), tatjana@ekoi.lt (T. Iezhova), d.murauskaitei@gmail.com (D. Murauskaitė), gedvalk@ekoi.lt (G. Valkiūnas).

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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).