New PCR primers targeting on a cytochrome b gene reveal diversity of Leucocytozoon lineages in an individual host

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Abstract

Avian haemosporidian parasites have received considerable attention in ecological and evolutionary as a result of its wide distribution and ease of detection. However, conventional PCR-based detection methods may sometimes underestimate haemosporidian-mixed infections, which are frequent in natural populations. This underestimation is due to differences in PCR sensitivity for detection of lineages within the mixed infections. Therefore, we designed new primers to amplify sequences that were not detected by the conventional primers and examined if our primers were useful for accurate detection of mixed infections. Blood samples were collected from 32 wild birds captured in Hokkaido, and 16 of these were positive for *Leucocytozoon* using the conventional primers, while 15 were positive using our primers. All positively amplified samples were sequenced, and found that the conventional primers detected 16% (5/32) multiple infections and none of them was novel lineage, whereas our primers detected 44% (14/32) multiple infections and ten of them were novel lineages. A phylogenetic analysis showed that the new primers can detect a wide range of *Leucocytozoon* lineages compared with that detected by the conventional primers. The results indicate that our primers are particularly suitable in revealing unique strains from multiple infections. Highly variable multiple infections in the same population of birds at the same location were found for the first time. We revealed high diversity of *Leucocytozoon* lineages in nature than expected, which would provide more information to better understand parasite diversity and host-vector interactions in wildlife.

1. Introduction

The avian malaria parasite (*Plasmodium*) and the related haemosporidians (*Haemoproteus* and *Leucocytozoon*), which are transmitted by arthropod vectors, are widely distributed in a great variety of avian host species. These parasites are considered to play a critical role in the dynamics of bird population through negative effects on survival (Alfonso et al. 2005). Therefore, detection of the genetic lineages of parasites within a host is important for ecological and evolutionary studies in wildlife.

PCR-based detection methods are widely used in surveys of haemosporidian wildlife infections. Application of these diagnostic tools revealed the remarkable genetic diversity of haemosporidian parasites. The first PCR-based protocol for detection of avian haemosporidia was reported by the Feldman research group (R.A. Feldman et al. 1995). This PCR targeted both parasite and host 18S rRNA genes but has not been used broadly as it only works for a small group of parasites belonging to the *Plasmodium* genus (R.A. Feldman et al. 1995). Subsequently, more general primers for both avian *Plasmodium* and *Haemoproteus* were reported by the Bensch group (Bensch et al. 2000) where a portion of the cytochrome b gene (*cob*) coded in the mitochondrial genome is targeted by PCR. Hellgren et al. slightly modified the Bensch protocol to amplify the target gene in *Leucocytozoon* (Hellgren et al. 2004), and these two protocols are still widely used for haemosporidian detection.

Although the PCR-based method markedly increases the detection of haemosporidian infections compared with that from microscopic examination of blood films, this occasionally underestimates

haemosporidian-mixed infections, which occur frequently in natural populations during co-existence of genetically similar parasite lineages (Pérez-Tris and Bensch 2005; Zehtindjiev et al. 2012). PCR-based detection in mixed infections using the conventional primers encounters an issue with sensitivity due to different primer affinities for parasite lineages. The sensitivity and specificity of haemosporidian detection varies with primers, and PCR does not always preferentially amplify the predominant parasite strain in the blood in mixed infections (Zehtindjiev et al. 2012; Bernotiene et al. 2016). Therefore, the use of different primers in parallel was recommended to estimate the diversity of malarial infections in wildlife (Bernotiene et al. 2016). However, the development of a primer set that can comprehensively detect multiple infections from the same haemosporidian genus has not yet been described.

In this study, we designed new primers targeting *cob*. We demonstrate that our primers have greater sensitivity to a wider range of parasite lineages than the conventional primers. Using our primers, we revealed a higher diversity of *Leucocytozoon* lineages in nature than expected. By detecting multiple infections, which have often been overlooked, we will be able to comprehensively examine novel lineages and accumulate important information on better understanding the remarkable diversity of parasites in wildlife populations.

2. Materials And Methods

Bird blood and DNA extraction

A total of 32 blood specimens were collected from 19 *Streptopelia orientalis* and 13 *Columba livia* birds in Obihiro, located in the southern part of Hokkaido in Japan, between April to May 2019. Bird species were identified using morphological characters of their heads. All animal experiments in this study were conducted in accordance with the guidelines for the use of animals of Obihiro University of Agriculture and Veterinary Medicine, Japan (Permit number: 19 − 14). DNA was extracted with NucleoSpin DNA RapidLyse (Takara Bio, Shiga, Japan) following the manufacturer's instructions. The extracted DNA was stored at − 30°C until use.

PCR and sequencing

To detect *Leucocytozoon, Haemoproteus*, and *Plasmodium* infections, the extracted DNA was amplified via nested PCR of the *cob* region from mitochondrial DNA. The first PCR was performed using primers CytB_HPL_intF1 and CytB_HPL_intR1, designed as previously described (Harl et al. 2019) (Supplementary Table 1). A 1 µL volume of the PCR product was used as a template for the second amplification with specific primers: HaemF and HaemR2 for *Haemoproteus* and *Plasmodium* and HaemFL and HaemR2L for *Leucocytozoon* (Bensch et al. 2000; Hellgren et al. 2004). All PCRs were performed in a 50 µL reaction volume containing 25 µL of KOD one PCR Master Mix (TOYOBO, Osaka, Japan), 1.5 µL of each primer (10 µM), 2 µL of template DNA (20–100 ng/µL, and 20 µL of deionized water. Conditions for both PCRs were as follows: 30 cycles at 98°C for 10 sec, 55°C for 5 sec, and 68°C for 5 sec. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). DNA sequencing was performed directly using the BigDye Terminator v3.1 Cycle sequencing Kit (Thermo Fisher Scientific, Waltham, MA) and an

ABI 3130 Genetic Analyzer (Thermo Fisher Scientific). Nucleotide sequences of each PCR product were confirmed using bi-directional sequences. The sequences obtained in this study have been deposited in DDBJ/EMBL/GenBank with the accession numbers described in Supplementary Table 3.

Isolation of natural-multiple infections

Double or multiple peaks in electropherograms of the second PCR products were defined as multiple infection by *Leucocytozoon* lineages in an individual bird (Supplementary figure). Cloning was performed using TArget Clone™ (TOYOBO) to isolate each PCR product. Briefly, PCR products were inserted into the pTA2 Vector (TOYOBO), which was transformed into *E. coli* HST08 Premium Competent Cells (Takara) using heat shock at 42°C. Cells were cultured on S.O.C. medium (Takara) at 37°C for 1 h in a shaking incubator and then spread on a LB-agar plate supplemented with 50 μg/mL ampicillin. The plate was incubated overnight at 37°C, and then, sixteen colonies for each isolate were picked and checked using colony PCR. Next, the colony PCR was performed in a 25 μL reaction volume containing 5 μL of 5 × PrimeSTAR GXL Buffer (Takara), 2 μL of dNTP Mixture, 0.75 μL of M13-20 and M13 Reverse primers (10 μM) (see Supplementary Table 1 for their nucleotide sequences) (Saiki et al. 1988), 1 μL of PrimeSTAR GXL DNA polymerase (Takara) and 16.25 μL of deionized water. PCR conditions were the following: 30 cycles at 98°C for 10 sec, 55°C for 5 sec, and 68°C for 30 sec. Ten colonies were selected from each isolation for sequencing. Plasmid DNA was extracted using Mag Extractor (TOYOBO), according to the manufacturer's instructions and sequenced as described above. The obtained nucleotide sequences were aligned with reference sequences deposited in the MalAvi database (Bensch et al. 2009) to identify lineages. We defined a new lineage as a sequence with one or more single nucleotide polymorphisms (SNPs) in the *cob* gene (Schumm et al. 2021).

Phylogenetic analyses

For construction of the *Leucocytozoon cob* gene phylogenetic tree, we chose 16 *Leucocytozoon* lineages found in this study and 29 reported lineages that infect species of the order Columbiformes. Sequences were identified using the MalAvi lineage name (Bensch et al. 2009). Several of the reported lineages demonstrated insufficient sequence length, so a 477-bp *cob* fragment was selected for phylogenetic analyses. All lineages detected in this study are represented in the phylogenetic analysis. The phylogenic tree was constructed in MEGA using neighbor-joining (NJ) methods (Kumar et al. 2018). The Kimura two-parameter model was used to estimate the evolutionary distance. To assess tree topology, bootstrap re-sampling (1,000 cycles) was performed for each method.

3. Result

PCR primers designed in this study

In 1 of 13 PCR products amplified using the previously reported *Haemoproteus* primer set, HaemF, and HaemR2, we identified *Leucocytozoon* sp. rather than *Haemoproteus* sp. by sequencing a part of *cob* (Bird ID S9 in Supplementary Table 2). This result led us to infer that some *Leucocytozoon* lineages exist

that are not amplified using HaemFL and HaemR2L (Hellgren et al. 2004). We thus confirmed whether the *cob* regions of *Leucocytozoon* that can be annealed by the HaemFL/HaemR2L primers are conserved. To do this, we aligned *cob* sequences from 12 *Leucocytozoon* lineages reported by Pacheco et al. (2018) (Fig. 1). We focused on conservation of *cob* regions that anneal with the HaemFL and HaemR2L primers. We found several mutations in this region, so we designed new primers (HaemFLn and HaemRLn) to amplify minor-lineage sequences that would be difficult to amplify with the conventional primers (Fig. 1) (see Supplementary Table 1 for their nucleotide sequences). Our new primers were designed as follows: i) at sites with mutations in more than one lineage, the minority base was used at the new primer sequence (Fig. 1) (T219C, C228T, T234C, T723A); ii) the highly conserved position at 715 (C) was chosen as the 3' terminal of HaemR2Ln the primer since the 3'-terminal region is critical for PCR (that of HaemR2L primer is not well conserved).

Prevalence of haemosporidian parasites

Samples were taken from a total of 32 individual birds, 19 *S. orientalis*, and 13 *C. livia*, for PCR analysis. In the *Haemoproteus*-specific PCR assay, 12 out of 32 birds were positive. These birds were all *S. orientalis* and were co-infected with *Leucocytozoon*, as described in Table 1 and Supplementary Table 2. In the *Leucocytozoon*-specific assay, 16 out of 32 birds were positive using the conventinal primers (HeamFL/HaemR2L), and 15 were positive using our primers (HeamFLn/HaemR2Ln). The infection ratio was 79% (15/19) and 8% (1/13) in *S. orientalis* and *C. livia*, respectively. All the samples with positive amplifications were sequenced, and multiple infections were detected by visualizing the double-base calling in sequence electropherograms. PCR with *Haemoproteus*-specific primers did not detect any multiple infections (data not shown). PCR with the HeamFL/HaemR2 primers detected multiple infections in 16% of the samples (5/32), whereas our primers HeamFLn/HaemR2n detected multiple infections in 44% (14/32) (Fig. 2), indicating that our primers are more suitable for detection of multiple infection.

Identification of haemosporidian lineages in single and multiple infection

Of the 12 samples that tested positive for *Haemoproteus*, 11 were identified as STRORI01 lineage, and the remaining one (Bird ID S10 in Supplementary Table 2) demonstrated an SNP at position 430 of *cob* (Supplementary Table 2). The PCR products that were identified as “multiple infections” by their sequencing electropherograms were cloned, and 10 clones from each PCR product were sequenced. SNPs were sequenced more than three times for confirmation. Three different sequences were found from Bird ID S3, six from S4, two from S13, two from S23, and four from S29 (Fig. 3a). We registered these as newly discovered lineages (STRORI06 to STRORI15). However, no novel lineages were identified from PCRs using the conventional primers. All of the 16-*Leucocytozoon*-PCR-positive specimens amplified using the conventional primer set belonged to one of the four previously reported lineages (Fig. 3a). In addition to the three lineages (AMO02, COLIV04, and STRORI05) that were reported, ten novel lineages were identified using our primers, HaemFLn/HaemRLn (Fig. 3b), which could detect all lineages detected by the conventional primers except for one (STRORI02) (Fig. 3). All the novel lineages were found in samples indicated to be multiple infections. Of the ten novel lineages, eight demonstrated the

same sequence found in more than two individual birds. In Bird ID S10, a STRORI05 lineage was detected with the conventional primer set, while the AEMO02 and COLIV04 lineages were detected using our primer set, indicating that detection of a number of lineages does depend on the primers used (Fig. 3).

Lineage diversity and phylogenetic analyses

To confirm the phylogenetic diversity of the lineages found in this study, a phylogenetic analysis was performed. Nine of the ten novel lineages were closely related and belong to the same phylogenetic group (Fig. 4). The new primers detected a wide range of *Leucocytozoon* compared with those detected using the conventional primers (Fig. 4). To show the variety of SNPs from all lineages found in this study, we compared them to AEMO02, which is the most widely infected lineage. Consequently, we identified 59 SNPs (Fig. 5). To see if unknown SNPs only found in novel lineages were present, we compared ten novel lineages with four known ones and determined that 13 new mutations were found only in the novel lineages. (Fig. 5).

4. Discussion

Our study found that *Leucocytozoon* exhibited a higher prevalence and diversity than expected, causing multiple infections. In comparison, the single *Haemoproteus* lineage of STRORI01 was detected in 11 samples although one sample (the bird ID S10) demonstrated a sequence that differed from STRORI01 by one nucleotide (Supplementary Table 2). The previous study has reported that the prevalence of *Leucocytozoon* was higher than that of *Haemoproteus* in all kinds of birds of Hokkaido (Yoshimura, 2014), as supported by this study (Table 1). This difference may be attributed to the host-vector-parasite interaction. Biting midges (*Culicoides*, Ceratopogonidae) and louse flies (Hippoboscidae) transmit *Haemoproteus*, mosquitoes (Culicidae) transmit *Plasmodium*, and blackflies (Simuliidae) transmit *Leucocytozoon*, but certain vectors remain unidentified for the great majority of described species (Atkinson et al. 1988; Bernotiene et al. 2019; Hellgren et al. 2008; Rn Malmqvist et al. 2004). The infection rate of vector-borne parasites may be closely related to surrounding environmental factors. Studies of the vertical distribution of blackflies showed that high-altitude forests are known as their preferred habitat (Imura et al. 2012; Chakarov et al. 2020). Also, climate demonstrates an effect on the development of parasites and vectors, and blackflies often prefer living in northern temperate areas (È Malmqvist et al. 1999; Gubler et al. 2001). Hokkaido is a subarctic region with vast forested regions, and these factors may contribute to a relatively large population of blackflies and a large diversity of *Leucocytozoon* infections. A low abundance or lack of appropriate insect vectors in Hokkaido due by the subarctic climate may be preventing the spread of *Haemoproteus* and *Plasmodium* parasites to other areas of Japan such as Minami-Daito Island, which exhibits high prevalence of *Haemoproteus* and *Plasmodium* (Murata et al. 2008). In this study, we examined haemosporidian infection in the same bird species at a single field site. To confirm the difference in genetic diversity between *Haemoproteus* and *Leucocytozoon*, we need to evaluate multiple bird species from a wider region to see if a similar conclusion would be obtained. In PCRs using our primers, we identified 10 novel lineages (Fig. 3a and 3b). A similar number of multiple infections have not been reported in the same population at this location. The new primers we

designed in this study would be particularly useful in uncovering unique lineages from multiple infections. The dependance of detection of mixed haemosporidian infections on PCR primers has been reported, and researchers noted that the diversity of the parasite is underestimated by PCR using the conventional primers (Bernotiene et al. 2016), which our results demonstrated.

The ability to identify more multiple parasite genotypes/lineages within the same host opens an important research area in bird-parasite interactions, and it will enable improved understanding of the diversity and structure of parasite communities, as well as the possibility to study the fitness effects and parasite interactions derived from co-infections (de Roode et al. 2004). Our phylogenetic analysis revealed that the parasite lineages detected by our primers are scattered across a wider range of *Leucocytozoon* than the lineages infecting Columbiformes registered in MalAvi (Fig. 3, Fig. 4). However, several *Leucocytozoon* lineages exist that could only be detected by using the conventional primers, so the combination of PCRs using these and our primers would provide a more detailed and comprehensive lineage information. To determine whether the primer sets, HaemFL/HaemR2L and HaemFLn/HaemRLn, are sufficient for investigation of mixed infections, further evaluation using these primers is needed.

Gene cloning is often applied as a useful method to isolate individual lineages from mixed infections; however, the lineage definitions need to be carefully defined since this method can encounter two expected types of PCR errors: single nucleotide mutations and jumping PCR artifacts (Pérez-Tris and Bensch 2005). To eliminate these possible errors in this study, we defined a new lineage when three or more identical sequences were detected from the sequenced clones. Ten sequences (STRORI06 to STRORI15) were defined as new lineages and registered in the MalAvi database (http://130.235.244.92/Malavi/). Also, we detected 62 novel clones, but we did not define them as novel lineages because they were detected only once or twice from the sequenced clones. This suggests that if the number of sequenced clones had been increased, more new lineages may have been defined. The investigation of the infection status of insect vectors using our *cob*-targeted primers would also be valuable to obtain a more detailed analysis of the genetic background of the blood sporozoites that parasitize these vectors. We believe that the use of our *cob* primers to investigate multiple *Leucocytozoon* infections would provide new insights into the ecological and evolutionary relationships among avian haemosporidian parasites and their hosts and arthropod vectors.

Declarations

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Conflicts of interest/Competing interests

The authors have no conflicts of interests/competing interests to declare that are relevant to the content of this article.

Availability of data and material

All data generated or analyzed during this study are included in this article and its supplementary information file.

Authors' contributions

Yui Honjo and Kenji Hikosaka designed research; Yui Honjo, Shinya Fukumoto and Kenji Hikosaka performed research; Yui Honjo analyzed data; and Yui Honjo, Hirokazu Sakamoto and Kenji Hikosaka wrote the paper. All authors read and approved the final manuscript.

Ethics approval

All animal experiments in this study were conducted in accordance with the guidelines for the use of animals of Obihiro University of Agriculture and Veterinary Medicine, Japan (Permit number: 19-14).

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1. Prevalence of haemosporidian infection detected in this study

Genus	Primer set *	Detected/Examined (%)	
		Streptopelia orientalis	*Columba livia*
Haemoproteus	HaemF, HaemR2	12/19 (63%)	0/13 (0%)
Leucocytozoon	HaemFL, HaemR2L	15/19 (79%)	1/13 (8%)
	HaemFLn, HaemR2Ln	15/19 (79%)	0/13 (0%)

* Nucleotide sequences of primers are described in Supplementary Table 1.

Figures

Figure 1

Alignments of a portion of the *cob* nucleotide sequence (positions, 213–239 and 708–737) of 12 *Leucocytozoon* species or isolates and the nucleotide sequences of primers used in this study. HaemFL/HaemR2L are primers reported by Hellgren et al. (2004) (a), and HaemFLn/HaemRLn are primers designed in this study (b). I in the nucleotide sequence of HaemR2L indicates inosine.

Figure 2

Difference in infection pattern in PCR assays using the conventional (HaemFL, HaemR2L) and new primers (HaemFLn/HaemRLn). Not detected means individuals that tested negative through nested PCR assay. Single and multiple infections are distinguished by presence of double or multiple peaks on sequence electropherograms.

Figure 3

Leucocytozoon lineage found by PCR using the conventional (HaemFL, HaemR2L) (A) and new primers (HaemFLn/HaemRLn) (B). Bird IDs are written in the left-hand side. Lineage names are given by MalAvi. Newly discovered lineages were confirmed more than three times and are shown in the red boxes. Previously reported lineages are shown in the blue boxes. Identical clones found less than twice that were not categorized as a newly found lineage and are shown in the gray boxes. GenBank accession numbers of each sequence are given in Supplementary Table 3.

Figure 4

NJ phylogenetic tree of partial sequences of *cob* (477 bp) from 16 *Leucocytozoon* lineages found in this study and of 29 reported lineages that infect species of the order Columbiformes. Sequences are identified by the MalAvi lineage name. Numbers above or below the branches indicate bootstrap values (1,000 bootstrap replicates). ▯ ; New lineages found using new primers, ● ; Reported lineages identified using new primers, ◁ ; Lineages identified using conventional primers.

Figure 5

Comparison of nucleotide variants of the lineages found in this study. Positions of mutations are listed. Novel lineages are in red. New mutations found in novel lineages are highlighted in light gray.

Supplementary Files

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- SupplementaryInformation.xlsx