New PCR primers targeting the 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 ecology and evolution as a result of their 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 we found that the conventional primers detected 16% (5/32) of multiple infections and none of them was a novel lineage, whereas our primers detected 44% (14/32) of 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 for 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 a higher diversity of *Leucocytozoon* lineages in nature than expected, which would provide more information to better understand parasite diversity and host-vector interactions in wildlife.

Keywords Avian haemosporidia · *Leucocytozoon* · Cytochrome b · Multiple infection · Lineage diversity

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 populations 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 (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 (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 (*cytb*) coded in the mitochondrial genome is targeted by PCR. Hellgren et al. (2004) slightly modified the Bensch protocol to amplify the target gene in
Leucocytozoon, 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 (Pérez-Tris and Bensch 2005; Zehtindijiev et al. 2012; Pacheco et al. 2018a). 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 (Zehtindijiev 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 cytb. 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 to better understand the remarkable diversity of parasites in wildlife populations.

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 and 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 cytb 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 s, 55 °C for 5 s, and 68 °C for 5 s. 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 bidirectional sequences. The sequences obtained in this study have been deposited in DDBJ/EMBL/GenBank with the accession numbers described in Supplementary Table 3.

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 cytb (Bird ID S9 in Supplementary Table 2). Similar results have been reported by the Szöllösi et al.’s (2008) group. Additionally, the metatranscriptomic analysis performed by the Galen et al.’s (2020) research group has indicated that a lot of lineages of Leucocytozoon that have been detected by PCR using the reported primers are existing. Our result and these previous reports 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 cytb regions of Leucocytozoon that can be annealed by the HaemFL/HaemR2L primers are conserved. To do this, we aligned cytb sequences from 12 Leucocytozoon lineages reported by Pacheco et al. (2018b) (Fig. 1). We focused on the conservation of cytb 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), and (ii) the highly conserved position at 715 (C) was chosen as the 3′ terminal of the primer HaemR2Ln since the 3′-terminal region is critical for PCR (the 3′-terminal region of the conventional primer HaemR2L is not well conserved).

### 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 *Escherichia 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 s, 55 °C for 5 s, and 68 °C for 30 s. 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 *cytb* gene (Schumm et al. 2021).

### Phylogenetic analyses

For construction of the *Leucocytozoon cytb* 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 *cytb* fragment was selected for phylogenetic analyses. All lineages detected in this study are represented in the phylogenetic analysis. The phylogenic trees were constructed in IQ-TREE web application, W-IQ-TREE, using maximum likelihood (ML) methods (Trifinopoulos et al. 2016) and in MEGA X using neighbor-joining (NJ) methods (Kumar et al. 2018). In the ML analysis, the TIM2+F+I+G4 model was chosen, and in the NJ analysis, the Kimura two-parameter model was used to estimate the evolutionary distance. To assess tree topology, bootstrap re-sampling (1000 cycles) was performed for each method.

### Result

#### 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 conventional primers (HeamFL/HeamR2L), and 15 were positive using our primers (HeamFLn/HeamR2Ln). 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

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**Fig. 1** Alignments of a portion of the *cytb* 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), and HaemFLn/HeamR2Ln are primers designed in this study. “I” in the nucleotide sequence of HaemR2L indicates inosine.
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 infections

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 a SNP at position T430A of cytb (lineage name; not identified) (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. When the clone possessing the same sequence was found a minimum of three times, we identified it as a new lineage. 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 AMO02 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 AEM002, which is the most common 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 substitutions were found only in the novel lineages (Fig. 5).

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). We examined haemosporidian infection in the same bird species at a single field site in this study. 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, B). 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. A previous study has reported that the prevalence of *Leucocytozoon* was higher than that of *Haemoproteus* in all kinds of birds of Hokkaido (Yoshimura et al. 2014), as supported by this study (Table 1).

This difference may be attributed to the host-vector-parasite interaction. Biting midges (*Culicoides*, Ceratopogonidae) 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; Bernotienė et al. 2019; Hellgren et al. 2008; 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 (Malmqvist et al. 1999; Gubler et al. 2001). Hokkaido is a subarctic region...
with vast forested area, 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).

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 an 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 and 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 of sensitivity and specificity of our primers using DNAs from many other avian host species 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 performed 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. Although we could not rule out the possibility that the new lineages obtained by cloning are artificial due to the uncertainties surrounding cloning, ten sequences (STRORI06 to STRORI15) were defined as new lineages by criteria described above 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.

Recently, metagenomics using the next-generation sequencing (NGS) technology has become increasingly popular when the researchers survey microbial communities. We consider this technology’s usefulness to address issues of identification of real diversity of Leucocytozoon lineages. In the study on haemosporidian lineage diversity, it has been reported that transcriptomes detect approximately 1500 single-copy orthologous loci from a phylogenetically diverse set of 33 haemosporidian mitochondrial lineages (Galen et al. 2020). In addition, a comparative study using NGS and Sanger sequence methods, reported by the other research group, has demonstrated that mixed infections involving dissimilar haemosporidian lineages are more likely to be overlooked (Yeo et al. 2022) and the Sanger sequence of PCR products does not always match the dominant NGS sequence. The investigation of the infection status of insect vectors using our cyt-b-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 our findings obtained by the use of our cyt-b primers would provide valuable information toward the future metagenomics to uncover the ecological and evolutionary relationships among avian haemosporidian parasites and their hosts and arthropod vectors.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-022-07667-5.

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

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

Declarations

Ethics approval and consent to participate 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 is not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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