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# Prevalence of avian haematozoa in wild birds in a high-altitude forest in Japan

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ABSTRACT

The infection dynamics of avian haematozoa, which includes the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, are complicated by a variety of environmental factors and host-parasite interactions. In Japan, the prevalence of haematozoa in wild birds has recently been determined in several local areas. However, no information on the annual prevalence of avian haematozoa in a single study site has been reported. Here, we investigated the long-term infection dynamics of haematozoa in wild birds inhabiting a mountain forest of Japan. Blood samples were collected from 415 wild birds captured in the Chichibu mountains in Saitama Prefecture at an altitude of 1650 m between 2007 and 2010. All obtained samples were examined for haematozoan infection using nested polymerase chain reaction (PCR) of the cytochrome *b* (cyt*b*) genes of haematozoa. A total of 62 out of 415 (14.9%) forest birds were PCR positive for haematozoa. Relatively high infection rates of *Leucocytozoon* were found among several bird species (*Parus ater*, 64.3%; *Parus montanus*, 81.8%) and may be due to the host preference of vector black flies and host nestling pattern in this forest. Phylogenetic analysis of amplified cyt*b* sequences revealed for the first time that a variety of lineages of avian haematozoa are distributed among wild bird hosts in a high-altitude forest stand in Japan. Notably, significant seasonal changes of the prevalence of avian haematozoa were not observed; however, continuous investigation will likely provide detailed information on host-parasite interactions, including local environmental factors, that influence the dynamics of avian haematozoan infections.



## 1. Introduction

Avian haematozoa, which are blood parasites that include the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, are transmitted by arthropod vectors and are widely distributed in captive and wild bird species around the world (Valkiunas, 2005). The prevalence of these vector-borne diseases is complicated by host–vector–parasite interactions (Knowles et al., 2010). For example, the type of arthropod vector, differ between biological communities (Lehane, 1991), and the abundance of vectors decreases with increasing vertical elevation, which has been shown to affect the prevalence of *Plasmodium* (van Riper et al., 1986). Climate also has effects on the development of parasite and vector (Gubler et al., 2001; Rogers and Randolph, 2006). Thus, the infection rate of vector-borne parasites may be closely related to surrounding environmental factors. To better understand the infection dynamics of haematozoa associated with environmental factors, it may be important to investigate the prevalence of haematozoa among local bird communities. Moreover, climate change may also have effects on vector

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distribution, leading to the emergence of infectious disease in new hosts.

A previous study showed that the distribution of *Aedes albopictus*, a vector mosquito species of avian malaria (Ejiri et al., 2008), has extended northwards during the past 50 years in Japan (Kobayashi et al., 2002), suggesting that infections of avian malaria may extend through both horizontal and vertical transmission. It is expected that longitudinal monitoring of the haematozoan prevalence among wild bird communities inhabiting specific local sites will enable detection of distributional changes of pathogens and determination of the time point of introduction and distribution processes of new haematozoa. Thus, this monitoring approach may potentially provide significant information concerning the infection dynamics of new vector-borne diseases.

Epidemiological studies conducted in many parts of the world have shown the location-specific prevalence of haematozoan parasites among wild birds with varying infection rates. For example, Murata (2002) reported the prevalence of haematozoa among injured or sick birds in Hyogo Prefecture, western Japan with 10.6% positive. Hagihara et al. (2004) detected infection of *Leucocytozoon lovati* in the rock ptarmigan (*Lagopus mutus japonicus*) inhabiting limited high mountain areas in Japan with 88.9% positive. Moreover, Nagata (2006) also surveyed avian haematozoa infection in 1553 wild birds across 36 species from northern (Akita), central (Ibaraki) and southern (Fukuoka and Iriomote) Japan resulting 14.5% positive. However, organized investigations on the prevalence of avian malaria in wild birds inhabiting a single local site in Japan for the purpose of evaluating the dynamics of haematozoa infection with consideration of seasonal changes have not been reported.

Here, we investigated the long-term infection dynamics of haematozoa in wild birds inhabiting a mountain forest of Japan over a three-year period to evaluate infectious factors such as seasonal prevalence.

## 2. Materials and methods

Wild birds were captured from May to November between 2007 and 2010 at Tsundashi Pass in the Chichibu mountains, Saitama Prefecture, Japan, at an altitude of 1650 m (N 35°55′17.6″, E 138°48′30.8″) using mist nets during overnight surveys (6–13 h per month). Blood samples were collected from the brachial vein and preserved in ethanol until subsequent processing in the laboratory. DNA was extracted from blood samples collected in 2010 using a DNA Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and by the phenol–chloroform method for all other samples. For the detection of haematozoa, extracted DNA was subjected to nested polymerase chain reaction (PCR) targeting the partial mitochondrial cytochrome *b* (cyt*b*) gene of haematozoa. The first PCR reaction included the Haem NFI-Haem NR3 primer for haematozoa (Hellgren et al., 2004) and was performed in a 25 μl reaction mixture containing 2.5 μl 10× Ex-Taq buffer (Takara, Ohtsu, Japan), 2 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 0.6 μM each primer, 0.625 U Ex-Taq (Takara), and 1 μl DNA template. The first PCR amplification program consisted of an initial denaturation step at 94 °C for 3 min followed by a total of 20 cycles consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s. The second PCR reaction was carried out using 1 μl of the PCR products amplified during the first reaction as template and included HaemF-HaemR2 primers for *Plasmodium*/*Haemoproteus* and HaemFL-HaemR2L primers for *Leucocytozoon* (Hellgren et al., 2004) with the same program used for the first PCR reaction, but with a total of 35 cycles. The amplified PCR products were visualized in agarose gels stained with ethidium bromide under ultraviolet light.

The PCR products were sequenced directly in both directions using BigDye™ Terminator Mix (Applied Biosystems, Forster City, CA) and were run on an ABI 3130-Avant Auto Sequencer (Applied Biosystems). Nucleotide sequences were aligned using the Clustal W program and compared with sequences in the GenBank database using the Basic Local Alignment Search Tool (NCBI website, http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses of the 305-bp cyt*b* gene sequences were performed using the neighbor-joining method and the PAUP 4.0 program with the Kimura two-parameter model to estimate the evolutionary distances. To assess tree topology, 1000 cycles of bootstrap resampling were performed.

## 3. Results

During the three-year study period, 415 wild birds consisting of 19 species were captured. A total of 62 of 415 (14.9%) birds were PCR positive for haematozoa over the three years. Six were positive for *Plasmodium* or *Haemoproteus* (1.4%), 56 for *Leucocytozoon* (13.5%), and no double-positive cases were found Table 1. Notably, no significant difference of infection rates in a single bird species was observed among seasons or between sexes.

Phylogenetic analysis revealed that four cyt*b* sequences from *Erithacus cyanurus* (Fig. 1; Ercy17–20) showed high similarity to those of *Plasmodium gallinaceum*. One sequence from *Cettia diphone* (Fig. 1; Cedi6) was classified into *Haemoproteus,* and another from *Troglodytes troglodytes* (Fig. 1; Trtr3) was grouped with *Plasmodium*. The other identified parasite lineages were classified into *Leucocytozoon*, but were not identical to *Leucocytozoon* cyt*b* sequences detected from host birds in Japan and deposited in GenBank. Seven of these lineages were detected from several bird species (indicated with shaded boxes in Fig. 1).

Twenty-six birds across 6 species (*C. diphone, Leiothrix lutea, E. cyanurus, Parus ater, Parus montanus,* and *Emberiza variabilis*) were recaptured during the study period. Six of the recaptured birds were found to be PCR positive at the time of each capture, and amplified sequences from blood samples revealed that three individuals had parasites (*C. diphone, P. ater*, and *E. variabilis*) with identical lineages over a period of 14 months or less (Fig. 1; Cedi4*, Paat5*, and Emva4*). Different lineages were found between the sampling points in the other 3 individuals, which consisted of 2 *P. montanus* and 1 *E. variabilis* (Fig. 1; Pamo3** and 6**, Emva1**). Of the recaptured birds, only one case of an