FULL PAPER *Parasitology*

Phylogenetic Comparison of *Leucocytozoon* spp. from Wild Birds of Japan

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(Received 18 July 2006/Accepted 28 September 2006)

ABSTRACT. Eight species of Japanese birds were found to be infected with *Leucocytozoon* species using microscopic analysis. We used PCR and sequence analysis of the mitochondrial cytochrome *b* gene (cyt *b*) to compare the genetic background among these detected protozoa species. In 20 individuals of 22 samples, a single amplified band was detected from 6 of 8 bird species; 9 Japanese rock ptarmigans (*Lagopus mutus japonicus*), 4 large-billed crows (*Corvus macrorhynchos*), 2 carrion crows (*C. corone*), 2 scops owls (*Otus scops*), 1 Japanese grosbeak (*Eophona personata*), and 2 brown-eared bulbuls (*Hypsipetes amaurotis*), respectively. Phylogenetic analysis based on the partial cyt *b* sequences revealed that all *Leucocytozoon* isolates in Japan closely grouped with other *Leucocytozoon* species previously reported in the literature. Among the Japanese isolates, the phylogenetic tree suggested that *L. lovati* from the Japanese rock ptarmigan may be basal to the parasites found in other bird species. Our study is the first to identify the molecular relationships among *Leucocytozoon* parasites in the avifauna of Japan.

KEY WORDS: cyt *b*, Japan, *Lagopus mutus*, *Leucocytozoon*, wild bird.

J. Vet. Med. Sci. 69(1): 55–59, 2007

Leucocytozoon spp. are well known protozoa of birds and some species exhibit virulent infectivity, especially in chickens and waterfowl [1, 8]. Within this genus, according to the high mortality of the hosts and the serious commercial loss for poultry production, *L. caulleryi* is the most important species for the veterinary field in Japan. Possible infected host birds must be immediately diagnosed for the infection and isolated *Leucocytozoon* should be classified to the species level for effective veterinary control against this protozoa.

Detection and description of *Leucocytozoon* species has been based on morphological analyses using microscopic observation of blood smears [3, 6, 13] which sometimes fails to detect the pathogen in cases of low parasitemia. Moreover, it has been suggested that there may be redundancy in the nomenclature of *Leucocytozoon* species [8]. The implementation of molecular methods will help in the clarification of these issues.

In Japan, several cases of *Leucocytozoon* in wild birds have been reported [17]. Although several host species were infected the observed *Leucocytozoon* could not be determined to the species level due to the difficulty of morphological characterization. Recently, Hagihara *et al.* [9] reported that wild Japanese rock ptarmigans (*Lagopus mutus japonicus*) were infected with *Leucocytozoon*. They classified those protozoa as *L. lovati* by observing gametocytes in blood cells, and also suggested the necessity for DNA analysis of this genus.

Genetic analysis of the genus *Leucocytozoon* has not been reported sufficiently as compared to other avian protozoa groups. *L. dubreuli* and *L. simondi* have been investigated based on the partial mitochondrial genome [18]. In addition, *Leucocytozoon* species were characterized in some birds in Sweden [11]. To clarify the relationships among *Leucocytozoon* species is significant in terms of biology, molecular epidemiology, and for the possible diagnosis of Leucocytozoonosis. Many genetic studies have been reported for other avian apicomplexan species such as *Plasmodium* and *Haemoproteus* using various gene targets and PCR [7, 18, 19, 22]. In this study, we present a molecular phylogeny of *Leucocytozoon* sp. distributed in Japan, based on the sequences of the cytochrome *b* (cyt *b*) gene from several wild bird blood samples.

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MATERIALS AND METHODS

Samples: All samples were collected and preserved as described by Murata [17] and Hagihara *et al.* [9]. Briefly, in total, 22 individual birds of 8 species were used for subsequent hematological investigations; 9 Japanese rock ptarmigans, 4 large-billed crows (*Corvus macrorhynchos*), 2 carrion crows (*C. corone*),2 scops owls (*Otus scops*), 2 copper pheasants (*Phasianus soemmerringui*), 1 Japanese grosbeaks (*Eophona personata*), 1 Ural owl (*Strix uralensis hondoensis*), and 2 brown-eared bulbul (*Hypsipetes amaurotis*). The Japanese rock ptarmigans were captured on Mt. Tateyama (36° 35'N, 137° 36'W) at about 2,400 m elevation in April and Mt. Jiigatake (36° 35'N, 137° 45'W) at about 2,650 m elevation in June, 2002. Other wild birds were mainly captured in several regions of Hyogo Prefecture, Japan, over a period from 1989 to 1999.

Blood samples were collected and stored and then samples were fixed and stained for microscopic observation. The remainders of the blood samples were used for the subsequent DNA extraction procedure.

PCR and sequencing: Blood samples were extracted using phenol-chloroform. Extracted DNA was dissolved in TE solution, and used for nested PCR. For primary amplification, primers DW2: 5'-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3' and DW4: 5'-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3' were designed as described previously [5, 18]. Secondary primers were designed from a highly conserved region of partial cyt b sequences between both *L. dubreuli* and *L. simondi* (GenBank accession nos. AY099063 and AY099064, respectively). The second round of PCR was performed using those newly synthesized primers; LCytbF: 5'-CAA ATT CTT ACT GGT GTA TTA TTA GC-3' and LCytbR: 5'-ATA ATA GAT AAT GAA TAA TCT CTT GG-3'.

The PCR reaction contained 2.5 μl of TaKaRa (Ohtsu, Japan) 10 × *Ex-Taq* buffer, 4 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.4 μM (each) primer, 1U of *TaKaRa Ex-Taq* and 1 μl of DNA template in a 25 μl reaction mixture. For the primary PCR, a total of 35 cycles was carried out, consisting of denaturing at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 1 min, with an initial denaturizing at 94°C for 4 min. The second PCR reaction was performed in a total of 40 cycles identical to the first PCR conditions, except that annealing temperature was at 50°C for 30 sec.

The amplification product was visualized in agarose gels stained with ethidium bromide. PCR products were subsequently sequenced in both directions. The reactions using BigDye™ terminator mix (Applied Biosystems, Foster City, California, U.S.A.), were run on an ABI3100 auto sequencer. Nucleotide sequences were aligned using the Clustal W program [20]. Phylogenetic analyses of about 465 bp sequences were performed using the neighbor-joining (NJ) and parsimony methods by PAUP programs. The Kimura two-parameter model was used to estimate the evolutionary distances. In the parsimony method, heuristic searches were used. Bootstrap re-sampling (1,000 cycles) was performed for each method to assess tree topology. Parasite lineages used for the phylogenetic comparisons were 4 avian *Plasmodium*, 5 *Haemoproteus*, and 12 lineages of *Leucocytozoon* from GenBank. A mammalian malaria parasite, *P. berghei*, was used as outgroup in the trees.

RESULTS

Using microscopy, all blood smear samples were found to be infected with *Leucocytozoon* at varying intensities as previously described [9, 17] Quantitative analysis of the protozoa in these samples was not addressed in this study. We were only able to identify samples from Japanese rock ptarmigans. These were classified as *L. lovati* (= *bonasae*) based on the morphology of gametocytes [9]. In other samples, we were unable to determine the species identifications due to the low parasitemias and insufficient quality of the gametocytes for morphological measurements.

As summarized in Table 1, 20 of 22 (91.0 %) samples were positive for *Leucocytozoon* using the nested PCR reactions. Samples from both 1 copper pheasant and 1 Ural owl were negative in all cases. Expected products were found from all amplified samples, showing a single band of approximately 850 bp after the second PCR reactions (data not shown). After the primary PCR, no amplification product was seen in ethidium bromide stained agarose gels.

No double peaks, which could indicate mixed infections or intraspecies polymorphisms, were found in any of the amplified DNA fragments in this study. There were no differences among individual amplified sequences within identical host species sampled from the Japanese rock ptarmigan (n=9), the carrion crow (n=2), the large-billed crow (n=4), and the scops owl (n=2). However, 2 isolates from the blown-eared bulbul harbored different sequences. Two phylogenetic trees were successfully constructed using two analytical methods. Both the neighbor joining and the maximum parsimony methods resulted in trees of the same topology, one of which is shown in Fig. 1. Maximum length for multiple alignments resulted in about 465bp, because some sequence data deposited in database were not so long enough to compare to our data. All acquired sequences from wild birds in this study showed a closer relation to *Leucocytozoon* species than *Plasmodium* or *Haemoproteus* species. Within *Leucocytozoon* samples, *L. lovati* was basal to other *Leucocytozoon* species.

Table 1. PCR results for the partial mitochondrial cytochrome *b* gene of *Leucocytozoon* from the infected bird species of Japan

Host bird species	Number of birds with *Leucocytozoon*	Number of birds with PCR positive	GenBank accession no.
Japanese rock ptarmigan (*Lagopus mutus japonicus*)	9	9	AB183550
Large-billed crow (*Corvus macrorhynchos*)	4	4	AB183553
Carrion crow (*C. corone*)	2	2	AB183552
Brown-eared bulbul (*Hypsipetes amaurotis*).	2	2	AB183556, AB183557
Scops owl (O*tus scops*)	2	2	AB183555
Japanese grosbeak (*Eophona personata*)	1	1	AB183554
Copper pheasant (*Phasianus soemmerringui*)	1	0	–
Ural owl (*Strix uralensis hondoensis*)	1	0	–
Total	22	20	

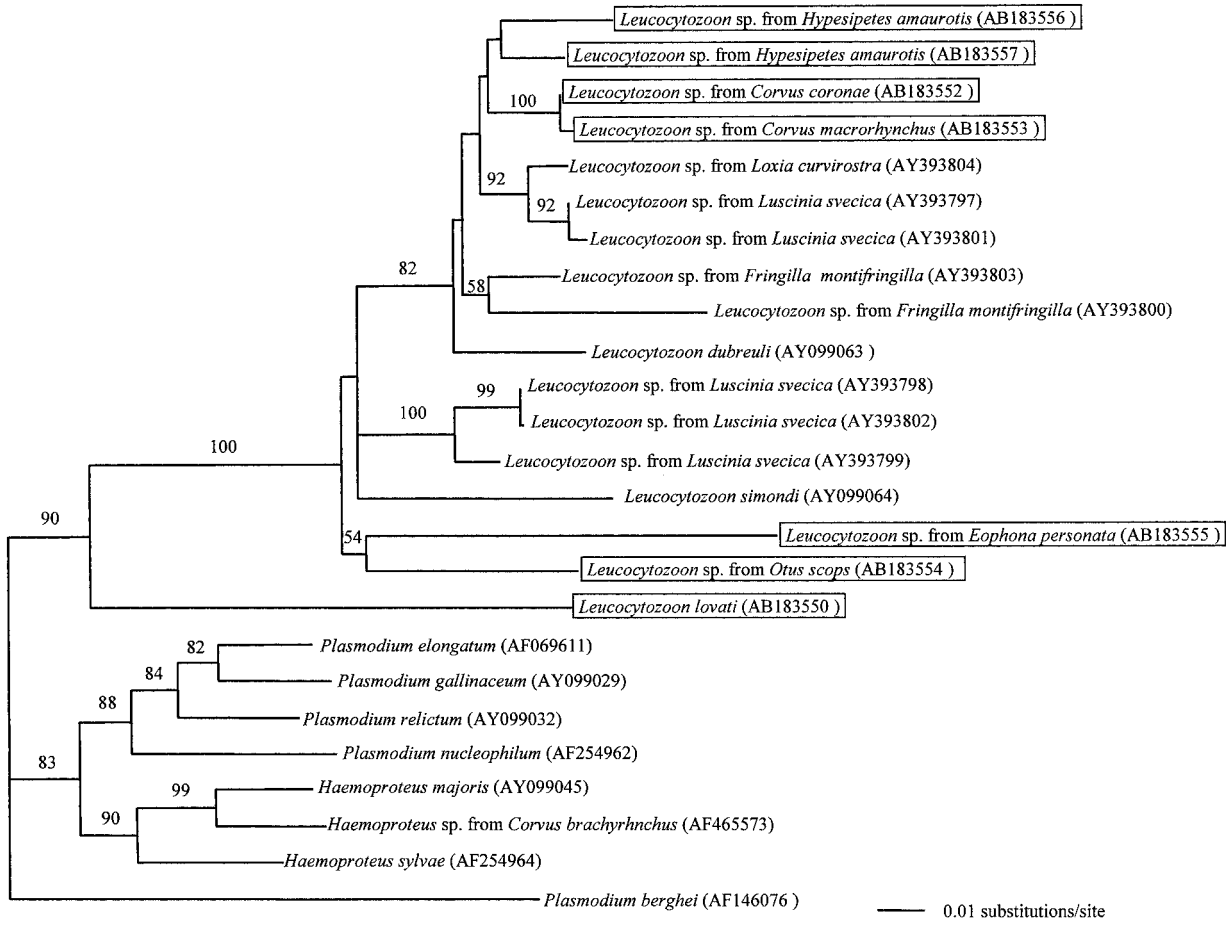

Fig. 1. A neighbor-jointing tree using partial cytochrome *b* gene DNA sequences of approximately 460 bp of *Leucocytozoon* species from wild birds of Japan (n=7), previously reported *Leucocytozoon* species (n=10) and other avian *Plasmodium* (n=4) and *Haemoproteus* species (n=3) obtained from GenBank. All samples are indicated by the parasite species names or host bird names with accession numbers. Species names within a box in the tree represent samples used in this study. Dotted lines in the tree separated three genera of avian Haemosporidia, *Leucocytozoon*, *Haemoproteus* and *Plasmodium* from top to bottom. *P. berghei* was used as the outgroup. Bootstrap values (>50%) are listed as percentages after 1,000 replicates.

DISCUSSION

In this study, we obtained partial mitochondrial cyt *b* gene sequences of *Leucocytozoon* species from wild bird of Japan for the first time. Because of its potentially severe impact on poultry production, research on *Leucocytozoon* in Japan has mainly focused on *L. caulleryi* infection in domestic chickens, with the objectives to develop protective immunity and vaccinations for the pathogen [14, 15]. *Leucocytozoon* infection has been reported among wild birds of Japan [9, 17], however, no classification and genetic analyses of detected *Leucocytozoon* sp. were performed in those studies. Murata [9] showed that infected birds harbored several species, and suggested the existence of several *Leucocytozoon* species in wild birds of Japan. Our data also suggest that there may have been several species and/or genotypes of *Leucocytozoon* in Japan. Although blood smears of both Copper pheasant and Ural owl showed positive for *Leucocytozoon*, no positive signals could be detected from DNA samples of those two bird species by present PCR. This could occur because primers used in this study might be mismatched to the sequences of those infected *Leucocytozoon* species and/or extracted DNA would contain less amount of the protozoa than to react enough to our PCR procedure.

All sequences of *L. lovati* acquired from the Japanese rock ptarmigan were identical (data not shown), suggesting that those *L. lovati* from this ptarmigan in this studied area have a similar genetic background at least in the mitochondrial cyt *b* gene. Similarly, every sequence from the large-billed crow (n=4) and the carrion crow (n=2) was identical with each other. These two crow species may be infected by *Leucocytozoon* with the same species or genotype. On the other hand, isolates from 2 brown-eared bulbuls had different sequences each other, suggesting that at least two species or genotypes could exist in this bird species in Japan.

Leucocytozoon isolates from other Japanese wild birds were distinct, forming separate clusters as shown in Fig. 1. This divergence may suggest possible host specificity of *Leucocytozoon* of each bird species. We failed to identify the species based on morphology of studied samples except from the Japanese rock ptarmigan. Further investigation will be necessary to evaluate whether those isolates from other wild birds could be different species or genotypes by referring to morphological features.

A complete molecular phylogeny of *Leucocytozoon* species has not been clearly established. Perkins and Schall [18] published a phylogenetic tree indicating that *L. dubreuli* and *L. simondi* separated into another cluster from avian *Plasmodium* and *Haemoproteus* groups. Our present study provides a further understanding of the phylogenetic relationship within the genus *Leucocytozoon* of Japan. As stated by Greiner [8], there are many potential synonyms among *Leucocytozoon* species. Molecular characterization of species will undoubtedly clarify the nomenclature. Our targeted gene, mitochondrial cyt *b*, may be used as one possible indicator for molecular classification of *Leucocytozoon*, however, other genes could also be candidates. For that purpose, a whole mitochondrial genome sequence of *Leucocytozoon* will be valuable, and our ongoing research on the sequence will appear elsewhere. Choosing suitable genes which could show species-specific restriction fragment length polymorphism (RFLP) will also allow the construction of a useful PCR-RFLP method for detecting and characterizing the pathogens as reported [2].

Insect vectors of the genus *Leucocytozoon* are *Simulium* black flies for most host species [21]. In this survey, we could not confirm the arthropod vectors of *Leucocytozoon*, however, several candidate arthropods were observed in the studied areas. This cyt *b* PCR detection can be applied to find *Leucocytozoon* not only from host birds but also from the insect vectors.

Our results suggest that *L. lovati* from the Japanese rock ptarmigan is genetically distinct from other *Leucocytozoon* species in Japan. Considering the historical distribution of this ptarmigan which has been regarded as a relic from the last ice age [10], this clear division seems to suggest the independent co-evolution of *L. lovati* with the Japanese rock ptarmigan separate from other hosts and parasites in Japan. *L. lovati* has been found in other ptarmigans in North Europe [12] and grouses in North America [4, 16]. Further comparative studies on the genetic divergence among *L. lovati* isolates could illustrate the historical distribution these haematozoa with their ancient host birds on a worldwide scale.

ACKNOWLEDGMENTS. The authors are very grateful to Drs. M. Kitahara and T. Sakanakura of the Institute of Alpine Environmental Research, Nagano, Japan for samples and Dr. R. N. M. Sehgal of the Department of Biology, San Francisco State University for his invaluable aid in revising the manuscript. K. Shinya and A. Motohashi are also gratefully thanked for their technical assistance. This study was supported by a Grant-in-Aid for Scientific Research (No. 16510179) from the Japan Society for the Promotion of Science and the Academic Frontier Project "Surveillance and control for zoonoses" from Ministry of Education, Culture, Sports, Science and Technology, Japan.

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