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ORIGINAL PAPER

Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan

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Abstract Several species of captive birds at zoological gardens of Japan were found to be infected with avian *Plasmodium*. However, incriminated vector mosquito species have not been identified yet. To indicate the competent vectors of avian malaria parasite, we collected mosquitoes at a zoological garden in Japan and examined for the avian malaria parasite DNA. Totally, 1,361 mosquitoes of 11 species were collected in the zoological garden of Kanagawa, the south of Tokyo in Japan in 2005. Captured mosquitoes were pooled by each species, date collected, and location and used for DNA extraction. Eight out of 169 DNA samples were positive for the nested PCR of avian *Plasmodium* cyt *b* gene. Estimated minimum infection rates of mosquitoes were 5.9 per 1,000. The PCR positive mosquito species were *Culex pipiens* group and *Lutzia vorax*. Some DNA sequences amplified from collected mosquitoes were identical to avian *Plasmodium* lineages detected from captive birds in the same zoological garden studied. Our results suggest that *C. pipiens* group and *L. vorax* could be incriminated vectors of avian malaria parasite transmitting in captive birds kept in the zoological garden in Japan.

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Introduction

In Japan, several species of wild and captive birds were found to be infected with various avian blood protozoa including *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* as well as in other countries (Murata 2002; Hagihara et al. 2004; Nagata 2006; Murata et al. 2008a, b). Some avian malaria parasite infections of the captive birds caused serious clinical condition and induced fatal results to the host birds in USA, Brazil, New Zealand, and Asia (Fleischman et al. 1968; Bennett et al. 1993; Clarke and Kerry 1993; Jones and Shellam 1999; Murata 2002; Grim et al. 2004; Alley et al. 2008; Belo et al. 2009); however, pathogenesis of captive birds in Japan have not been investigated in detail. Furthermore, pathology of these avian *Plasmodium* in wild birds were still unclear yet especially in the endangered species of Japan. For the prevention and control of the avian hematozoa infections in Japan, the transmission cycles of these blood protozoa should be investigated for the host birds and vectors. Blood sucking arthropods such as mosquitoes (Diptera; Culicidae) of *Culex* spp. and *Aedes* spp. play an important role as the vectors of avian *Plasmodium* spp. (Bennett 1987; Valkiūnas 2005). Prevalence of vector-borne diseases is principally determined by biting density and infection rate of vector population (Stokstad 2004). Thus, surveillance of the incidence of avian *Plasmodium* spp. in vector mosquitoes is important to establish preventive measures for avian malaria infection not only in wild birds but also in captive birds.

Recently, suspected arthropod vectors of both avian *Plasmodium* (Ejiri et al. 2008) and of *Leucocytozoon* (Sato et al. 2009) have been suggested for Japanese wild birds, respectively. However, prevalence of avian malaria in mosquitoes inhabiting at artificial environments such as zoological gardens in Japan has not been reported so far. Several cases of avian malaria parasite infections have been found from captive birds in a zoological garden of Japan (Murata et al. 2008a) suggesting the occurrence of infective vector arthropods. In this study, to indicate the competent vectors of avian malaria parasite whose host might be captive birds in a zoological garden of Japan, we collected mosquitoes and carried out the detection of avian malaria parasite DNA from the mosquitoes.

Materials and methods

Mosquitoes were collected monthly in a zoological garden of Kanagawa, Japan from May to August 2005. The zoo is 40.2 ha in area and located in the south of Tokyo, 35.49′ N, 139.52′ E and surrounded by secondary forests apart from human residential areas. A total of 41 mammal species, 22 bird species, and three reptile species are kept and exhibited in the zoo. CDC traps, gravid traps, and a sweeping net were used for mosquito collection. Captured mosquitoes were identified to species following morphological keys (Tanaka 1979; Toma and Miyagi 1986). All the collected mosquitoes were kept at −20°C until DNA extraction.

DNA extraction from mosquitoes and a nested PCR amplification of partial cyt *b* gene of avian malaria mitochondrial genome were implemented as described previously (Ejiri et al. 2008). Briefly, after DNA extraction, we used the DW2 and DW4 primers (Perkins and Schall 2002) for the first PCR, APFN (5′-CTT ATG GAA TTA TGG ATT TCT TTT AGG-3′) and APRN (5′-ATA ATA AAG CAT AGA ATG AAC ATA TAA ACC-3′) primers for the second. PCR products were subsequently sequenced in both directions using BigDye™ terminator mix (Applied Biosystems, Foster City, CA, USA). Phylogenetic analyses using about 433-bp sequences were performed as described previously (Ramsey et al. 1986). Briefly, using the neighbor-joining (NJ) method by PAUP program, the Kimura two-parameter model was utilized to estimate the evolutionary distances. Bootstrap re-sampling (1,000 cycles) was performed for each method to assess tree topology. Parasite lineages used for the phylogenetic comparisons included four known avian malaria species, *Plasmodium cathemerium*, *Plasmodium gallinaceum*, *Plasmodium relictum*, and *Plasmodium juxtanucleare* with other avian *Plasmodium* from GenBank and other avian *Plasmodium* lineages were obtained from personally communicated DNA sequence data of captive birds kept in the zoological garden. A lineage of lizard malaria parasite, *Plasmodium*. *floridense*, was used as out group in the tree.

To estimate infection rate of the mosquitoes examined, the minimum infection rate (MIR) of each mosquito species was calculated as previously described (White et al. 2006). MIR has been utilized to estimate an infection rate by presuming that at least one individual of the pooled sample could be infected. The formula of MIR is as follows; MIR = number of PCR positive/number of collected mosquitoes × 1,000.

Results

Prevalence of parasites in mosquitoes

Total of 1,361 mosquitoes of 11 species in six genera including *Aedes albopictus*, *Aedes japonicus*, *Armigeres subalbatus*, *Culex bitaeniorhynchus*, *Culex pipiens* group, *Culex sasai*, *Culex tritaeniorhynchus*, *Culex* (*Culiciomyia*) sp., *Lutzia vorax*, *Tripteroides bambusa*, and *Uranotaenia novobscura* (Table 1). Among them *C*. *pipiens* group was the most dominant species throughout the study. Totally, 169 DNA samples were obtained and eight out of 169 samples were positive for avian malaria parasite cyt *b* gene. Over all infection rate of avian *Plasmodium* parasite was estimated as MIR of 5.9 per 1,000. Samples of two mosquito species, *C*. *pipiens* group (four DNA samples) and *L*. *vorax* (four DNA samples) were PCR positive with MIRs of 5.2 and 51.3 per 1,000, respectively (Table 1).

Molecular phylogenetic analyses

A phylogenetic tree was successfully obtained from the amplified sequences of mosquito samples, including at least five lineages of *Plasmodium* closely related to avian malaria protozoa in the collected mosquitoes (Fig. 1). Two lineages from both *C*. *pipiens* group and *L*. *vorax* were completely identical to the avian *Plasmodium* sp. lineage amplified from captive birds kept in the same zoological garden, namely, white-eared pheasant (*Crossoptilon crossoptilon*) and common crane (*Grus grus*). Other two lineages detected from *C*. *pipiens* group and *L*. *vorax* were completely identical to *P*. *relictum* lineage (DQ659544) amplified from carrion crow (*Corvus corone*) in Japan. One lineage detected from *L*. *vorax* was identical to avian *Plasmodium* sp. lineage amplified from captive birds, brown-eared bullbul (*Hypsipetes amaurotis*), Himalayan

Table 1 Minimum infection rate (MIR) of avian *Plasmodium* spp. DNA sequences from the mosquitoes collected in study location using nested PCR

Species	No. of mosquitoes collected	No. of pools examined	No. of pools positive	Minimum infection rate (per 1,000)
Aedes albopictus	330	40	0	0
A. japonicus	104	19	0	0
Armigeres subalbatus	39	13	0	0
Culex bitaeniarhynchus	11	5	0	0
C. pipiens group	763	63	4	5.2
C. sasai	12	3	0	0
C. tritaeniorhynchus	7	4	0	0
Culiciomyia sp.	6	3	0	0
Lutzia vorax	78	12	4	51.3
Tripteroides bambusa	10	6	0	0
Uranotaenia novobscura	1	1	0	0
Total	1,361	169	8	5.9

Minimum infection rate (MIR)=number of positive pool / number of mosquitoes collected×1,000

Monal Pheasant (*Lophophorus impejanus*), Humboldt Penguin (*Spheniscus humboldti*), and Temminck's tragopan (*Tragopan temminckii*). While another lineage was also detected from *L. vorax* (AB474379) with a few base substitutions to other lineages from birds in the same location.

All the obtained sequences from the mosquitoes were deposited to GenBank, having serial accession numbers from AB474376 to AB474379, AB4743781, and AB474382.

Discussion

In the present study, we detected avian *Plasmodium* DNA sequences from the mosquitoes collected in zoological garden of Japan. Detection of avian *Plasmodium* from mosquitoes was described on an oceanic island of Minami Daito Island in Japan (Ejiri et al. 2008). However, the island is located far from the main land of Japan with different mosquito fauna (Miyagi 1977). There are almost no concrete studies on the prevalence of avian malaria parasite in mosquitoes in the main land of Japan. The present study is the first one which suggests that mosquitoes inhabiting at the zoological garden located in urban area could be vectors of avian malaria in Japan. Moreover, we observed firstly the mosquito fauna among zoological gardens of Japan, showing that *C. pipiens* group and *A. albopictus* were dominant in studied area as well as previous report in urban areas surrounding Tokyo, Japan (Tsuda et al. 2006).

Among the mosquitoes examined in this study, MIR of avian malaria parasite was estimated as 5.9 per 1,000. Recently, we have reported MIR of collected mosquitoes in natural environment, Minami Daito Island of Japan was 12 per 1,000 (Ejiri et al. 2008). Another study in a secondary forest near residential area in the same prefecture as the present study showed that MIR was seven per 1,000 (Shirotani et al. 2009). No significant difference in MIR was found among the three study areas. Comparing to human malaria vectors, vector species of avian malaria in Japan have not been clarified in detail. Although six species have been incriminated as avian malaria vectors so far in Japan (Ejiri et al. 2008; Shirotani et al. 2009), intensive field studies on avian malaria vectors will be required to understand the transmission cycle and prevalence of this vector-borne protozoa infection throughout Japan.

Amplified DNA sequences from two mosquito species (*L. vorax* and *C. pipiens* group) found in this study corresponded to those of avian *Plasmodium* lineages previously detected from the captive birds of the same zoological garden (Fig. 1). Therefore, it is suggested that transmission of avian *Plasmodium* could occur between those two mosquito species and host bird species within the zoological garden. In addition to previously incriminated four vector species of *A. albopictus*, *Lutzia fuscanus*, *Culex quinquefasciatus*, and *Mansonia* sp. on Minami Daito Island, the present study indicated *C. pipiens* group and *L. vorax* as vectors of avian *Plasmodium* in main land of Japan. Further examinations if these mosquitoes harbor infective sporozoites of *Plasmodium* and which host bird species could be the blood meal of the vector mosquitoes are necessary to demonstrate studied mosquitoes as vectors of the targeted pathogens. Detection of avian *Plasmodium*

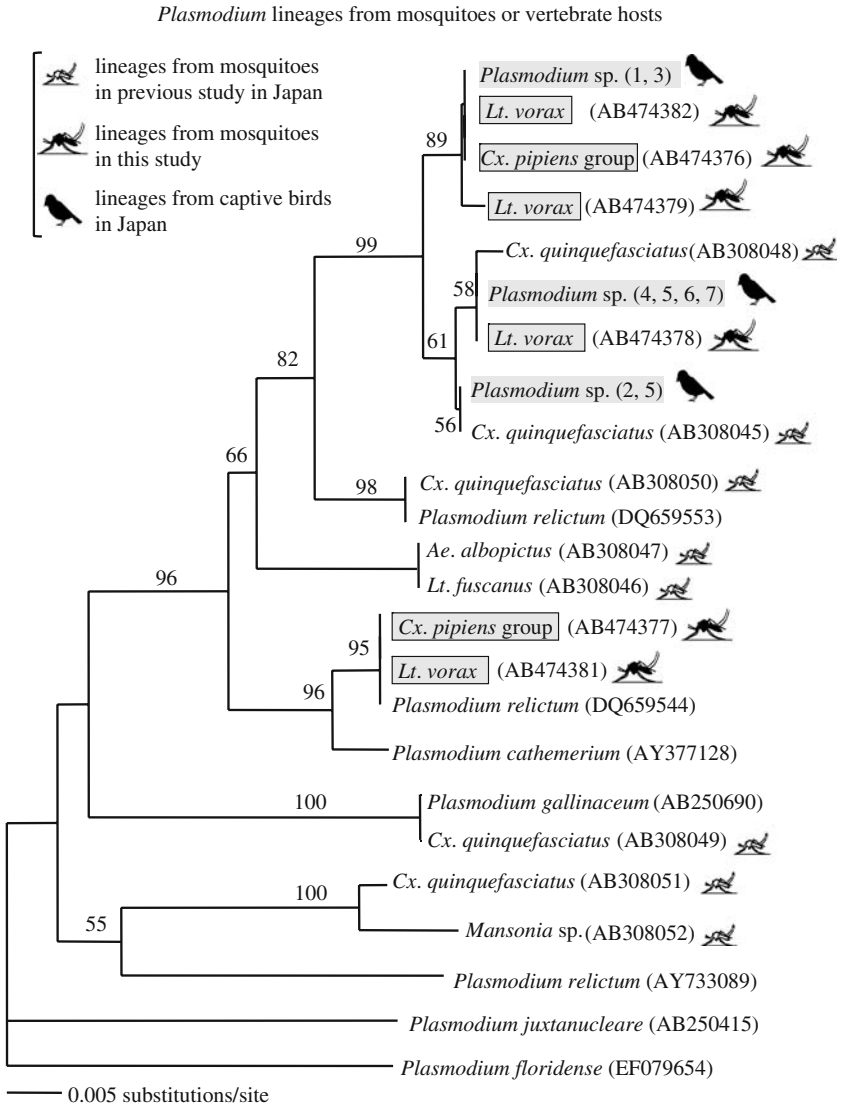

Fig. 1 Phylogenetic status of detected lineages from mosquitoes collected in a zoological garden of Japan: phylogenetic relationship among amplified avian *Plasmodium* lineages from the mosquitoes collected in zoological garden of Kanagawa using NJ method with cyt *b* sequences. *Numbers in branches* indicate bootstrap values on 1,000 replicates. Avian malaria lineages detected from the mosquitoes in this study are indicated by species names within *boxes* and with *black mosquito silhouette* in the tree. And species names with *white mosquito silhouette* and *accession number* in the tree indicate the lineages detected from the mosquitoes in the previous study of Minami Daito Island (Ejiri et al. 2008). *Plasmodium* sp. with *bird silhouette* indicate avian *Plasmodium* lineages from personally communicated DNA sequence data of captive birds (*1 Crossoptilon crossoptilon*, *2 Chrysolophus amherstiae*, *3 Grus grus*, *4 Hypsipetes amaurotis*, *5 Lophophorus impeyanus*, *6 Spheniscus humboldti*, *7 Tragopan temminckii*). Operational taxonomic units with *gray background* indicate the lineages detected in the same zoological garden of Kanagawa. *Cx Culex*, *Lt Lutzia*

from bird hosts and vectors in other places may be also needed to reveal the present status of the prevalence of avian malaria in Japan.

In this study, we suggested that *C. pipiens* group could transmit pathogens such as avian malaria protozoa to host bird species. Both humans and birds have been known as blood source of this mosquito species (Magnarelli 1977; Karoji et al. 1980), suggesting that *C. pipiens* group may serve as bridge vectors of the pathogens from birds to human such as West Nile virus (WNV) (Andreadis et al. 2001; Anderson et al. 2004). Therefore, to demonstrate the whole transmission cycle of avian malaria protozoa may be important to evaluate the risk of WNV infection in Japan. Moreover, risk assessment of vector-borne diseases in zoological garden might be significant in public health aspect, namely, if captive birds are infected with WNV, those mosquitoes in the facilities could be vectors and/or bridge vectors to humans.

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