**FULL PAPER** *Parasitology*

# Detection of Avian *Plasmodium* spp. DNA Sequences from Mosquitoes Captured in Minami Daito Island of Japan

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ABSTRACT. Several species of birds in Minami Daito Island, an oceanic island located in the far south from the main islands of Japan, were found to be infected with avian *Plasmodium*. However, no vector species of the avian malaria in this island have been revealed yet. To speculate potential vectors, we collected mosquitoes there and investigated using a PCR procedure whether the mosquitoes harbor avian malaria or not. Totally 1,264 mosquitoes including 9 species were collected during March 2006 to February 2007. The mosquitoes collected were stored every species, sampled date and location for DNA extraction. Fifteen out of 399 DNA samples showed positive for the partial mtDNA cytb gene of avian *Plasmodium*. Estimated minimum infection rate among collected mosquitoes was 1.2% in this study. Four species of mosquitoes; *Aedes albopictus, Culex quinquefasciatus, Lutzia fuscanus and Mansonia* sp. had avian *Plasmodium* gene sequences. Detected DNA sequences from *A. albopictus* and *L. fuscanus* were identical to an avian *Plasmodium* lineage detected in bull-headed shrike (*Lanius bucephalus*) captured in the island. Different sequences were detected from *C. quinquefasciatus*, which were corresponding to an avian *Plasmodium* from a sparrow (*Passer montanus*) and *Plasmodium gallinaceum*. Our results suggest that *A. albopictus, Lutzia fuscanus, C. quinquefasciatus*, and *Mansonia* sp. could be potential vectors of avian malaria in Minami Daito Island. This study was the first report of molecular detection of avian *Plasmodium* from mosquitoes in Japan.
KEY WORDS: bird, Japan, mosquito, *Plasmodium*, vector.

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Recently many studies have been conducted on prevalence of avian blood protozoa such as the genera *Plasmodium, Haemoproteus* and *Leucocytozoon* using molecular biological methods in worldwide scale [4, 5, 7, 12–14, 19, 27, 31, 34], but a few in Asian countries including Japan. PCR based molecular diagnoses revealed that wild birds of Japan were infected with avian blood protozoa which might be transmitted by mosquitoes [27]; however, further question that which species of mosquitoes could be the vectors still remains unclear. Prevalence of vector borne diseases is usually influenced by the distribution range of vector arthropods and incidence of the disease pathogens harbored in the vector shows correlation with the numbers of the pathogen positive vector mosquitoes [35]. Many detection case reports on avian malarial DNA by PCR from the host birds are reflecting the progress of sensitivity and convenience of this molecular technique. There have been also PCR positive results from naturally infected vector mosquitoes of human *Plasmodium* [24, 41]; however, no evidence has been described on the detection of avian malaria parasites from vectors so far.

Following the former studies on the prevalence of avian blood protozoa in Japan [11, 25], further molecular biological analyses of avian *Plasmodium* and *Leucocytozoon* have been reported on wild birds of Japan, respectively [27, 34]. More recently, Murata *et al.* (2008) also showed that avian malaria distributed even in a particularly isolated environment of an oceanic island, Minami Daito Island, Japan [26]. This island remains isolated from the main islands of Japan and has some endemic bird species and/or subspecies such as Borodino bush warbler (*Cettia diphone restrictus*), Varied tit (*Parus varius orii*), Borodino islands white-eye (*Zosterops japonicus daitoensis*), Daito scops owl (*Otus elegans interpositus*), Borodino islands bulbul (*Hypsipetes amaurotis borodinonis*) and bull-headed shrike (*Lanius bucephalus*) [1]. Because of interspecies competition, the ecosystem of oceanic islands seems to be evolutionally naive, so the birds inhabiting these islands may easily suffer from not only the invasion of alien birds but also foreign infectious diseases [3, 38, 43, 45]. One prominent example is the situation of native birds in Hawaii Islands. These well known oceanic islands were primarily free from mosquitoes and avian *Plasmodium* before the arrival of Europeans; however, avian malaria of *P. relictum* and a possible vector mosquito, *Culex quinquefasciatus*, had been introduced with the human migration to this island area [20]. Unconscious but artificial introduction of *P. relictum* and *C. quinquefasciatus* eliminated or damaged seriously some native birds of Hawaii [39]. Since, not only in Hawaii, newly introduced infectious diseases could affect for wild birds living in isolated envi-

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ronment [18, 38, 40], the identification of transmission cycle of infectious diseases might be important.

As mentioned above, the native birds infected with avian malaria in Minami Daito Island of Japan have been reported with relatively high prevalence [26]. Furthermore, several mosquito species were identified in this isolated oceanic island [22], suggesting that they could be potential vectors of avian malaria there. So, detection of avian malaria protozoa from possible vectors could illustrate the transmission cycle in this particular oceanic island of Japan with similar environmental situation to Hawaii. In this study, we present candidates for vector of avian malaria in Minami Daito Island of Japan, which were confirmed by modified molecular biological procedures.

## MATERIALS AND METHODS

Mosquitoes were collected from several locations in Minami Daito Island of Japan (25°51'N, 131°15'E) from March 2006 to February 2007. CDC traps, gravid traps and a sweeping method were utilized for collection. Captured mosquitoes were morphologically classified and kept at –20°C in 1.5 m*l* tubes until DNA isolation.

After species identification of the collected mosquitoes, they were pooled as consisting of 1 to 5 individuals by its species, captive date and place. Samples were disrupted and extracted DNA by REDExtract-N-Amp Tissue PCR kit (SIGMA) following manufactures' instruction. We used the primers for amplification of partial cytb gene of avian mitochondrial (mt) DNA. For the first PCR, DW2 and DW4 primers were utilized as previously described [31], and for the second amplification, our originally designed primers, 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'), derived from relatively conserved region of several avian Plasmodium previously registered in GenBank were used. Each solution for PCR reaction was contained 2.5 $\mu l$ of 10 × buffer , 4 mM of $MgCl_2$, 200 mM of each dNTP, 0.4 $\mu$M of each primer, 1 U of Ex-Taq polymerase (TAKARA, Japan), and 1 $\mu l$ of the DNA in total volume of 25 $\mu l$. For the primary PCR, a total of 35 cycles was carried out, consisting of denaturing at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min, with an initial denaturizing at 94°C for 3 min. The second PCR reaction was performed as well as the first PCR reaction using 1 $\mu l$ of the first PCR reaction.

The amplified products were 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 ABI3130 (Applied Biosystems, Foster City, California, U.S.A.) auto sequencer. Phylogenetic analyses of about 478 bp sequences were performed as described previously [34]. 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 elongatum, P. gallinaceum, P. relictum* and *P. juxtanucleare* with other avian *Plasmodium* from GenBank and from personally communicated data obtained from wild birds of Minami Daito Island. A lineage of human malaria parasite, *P. falciparum*, was used as out group in the tree.

To evaluate infection rate of the mosquitoes studied, the minimum infection rate (MIR) of each mosquito was calculated as previously described [44]. MIR was utilized to estimate an infection rate by presuming that at least one individual of the pooled sample was infected. The formula of MIR is as follows; MIR = number of PCR positive/number of collected mosquitoes × 100.

## RESULTS

One thousand and two hundred sixty (1,264) mosquitoes consisting of 5 genera with 9 species (*Aedes albopictus, A. daitensis, A. togoi, Armigeres subalbatus, C. quinquefasciatus, C. rubithoracis, Lutzia fuscanus, Mansonia uniformis and Mansonia* sp.) were captured and identified. Among collected samples, *C. quinquefasciatus* was the most dominant species through studied period. Detailed distribution of collected mosquito species and individual numbers in this island will be described elsewhere. Totally 399 DNA samples were obtained from the 1,264 mosquitoes mentioned above. Fifteen out of 399 samples were positive for the parasite cytb gene. Samples of 4 species of mosquitoes, *A. albopictus, C. quinquefasciatus, L. fuscanus* and *Mansonia* sp., were PCR positive. MIRs of respective mosquito species were as follows: *A. albopictus* (1.2%), *C. quinquefasciatus* (1.1%), *L. fuscanus* (20%) and *Mansonia* sp. (2.8%) (Table 1). No avian malaria sequences were detected from *A. daitensis, A. togoi, Ar. subalbatus, C. rubithoracis* and *M. uniformis* (Table 1). All obtained nine sequences from the mosquitoes were deposited to GenBank, having accession numbers from AB308044 to AB308052.

A phylogenetic tree was successfully obtained from the amplified sequences of mosquito samples, showing that at least seven lineages of *Plasmodium* were included in the collected mosquitoes (Fig. 1). Two avian malaria lineages from both *A. albopictus* and *L. fuscanus* were completely identical to avian *Plasmodium* sp. lineage amplified from bull-headed shrike lived in the same island. One lineage amplified from *C. quinquefasciatus* was identical to that of an established avian malaria species, *P. gallinaceum*, while another lineage detected from *C. quinquefasciatus* was completely identical to that of an avian *Plasmodium* sp. from a tree sparrow lived in this island. Other lineages showed relatively high similarities to those of previously sequenced avian *Plasmodium* spp.

## DISCUSSION

Blood sucking arthropods like mosquitoes play important

Table 1. Infection rates of avian *Plasmodium* spp. DNA sequences from the mosquitoes collected in Minami Daito Island using nested PCR

| Mosquito species | No. of collected mosquitoes | No. of pooled samples | No. of PCR positive | Minimum infection rate (%) |
|---|---|---|---|---|
| *Aedes albopictus* | 81 | 46 | 1 | 1.2 |
| *Aedes daitensis* | 3 | 3 | 0 | 0 |
| *Aedes togoi* | 2 | 2 | 0 | 0 |
| *Armigeres subalbatus* | 4 | 4 | 0 | 0 |
| *Culex quinquefasciatus* | 1,066 | 294 | 12 | 1.1 |
| *Culex rubithoracis* | 4 | 3 | 0 | 0 |
| *Lutzia fuscanus* | 5 | 5 | 1 | 20 |
| *Mansonia uniformis* | 63 | 22 | 0 | 0 |
| *Mansonia* sp. | 36 | 20 | 1 | 2.8 |
| Total | 1,264 | 399 | 15 | 1.2 |

roles for the transmission of vector borne diseases including representative zoonoses of human malaria, dengue fever and West Nile fever [17, 29, 32]. Identification of arthropod species as vector is also essential to reveal the transmission cycles of vector borne diseases. Evaluation of positive rates of those etiologic agents in the vectors is necessary to estimate the prediction of prevalence and the risk of infection for the diseases. Direct detection of infective stage protozoa from their vectors is accompanied with difficulties in the requirement for trained skills and in the case of false positive because prevalence of pathogens in nature tends to be relatively low levels [15]. Although several studies showed advantages of molecular detection of various Plasmodium genes from mosquitoes using PCR [2, 6, 21, 36, 37], no molecular based investigations have been reported on the detection of avian malarial protozoa from wild mosquitoes of Japan, neither even on morphological detection of sporozoites. In this study, we detected avian *Plasmodium* DNA sequences from the wild mosquitoes collected in Japan for the first time.

One point two (1.2)% of 1,264 mosquitoes collected in Minami Daito Island were considered to be infected with any avian Plasmodium. Among the studied mosquito species in the island, 1.1% of *C. quinquefasciatus* were positive for avian malaria PCR, suggesting relatively lower infection rate compared with the rate of 15% in the same vector species in Hawaii [45]. Higher positive rate in Hawaii may associate with the fact that mosquitoes found there had been accidentally introduced by human migration to the islands. We have no robust evidence whether the mosquitoes in Minami Daito Island had been introduced such as in Hawaii or not, however, the first Japanese migrants to this desert island in 1900 had suffered from mosquito bites [22], suggesting that some mosquito species originally distributed in this island. It is reported that the infection rates of human malaria in wild mosquitoes are generally less than 10% in most endemic areas by direct detections of sporozoites and/or oocysts from vectors [33, 42]. Therefore, lower infection rates in the vectors in Minami Daito Island (1.2%) might associate with their origin compared to the artificially introduced vector in Hawaii (15%). Detection sensitivity must be strongly considered because recent remarkable progress of ELISA and/or PCR methods enables more feasible results [2, 9, 37]. In the present study, annual prevalence of positive rate in vectors still remains unclear. Further improvement and modification to our detection methods will contribute to demonstrate accurate transmission factors such as infection rates of hosts and vectors in this island.

Complete identity of the amplified DNA sequences of avian *Plasmodium* from two mosquito species (*L. fuscanus* and *A. albopictus*) and that from the host bird, bull-headed shrike, suggests that transmission of avian *Plasmodium* could occur between those two mosquito species and host bird species in Minami Daito Island. Murata *et al.* (2008) reported *Plasmodium* infection of wild birds such as bull-headed shrike, tree sparrow and Borodino islands white-eye by light microscopy in Minami Daito Island, and predicted further detection of different avian malaria there [26]. More evidences such as these mosquitoes harbor infective sporozoites of *Plasmodium* are required to prove these mosquitoes being as vectors of avian malaria, however, it was considered that *L. fuscanus* and *A. albopictus* could play as vectors for avian malaria in the island. Several reports on the analysis of blood meal of vector mosquitoes [16, 23, 28] could be usable for further investigations. The other two lineages from *C. quinquefasciatus* corresponded to an avian *Plasmodium* lineage detected in tree sparrow and, surprisingly, to that of *P. gallinaceum*. In Japan, no occurrence of *P. gallinaceum* has been reported in nature. Only presentation of the amplified sequence of *P. gallinaceum* may be not enough to state the existence of this highly pathogenic avian malaria [30] in this island. More detailed investigations will be also necessary to assess the possibility of introduction of *P. gallinaceum* into main islands of Japan.

Phylogenetic analysis in the present study revealed that the lineages from *C. quinquefasciatus* and *Mansonia* sp. also associated with those from avian *Plasmodium*, suggesting that there might be at least eight lineages or more of avian *Plasmodium* in this island. It has been clearly shown that *C. quinquefasciatus* is a vector for avian malaria by *P. relictum* in Hawaii [45]. So, this mosquito species likely to served as a vector in the present studied island. Hawaiian

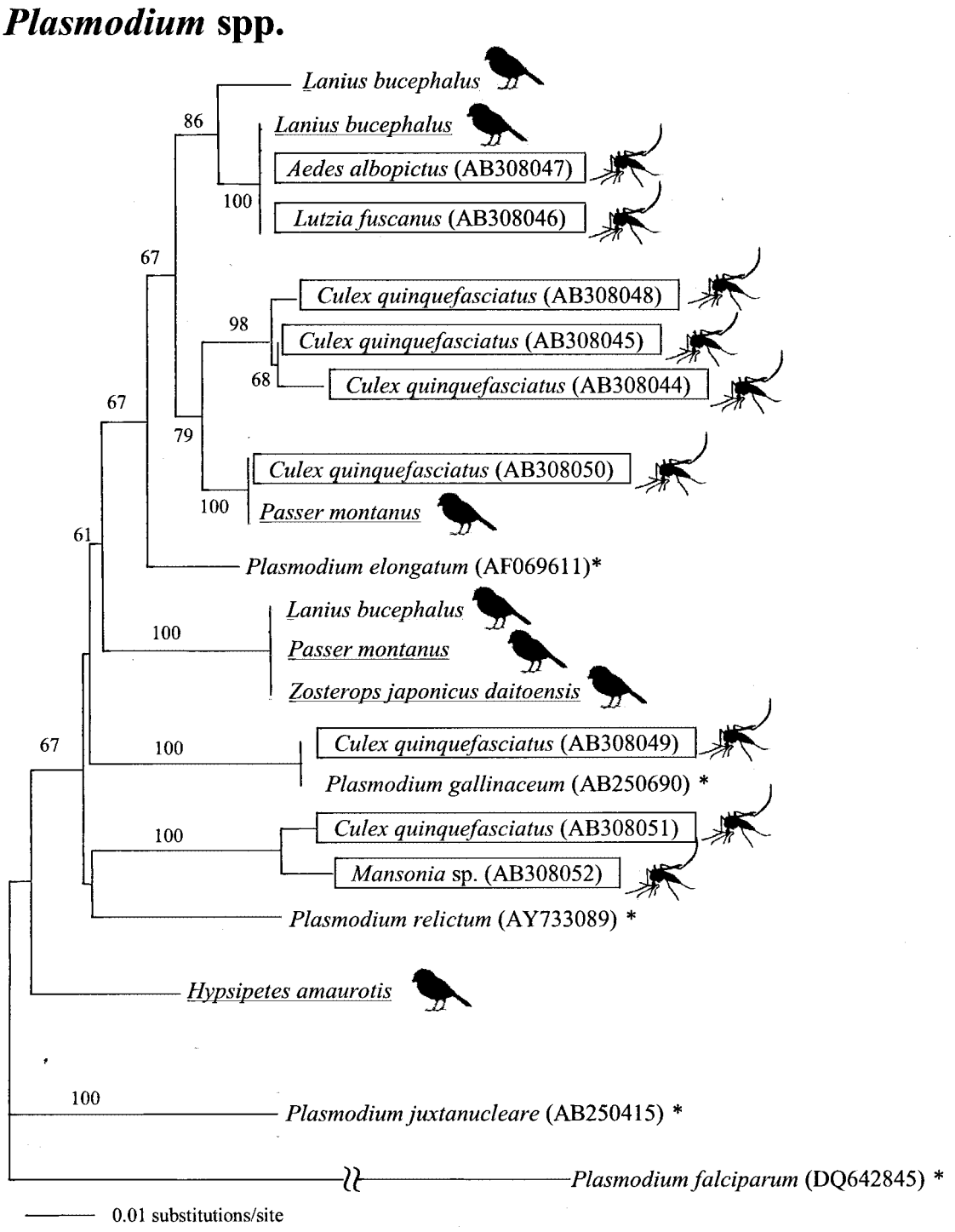


Fig. 1. Phylogenetic relationship among amplified avian *Plasmodium* lineages from the mosquitoes collected in Minami Daito Island of Japan using NJ method with cyt *b* sequences. Numbers in branches indicate bootstrap values on 1000 replicates. All OTUs are indicated by the species names of mosquitoes or malarial parasites with accession numbers (*) or host birds (underlined). Species names within boxes with mosquito silhouette in the tree indicate the lineages detected from the mosquitoes. Underlined species names with bird silhouette correspond to personally communicated lineage sequences detected from the wild birds of Minami Daito Island. Asterisked protozoa sequences were obtained directly from GenBank.

avian *Plasmodium* has been regarded as genetically one species from the result of the past introduction of both pathogen and vector. Murata *et al*. (2008) reported that the infection of several protozoan species from the birds inhabiting this island [26]. Our present lineage detection of multiple avian *Plasmodium* from mosquitoes also supported it. Comparative studies for genetic relationship among lineages of avian protozoa in other places of Japan will be necessary to consider the origin of avian malaria protozoa in Minami Daito Island. It is also important to understand genetic background whether the pathogens in one area are introduced ones or not. This island is an important place for migratory birds to rest [1], hence it is considered that those birds have a possibility to serve as transporters of pathogens. Global-Warming will change the distribution of vectors with consequent introduction and spreading of pathogens [10]. Therefore, demonstration of the whole transmission cycle among birds, vectors and pathogens is the most essential to derive the prevention of avian malaria in Japan. Further attention also should be paid for vector borne bird pathogens other than avian *Plasmodium*, such as West Nile virus and avian pox virus because those pathogens could be transmitted by the same mosquito species collected in this study. Our detection method will be utilized not only for evaluating avian *Plasmodium* in mosquitoes in mainlands of Japan but also for other vector borne diseases in Japan as a potential monitoring tool.

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