FULL PAPER *Parasitology*

Molecular Identification of Avian Haemosporidia in Wild Birds and Mosquitoes on Tsushima Island, Japan

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ABSTRACT. We investigated for the first time the prevalence of avian haemosporidia of genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* among birds and mosquitoes on Tsushima Island of Japan, which is located between Japan and the Korean Peninsula. Of 55 wild birds belonging to 33 species, 16 (29.1%) tested positive for haemosporidia as follows: *Plasmodium* spp. (11/55; 20.0%); *Haemoproteus* spp. (2/55; 3.6%); and *Leucocytozoon* spp. (3/55; 5.5%). A genetic lineage isolated from the Eurasian Sparrowhawk (*Accipiter nisus*) was identical to that of the known avian malaria parasite *P. circumflexum*. Several genetic lineages were identical or closely related to the parasite lineages that were previously detected in birds and mosquitoes in Japan and Korea. Another single identical genetic lineage was also detected in both migratory and resident birds. A total of 753 mosquitoes from 12 species were collected; and one fully fed *Aedes albopictus* was positive for avian *Plasmodium*(1/753; 0.13%) which is identical to a genetic lineage detected in both mosquitoes in Japan and birds in Korea. Blood-meal identifications of blood-fed mosquitoes showed direct contact between the mosquitoes and 4 species of mammals including humans, cattle, rodents and the endangered Tsushima leopard cat (*Prionailurus bengalensis euptilura*). Migratory birds use Tsushima Island as a site for wintering, breeding and resting, and our results suggest the transmission of avian haematozoa between resident and migratory birds during their stay on Tsushima Island.
KEY WORDS: avian haemosporidia, Japan, migratory bird, mosquito, vector.

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Avian haemosporidia including *Plasmodium, Haemoproteus* and *Leucocytozoon* are parasitic protozoa, which can occasionally cause harmful effects, such as lower reproductive output, poor physical condition and hypertrophy of internal organs in several passerine birds [25] and high mortality in Hawaiian endemic birds naïve to avian *Plasmodium* [1]. The prevalence of these vector-borne avian protozoa in Japan has been reported in both birds and mosquitoes [3–7, 16–18, 21, 22, 28]. These results could be especially important for conservation of endangered wild bird species, but the study areas were limited and the epidemiological data were insufficient to understand the transmission dynamics and assess the infection-risk for these vector-borne etiological agents in Japan.

Migratory birds could be important carriers of vector-borne diseases such as avian malaria [26] and West Nile fever [13] in the wild. Recent climate change could have affected the population structure of migratory birds [12], influenced the dispersal range of vectors and lengthened the expected life of arthropod vectors of infectious disease. These effects could extend to possible changes in transmission areas and seasonal occurrence of vector-borne diseases [8]. Furthermore, dispersal of infective vectors could enhance the transmission rate and risk of infection by the avian malaria parasite in both naïve and even adapted host birds [8]. Therefore, surveillance of vector-borne pathogens in wild birds is important for conservation ecology and disease control.

Tsushima Island is located between the southwest end of mainland Japan and the south end of the Korean Peninsula (138 km from Japan and 49.5 km from Korean Peninsula) (Fig. 1). This island is 82 km long and 18 km wide, and 89% of the ground is covered with mountain forests. Because of the effects of the warm Tsushima current, the monthly temperature difference is small throughout the year, ranging from 12.4 to 19.1°C, but the seasonal northwest wind produces a cold winter (average temperature is 6.8°C from December to February). The annual mean precipitation and temperature between 1995 and 2010 were 1,480 mm and 15.7°C, respectively. Because of these geographical and climatic features, Tsushima Island provides unique ecological habitats. Many endemic and/or local species, such as the Tsushima leopard cat (*Prionailurus bengalensis euptilura*), inhabit this niche environment. Forty-nine families of 217 species of wild birds were recorded on Tsushima Island, and migratory birds use this island for breeding, overwintering, or just for resting during their long migration [38, 39].

A recent study on the migration routes of birds by satellite tracking showed migration through the Korea/Tsushima strait [11]. The migratory behavior of birds has been suggested as an important ecological factor that spreads the geographical

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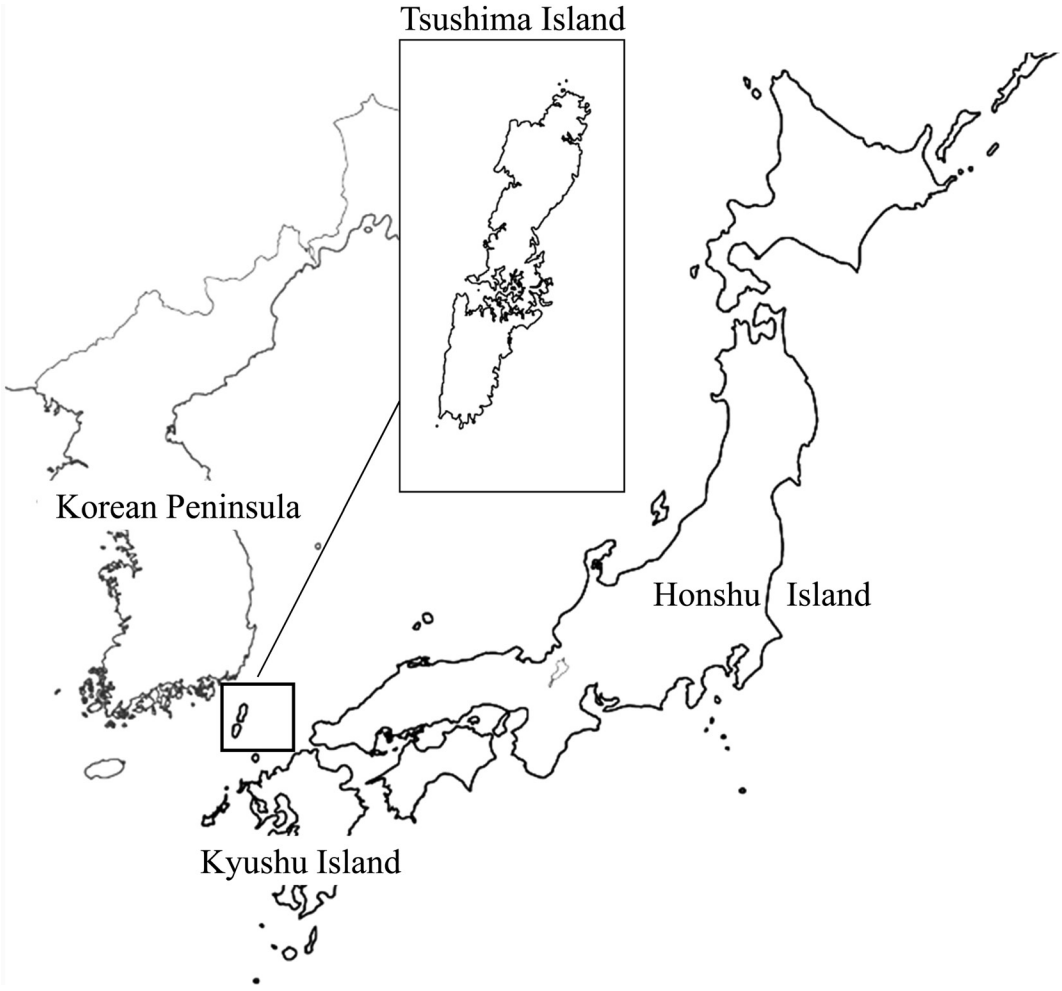

Fig. 1. Location of Tsushima Island, Japan.

distribution of avian haemosporidia [26, 36]. Mosquitoes of genus *Culex* include well-known or suspected vectors of the avian malaria parasite in Japan [3, 5–7,16–18], and some *Culex* species inhabit Tsushima Island [20]. Migratory birds infected with avian malaria parasites can be expected to carry the parasite from their wintering sites and transmit or exchange it through vector mosquitoes that inhabit Tsushima Island. However, the prevalence of avian malaria parasites in both bird and mosquito hosts from Tsushima Island has not been reported. In this study, we investigated the prevalence of avian malaria parasites in both bird and mosquito hosts to elucidate the basic ecological relationship between the parasites, avian hosts and mosquitoes on this geographically unique island of Japan.

MATERIALS AND METHODS

Sample collection of birds: Dead specimens of birds were collected and kept frozen at −20°C from January 2008 to July 2011 at Tsushima Wildlife Conservation Center (TWCC). Those birds were all found in this island. Small portions of the liver were kept at −20°C in 1.5 m*l* tubes until DNA isolation.

DNA extraction and molecular detection of avian haemosporidia from bird samples: DNA was extracted using the QIAamp®DNA Micro Kit (QIAGEN, Valencia, CA, U.S.A.). Extracted DNA was used for nested-PCR to detect the partial cyt*b* gene of the avian malaria mitochondrial genome, as described previously [5, 17, 18]. Briefly, primers DW2 and DW4 [31] were used for the first PCR and HAEMNFI and HAEMNR2 [35] were used for the second PCR to detect *Plasmodium* spp. and/or *Haemoproteus* spp., and LcytbF and LcytbR were used for *Leucocytozoon* spp. [28]. We also used the primer set of HAEMNFI and HAEMNR3 [9] for the first reaction and HAEMF and HAEMR2 [2] for the second to amplify *Plasmodium* spp. and *Haemoproteus* spp. To detect *Leucocytozoon* spp., the primer set of HAEMFL and HAEMR2L [9] was used for the second PCR.

Collection of mosquitoes: Mosquitoes were collected at a forest area adjacent to TWCC, in which wild birds would distribute from May to September 2010 with both CDC traps and a sweeping net. Collected mosquitoes were identified

into species according to morphological keys [32, 34] and kept at −20°C until extraction of DNA. Mosquito specimens were separated using microscissors into the head-thorax part and the abdomen under a microscope [3, 5]. One to five mosquitoes were pooled for DNA extraction by species, collection date, place and presence of a blood-meal.

DNA extraction and molecular analyses of mosquito samples: Samples were disrupted, and DNA was extracted using the REDExtract-N-Amp Tissue PCR kit (SIGMA, St. Louis, MO, U.S.A.) according to manufactures' instructions. Extracted DNA from the mosquitoes was used for nested-PCR amplification of the partial cyt*b* gene of the avian malaria mitochondrial genome as described previously [7]. Briefly, we used the DW2 and DW4 primers [31] for the first reaction and HAEMF and HAEMR2 [2] for the second. Detected amplicons from wild birds and mosquitoes were sequenced, and detected lineages were analyzed for phylogenetic relationship using 273-bp sequences as described previously [7].

To evaluate the rate of infection among the studied mosquitoes, the minimum infection rate (MIR) of each mosquito was calculated as previously described [37] under the assumption that only one individual of the pooled sample was infected. The formula for calculation of MIR is as follows: MIR=number of PCR-positive/total number of collected mosquitoes × 1,000.

The blood-source animals of the mosquitoes were also identified in the fully fed mosquitoes by PCR amplification of the vertebrate cyt*b* sequence from avian- and mammalian-derived DNA as described previously [3, 17, 29]. When no PCR products were obtained, another primer set (VerU-1 and VerU-2) was used to amplify the vertebrate 16S ribosomal RNA region universally for both birds and mammals [3, 17].

RESULTS

A total of 55 wild birds belonging to 33 species across 12 orders were examined; and lineages from 3 genera of haemosporidia, *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp., were detected (Table 1). In total, haematozoa were found in 29.1% of 55 individuals belonging to *Anseriformes, Ciconiiformes, Passeriformes, Falconiformes, Gruiformes* and *Columbiformes*. The prevalence of haematozoa was 20%, 3.6% and 5.5% for *Plasmodium* spp. *Haemoproteus* spp. and *Leucocytozoon* spp., respectively; and no mixed infections were detected.

A total of 753 mosquito specimens from the 12 species *Aedes albopictus, Ae. flavopictus, Ae. japonicus, Ae. nipponicus, Ae. seoulensis, Ae. togoi, Ae. vexans nipponii, Armigeres subalbatus, Culex mimeticus*, *Cx. pipiens pallens, Cx. tritaeniorhynchus and Tripteroides bambusa* were collected (Table 2). *Aedes nipponicus* and *Cx. tritaeniorhynchus* were the predominant species in the study area. In total, 320 DNA samples were obtained from the collected specimens. This is the first collection of adult *Ae. seoulensis* recorded in Japan. Only one fully fed *Ae. albopictus* captured in August 2010 was positive for the avian malaria parasite by PCR (MIR=0.13%). This is the first report of detection of avian *Plasmodium* DNA in mosquito on Tsushima Island.

Twenty-four blood-fed mosquitoes representing 6 species were collected, and 21 blood-meals were successfully identified as originating from 4 mammalian species including humans (*Homo sapiens*), cattle (*Bos taurus*), mice (*Mus musculus*) and the Tsushima leopard cat (*Prionailurus bengalensis euptilurus*) (Table 2). The most common blood-source animal was *Bos taurus*. Two blood-fed mosquitoes had each fed on 2 blood-source species: *Bos taurus* and *Homo sapiens*, and *Homo sapiens* and *Mus musculus*, respectively.

Phylogenetic analysis revealed three types of avian haemosporidia on Tsushima Island in the following species *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. (Fig. 2). The genetic lineage (TsB45) detected from the Eurasian Sparrowhawk (*Accipiter nisus*) was completely identical to that of *P. circumflexum*(AF495576) amplified from the song thrush (*Turdus philomelos*) in Sweden. The genetic lineages amplified from the common moorhen (*Gallinula chloropus*: TsB10), grey heron (*Ardea cinerea*: TsB14) and mallard (*Anas platyrhynchos*: TsB17) have not been reported from Japan, but are identical to the genetic lineage detected in the great tit (*Parus major*: DQ659590) in Sweden and black-faced bunting (*Emberiza spodocephala*: EF380136) from Korea. In addition, 2 *Plasmodium* lineages detected from the grey heron (*Ardea cinerea*: TsB1, TsB24) are closely related to the lineage amplified from mosquitoes of Japan, *Lutzia vorax* and *Culex pipiens pallens*. The genetic lineage amplified from mosquito in this study (Ts143h) was identical to that from the birds of Japan and Korea.

DISCUSSION

Important roles of migratory birds have been suggested in the dispersion and/or outbreaks of several zoonoses, such as bird flu [15, 19] and West Nile fever [27]. As avian haemosporidia are also carried by infected migratory birds, their transmission cycles and prevalence should be determined to provide fundamental information for evaluating the present status of pathogens. In this study, we revealed for the first time the prevalence of avian haemosporidia on Tsushima Island, which is located between the Korean peninsula and Japan and lies on an avian migratory route. Compared with previous epidemiological studies of birds on the main island of Japan, the average prevalence of 29.1% observed in this study is higher than the previously reported 10.6% [21] or 14.5% [23]. Murata *et al.* [22] reported the high infection rate of 59.6% in 183 wild birds of 4 species on a remote island of Japan. The lower infection rates found in this survey are probably due to the larger land mass and the colder climate for vectors than on Minami-Daito Island. Murata *et al.* [22] assumed that the high infection rates in insular birds were affected by the density and /or inherited factors of the host birds, and also by the favorable climate for the vectors. Another explanation for the differences in prevalence of avian haemosporidia may partly be the application of different detection methods, such as microscopy and/or PCR. Another possible reason for the difference in prevalence between the present and previous studies is the different ma-

Table 1. Results of detection of *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp. from birds collected on Tsushima Island, Japan

Bird Species		range distribution in Tsushima	No of bird examined	Detected parasite genera		
Order	Species			*Plasmodium*	*Haemoproteus*	*Leucocytozoon*
Podicipediformes	*Tachybaptus ruficollis*	*RB*	2			
Cuculiformes	*Cuculus saturatus*	*PV*	1			
Anseriformes	*Anas crecca*	*WV*	1			
	Anas platyrhynchos	*WV*	3	2		
Galliformes	*Phasianus colchicus karpow*	*RB*	2			
	Bambushicola thoracica	*RB*	2			
Ciconiiformes	*Ardea cinerea*	*RB,PV*	6	3		
	Ixobrychus eurhythmus	*IV*	1	1		
	Nycticorax nycticorax	*RB*	3			
Passeriformes	*Fringilla montifringilla*	*WV*	1			1
	Cettia diphone	*RB*	2			
	Regulus regulus	*WV*	2	1		
	Luscinia cyane	*PV*	1			
	Parus major	*RB*	1			
	Riparia riparia	*PV*	1			
	Hirundo rustica	*MB*	1			
	Corvus corone	*RB*	1			1
	Hypsipetes amaurotis	*RB*	3	2		
	Bombycilla japonica	*PV*	1			
	Carduelis spinus	*WV*	1			
	Troglodytes trogolodytes	*RB*	1			
	Zosterops japonicus	*RB*	2			
Falconiformes	*Accipiter gentilis*	*PV*	1			
	Accipiter nisus	*WV*	3	1		1
	Falco peregrinus	*RB,WV*	1			
Charadriiformes	*Synthliboramphus antiquus*	*WV*	1			
	Larus argentatus	*WV*	2			
	Hydrophasianus chirurgus	*AV*	1			
Gruiformes	*Gallinula chloropus*	*RB*	2	1		
	Grus vipio	*PV*	1			
Columbiformes	*Streptopelia orientalis*	*RB*	2		2	
Strigiformes	*Otus scops*	*PV*	1			
Coraciiformes	*Alcedo atthis*	*RB*	1			
Total			55	11	2	3
Infection rate			29.1%	20.0%	3.6%	5.5%

RB:resident breeder, *MB*:migrant breeder, *WV*:Winter Visitor, *PV*:Passage Visitor, *IV*:Irregular visitor, *AV*:Accidental visitor.

terials used for the PCR-detection; we used avian liver DNA, whereas previous molecular biological studies used blood DNA. Regarding the transmission of haemosporidia by vector mosquitoes on Tsushima Island, it is difficult to argue whether those protozoa detected in wild birds had reached the infectious stage to be transmitted by mosquitoes, since we did not examine blood from the frozen bodies of birds. Despite the differences in detection methods in the present study, it is certain that these birds had the parasites, and intensive examinations of bird samples by both PCR detection and observation of blood smears will be required in future to confirm transmission of haemosporidia on Tsushima Island.

The predominant species of mosquitoes collected in this study was *Culex tritaeniorhynchus* followed by other species belonging to genus *Aedes*, such as *Ae. nipponicus* and *Ae. albopictus*. All mosquito species collected in this study were included in the check list of mosquito species collected from Tsushima Island, except for the adult females of *Ae. seoulensis* and *Cx. mimeticus.* The former and latter species were collected for the first time in Japan and on Tsushima Island [14, 20, 30]. Mosquitoes known to be vectors of avian *Plasmodium*, such as *Cx. pipiens pallens* and *Ae. albopictus* [3, 5–7, 16, 17], were collected, and we showed for the first time the approximate seasonal distribution from May to September on Tsushima Island. Identification of the species of mosquitoes in the study area is necessary, but determining the seasonal distribution of suspected vectors of pathogens could be even more important to explain the dynamics of transmission.

The results of parasite detection from bird samples showed

Table 2. Results of blood-meal identification and detection of avian malaria parasites in mosquitoes collected on Tsushima Island, Japan

Mosquito species	No. of mosquitoes			Blood source animals identified				
	Collected	Unfed	Fully fed	Human	Cattle	Mouse	Wild cat	Unidentified*
Aedes albopictus	93	85	8	2	1	2	1	2
Aedes flavopictus	21	19	2	1	1	0	0	0
Aedes japonicus	30	28	2	1	2	0	0	0
Aedes nipponicus	167	163	4	3	1	0	0	0
Aedes seoulensis	4	4	0	0	0	0	0	0
Aedes togoi	3	3	0	0	0	0	0	0
Aedes vexans nipponii	1	1	0	0	0	0	0	0
Armigeres subalbatus	131	124	7	0	6	0	0	1
Culex mimeticus	2	2	0	0	0	0	0	0
Culex pipiens pallens	15	15	0	0	0	0	0	0
Culex tritaeniorhynchus	279	278	1	1	0	1	0	0
Tripteroides bambusa	7	7	0	0	0	0	0	0
Total	753	729	24	8	11	3	1	3

*Blood source PCR was positive but not identified into species.

that the most prevalent avian haemosporidia on Tsushima Island was *Plasmodium* spp.(20.0%)*Haemoproteus* spp. and *Leucocytozoon* spp. were detected for the first time from wild birds of Tsushima Island. The genetic relationships between avian *Plasmodium* lineages, avian hosts and the suspected vector mosquitoes are shown in the phylogenetic tree (Fig. 2), which indicates the presence of a variety of avian *Plasmodium* lineages in wild bird communities on Tsushima island that are transmitted inside and/or outside Japan. The genetic lineages of avian *Plasmodium*(TsB10, TsB14 and TsB17) detected in the common moorhen, grey heron and mallard in this study had been found in wild birds in Sweden and Korea, suggesting a wide geographic distribution across Eurasia. This genetic lineage has not been found in Japanese mosquitoes; therefore, it is most likely that those birds had been infected with this genetic lineage somewhere outside Japan before flying to Tsushima Island. The genetic lineage (TsB45) was identical to *P. circumflexum* and was detected for the first time in wild birds such as the Eurasian Sparrowhawk in Japan. The genetic lineage (Ts143h) amplified from *Ae. albopictus* in this study has been found in birds from Japan and Korea. The genetic lineages detected in both wild birds and mosquitoes in Japan have probably established their transmission cycle in Japan.

About 89% of the mosquitoes collected belonged to 4 species, *Ae. albopictus*, *Ae. nipponicus*, *Ar. subalbatus* and *Cx. tritaeniorhynchus*. The number of mosquitoes in the *Cx. pipiens* group, a primary vector of the avian *Plasmodium* parasite in mainland Japan, was only 15 (2%) in this study. An entomologic study of the vector situation for human filariasis on Tsushima Island conducted in the 1960s reported a low density of *Cx. pipiens* group [24]. The infection rates of mosquito populations with avian *Plasmodium* parasites ranged between 1% and 5% in previous studies conducted in Japan [3, 6, 7, 16, 17]; therefore, a sample size less than 100 is insufficient to obtain a female infected with the avian *Plasmodium* parasite. Application of different collection methods for mosquitoes, such as dry ice traps and/or gravid traps, will be required in future studies to clarify the vector mosquitoes for the avian *Plasmodium* parasite on Tsushima Island.

About 67% of the blood-fed mosquitoes collected in this study were *Aedes* mosquitoes, and one blood-fed *Ae. albopictus* was positive for avian *Plasmodium* by PCR (MIR=0.13%). Table 2 shows that the *Aedes* mosquitoes fed exclusively on mammals. Recent studies of the feeding pattern of *Ae. albopictus* conducted in Japan reported similar results: the mosquitoes preferred to feed on mammals, especially humans [17, 29]. The previous studies also indicated that the feeding behavior of *Ae. albopictus* is opportunistic and that they are able to feed on a variety of animals such as birds, amphibians, and reptiles depending on the availability of blood-source animals [10, 17, 29, 33]. Furthermore, *Plasmodium* DNA from *Ae. albopictus* has been detected on an oceanic island [7], indicating direct contact between infected birds and *Ae. albopictus*. To clarify the ecological relationships between *Ae. albopictus*, the avian Plasmodium lineage, and the bird hosts on Tsushima Island, additional molecular ecological studies of mosquitoes and birds will be required in the future.

In conclusion, we found a variety of avian blood parasites in wild birds on Tsushima Island as well as evidence of direct contact between infected birds and mosquitoes inhabiting the island, although the number of samples positive for the avian malaria parasite was small and the results are not conclusive at present. Further investigations not only on this island but also in neighboring areas around Japan and Korea will reveal the geographically and ecologically unique nature of Tsushima Island as an important contact zone among migratory birds, vector mosquitoes, and avian malaria parasites.

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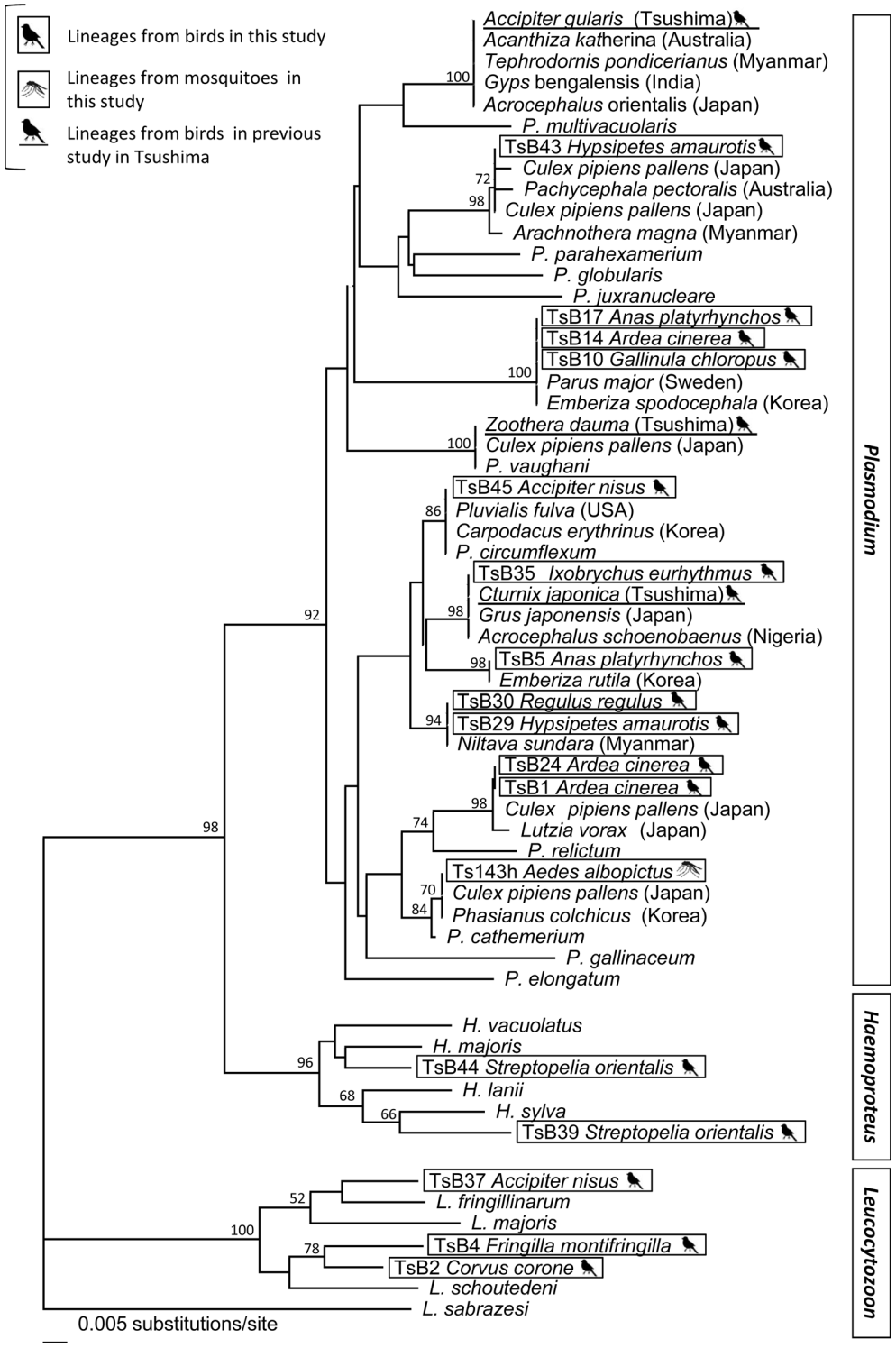

Fig. 2. Phylogenetic relationships among amplified avian haemosporidia linages in birds and mosquitoes collected on Tsushima Island, Japan.

Cyt*b* sequences of avian haemosporidia and the neighbor-joining method were used. Numbers of branches indicate bootstrap values based on 1,000 replicates. Parasite lineages detected in this study are boxed with the sample number, names and silhouette of birds or mosquitoes. Also, the lineages detected from Tsushima are under-lined with the names and silhouettes of the host birds.

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