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Parasitology

A long-term field study on mosquito vectors of avian malaria parasites in Japan

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ABSTRACT. Avian malaria is a mosquito-borne disease of birds caused by avian *Plasmodium* spp. in worldwide scale. Some naïve birds show serious symptoms which can result in death. Surveillance of vectors and parasites are important to understand and control this disease. Although avian malaria has been found in Japan, detailed prevalence and dynamics remained understudied. We aimed to observe annual changes in the abundance of mosquitoes and the prevalence of avian *Plasmodium* parasites in Japan. Mosquitoes were collected using dry ice traps over a 10-year period, at a fixed research area located in Kanagawa prefecture. Collected mosquitoes were investigated for the species composition, population size and prevalence of avian *Plasmodium* by PCR. Mosquitoes belonging to 13 species in 7 genera were collected (n=8,965). The dominant species were *Aedes* (*Ae.*) *albopictus* and *Culex* (*Cx.*) *pipiens* group (gr.). Seven avian *Plasmodium* lineages, all of which were previously known, were detected from *Cx. pipiens* gr., *Ae. albopictus*, and *Tripteroides bambusa*. Three genetic lineages were dominant and were probably transmitted by *Cx. pipiens* gr. whose could be the primary vector of these parasites. Annual variations in the seasonal prevalence of mosquitoes and avian *Plasmodium* were revealed for the first time during recent 10 years in Japan. Namely, avian *Plasmodium* occurrence in the vector population peaked often in June to July and September to October when the density of the vector population was presumably high enough for the transmission of avian *Plasmodium* upon appearance of infected birds.

KEYWORDS: avian malaria, *Culex pipiens*, Japan, mosquito, *Plasmodium*

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Mosquitoes (Diptera: Culicidae) are important arthropod vectors for the transmission of many vector-borne diseases such as human malaria, Japanese encephalitis, Dengue fever, West Nile fever, and avian malaria [1, 3, 7, 26, 39, 42]. Female mosquitoes can transmit those pathogens to vertebrate hosts during their blood-feeding activities and can also obtain pathogens from infected vertebrate hosts. To control vector-borne diseases, it is important to understand the general occurrence of mosquitoes in each area as well as the prevalence of pathogens which mosquitoes harbor. Moreover, it is also noteworthy to realize that recent climate changes have also affected the biology of mosquitoes in many ways such as the extension of distribution and active period, and increase of population density caused by the shortening of developmental period [3, 11, 13]. These effects strongly suggest that long-term and comparative mosquito surveillance are necessary to estimate the probable risks of vector-borne diseases.

Avian malaria is a disease of birds caused by avian *Plasmodium* parasites and is transmitted by mosquitoes mainly belonging to the genera *Culex*, which are distributed throughout the world [38]. This vector-borne protozoal disease can cause serious symptoms or even death in host birds. [2, 4, 6, 12, 15, 19, 31]. Several studies showed a relatively high prevalence of avian malaria in wild and captive birds in Japan [17, 18, 31, 32]. Entomological studies on mosquito vectors of avian malaria also showed that *Culex* mosquitoes in Japan harbor several avian *Plasmodium* lineages and are vectors of avian malaria in Japan [7, 10, 20, 21, 24, 34]. However, no long-term studies monitoring the situation for over the years have been reported thus far, and changes in the occurrence of mosquitoes and of the prevalence of avian *Plasmodium* remain unknown.

Most of the Japanese archipelago belongs to the temperate zone and the environment is broadly classified into coastal area, rural area, urban area, and forest area. Each environment might have a distinct transmission cycle of avian *Plasmodium* maintained in native birds. The *Plasmodium* lineages and vector species are also different depending on the environment. Previously reports of the avian malaria prevalence in mosquitoes in Japan included zoos [9, 10], a subtropical island [7], wetlands [10, 21], coastal areas [24],

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a city park [24] and a college educational farm in the rural area [34]. Many of these locations also serve as important landing zones for migratory birds. Some results indicated that the dominant *Plasmodium* lineages were consistently found in mosquito vectors, although there were significant annual variations in mosquito density and the prevalence of *Plasmodium* lineages in mosquitoes [21, 25]. Lalubin *et al*. [25] showed antagonistic dominance patterns during their survey, but no seasonal surveillances have been reported in Japan except Kim and Tsuda [20]. To confirm these findings and to deepen our understanding of avian *Plasmodium* transmission in wild bird communities, we investigated the occurrence of mosquitoes and the prevalence of avian malaria parasites in these mosquitoes, over a 10-year period at a fixed study area in Japan as a follow-up surveillance for previous one-seasonal research at the same study area as Shirotani *et al.* [34].

MATERIALS AND METHODS

Study site

The study site was the college educational farm (28 ha) and the neighboring forest (5 ha) of Nihon University, located in Kanagawa, Japan (35°22'35.20" N, 139°27'55.16" E). The farm and forest are surrounded by residential area. Inside of the farm and forest is a typical environment very similar to that of rural areas in Japan. Over 30 cows and 200 pigs are bred in the farm, while the forest includes rice fields, small streams and a grove. Some wild birds such as Hawfinch (*Coccothraustes coccothraustes*), Japanese tit (*Parus minor*), Pale thrush (*Turdus pallidus*) and Brown-eared bulbul (*Hypsipetes amaurotis*) have been confirmed to inhabit the study site.

Mosquito collection and DNA extraction

Mosquitoes were collected from April 2006 to March 2016. Mosquito collection was carried out three times per month using battery-operated CDC-type traps, enhanced with 1 kg each of dry ice for attracting mosquitoes. Four different environments were selected for collection sites, and one trap was set per environment. The first trap was surrounded by shrub and nearby rice fields, and set at a height of 2 m. The second trap was set in open dirt and nearby rice fields. The third trap was surrounded by tall trees and nearby a river, and set at a height of 1 m. The fourth trap was set in open space and nearby cattle stall at a height of 1 m. The dry ice was placed within a cooler bag to extend the duration of CO_2 release. We set these traps in the evening and let them operate for 24 hr. If the trap had problems, we tried again another day. Furthermore, we calculated the number of mosquitoes per trap per day in each month for standardization.

Collected mosquitoes were morphologically identified into species [35]. The mosquitoes were then separated into two parts, head–thorax and abdomen, in order to distinguish the developmental stages of avian *Plasmodium* parasites in the mosquitoes by PCR [22, 23]. Unfed females of *Cx. pipiens* gr. and *Aedes* (*Ae.*) *albopictus* were pooled, 1 to 5 and 1 to 10 mosquitoes per pool, respectively. Blood-fed and gravid females of the above species, and members of additional species, were processed individually. DNA was extracted from each sample using REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO, USA).

Detection of avian malaria parasites by nested PCR

Avian *Plasmodium* DNA was amplified by nested PCR for the partial cytochrome *b* gene of the avian malaria mitochondrial genome, as previously described [5, 7]. From 2006 to 2012, we used DW2 and DW4 primers for the 1st PCR [33], and APFN and APRN for the 2nd PCR [7]. From 2013 to 2016, we used HEAMNF and HEAMNR2 for the 2nd PCR instead of APFN and APRN [41] to expect more lineage detection. All amplified fragments were sequenced in both directions using Big Dye ™ terminator mix (Applied Bio systems, Foster City, CA, USA). Detected lineages were identified using the Basic Local Alignment Search Tool (BLAST) in the GenBank database (www.ncbi.nlm.nih.gov/BLAST/) and MalAvi [5]. To estimate infection rate of the mosquitoes examined, the minimum infection rate (MIR) of each mosquito species was calculated as previously described [43]. MIR has been utilized to estimate the infection rate by presuming that at least one individual in the positive pooled sample is infected. The formula of MIR is as follows: MIR=number of PCR positive/number of collected mosquitoes ×1,000.

RESULTS

A total of 8,965 mosquitoes were collected and identified, resulting in 13 species from seven genera: *Cx. pipiens* gr., *Cx. tritaeniorhynchus, Cx. bitaeniorhynchus, Cx. orientalis, Lutzia vorax, Ae. albopictus, Ae. japonicus, Ae. nipponicus, Tripteroides* (*Tr.*) *bambusa, Armigeres subalbatus, Anopheles* (*An.*) *sinensis, An.* sp., and *Uranotaenia novobscura* (Table 1). While the species composition varied from year to year during the study, *Ae. albopictus* and *Cx. pipiens* gr. were the dominant species throughout the study period, ranging from 24.4% to 63.6% and 30.5% to 73.2% of the total collected mosquitoes per year, respectively. Most of *Plasmodium* DNA (94.7%) was detected from *Cx. pipiens* gr. In addition, avian *Plasmodium* DNA was detected from *Ae. albopictus* and *Tr. bambusa* in 2015. To compare the annual variation in seasonal prevalence of *Cx. pipiens* gr. and avian *Plasmodium* parasites in mosquitoes, the month of their appearance, peak and end are presented for each year of the study (Figs. 1, 2). The appearance of the first female of *Cx. pipiens* gr. was generally observed in April, with the exception of 2007 and 2014 in January (Fig. 1a). The density of *Cx. pipiens* gr. generally peaked in June or July and then decreased until around November or December (Fig. 1b). We investigated the relationship between mosquito density and *Plasmodium* prevalence in *Cx. pipiens* gr., in which *Plasmodium* DNA was most detected. The appearance of avian *Plasmodium* parasites in *Cx. pipiens* gr. varied from year to year but could be constantly observed between April and July (Fig. 2a). The peak of MIR in *Cx. pipiens* gr. was generally observed between June to July and September to October. No avian *Plasmodium* DNA was detected during November through March of all years (Fig. 2b).

Table 1. Mosquito species and numbers examined in this study, the number of mosquito samples positive for avian *Plasmodium* DNA, and the minimum infection rate of mosquitoes from a study site at Kanagawa, Japan, between 2006 and 2015

Mosquito species collected		Year of mosquito collection										Total
		2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	
Culex pipiens gr.	*n*	204	196	144	74	141	142	261	603	843	532	3,140
	m	19.6	25.5	27.8	0	21.3	14.1	0	33.2	24.9	22.6	23.2
	t	1.4	1.2	1.1	0.5	1.0	1.0	1.8	4.4	5.9	3.8	2.2
Aedes albopictus	*n*	173	94	175	49	358	302	755	1,663	994	613	5,176
	m	0	0	0	0	0	0	0	0	0	4.9	0.6
	t	1.2	0.6	1.3	0.4	2.5	2.0	5.2	12.2	6.9	4.3	3.6
Aedes japonicus	*n*	0	2	93	3	5	7	3	14	66	4	197
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	<0.1	0.7	<0.1	<0.1	<0.1	<0.1	0.1	0.4	<0.1	0.1
Tripteroides bambusa	*n*	27	13	14	14	43	34	0	56	60	41	302
	m	0	0	0	0	0	0	0	0	0	24.4	3.3
	t	0.2	0.1	0.1	0.1	0.3	0.2	0	0.4	0.4	0.3	0.2
Armigeres subalbatus	*n*	1	3	2	4	8	8	12	6	17	16	77
	m	0	0	0	0	0	0	0	0	0	0	0
	t	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1	<0.1	0.1	0.1	0.1
Culex tritaeniorhnchus	*n*	1	0	0	0	17	5	0	4	10	0	37
	m	0	0	0	0	0	0	0	0	0	0	0
	t	<0.1	0	0	0	0.1	<0.1	0	<0.1	0.1	0	<0.1
Culex bitaeniorhynchus	*n*	0	0	0	0	0	0	0	6	5	1	12
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	0	0	0	<0.1	<0.1	<0.1	<0.1
Lutzia vorax	*n*	0	0	0	0	0	0	0	2	4	1	7
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	0	0	0	<0.1	<0.1	<0.1	<0.1
Anopheles sinensis	*n*	0	0	0	0	0	0	0	0	1	0	1
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	0	0	0	0	<0.1	0	<0.1
Uranotaenia bimaculata	*n*	0	0	0	0	0	0	0	2	1	0	3
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	0	0	0	<0.1	<0.1	0	<0.1
Culex orientalis	*n*	0	0	0	0	0	0	0	0	1	0	1
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	0	0	0	0	<0.1	0	<0.1
Aedes nipponicus	*n*	0	0	0	0	1	0	0	0	0	0	1
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	<0.1	0	0	0	0	0	<0.1
Anopheles group	*n*	0	0	0	1	0	0	0	0	0	0	1
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	<0.1	0	0	0	0	0	0	<0.1
Unknown	*n*	0	0	0	0	3	1	0	0	6	0	10
	m	0	0	0	0	0	0	0	0	0	0	0
	t	0	0	0	0	<0.1	<0.1	0	0	<0.1	0	<0.1
Total	*n*	406	308	428	145	576	499	1,031	2,356	2,008	1,208	8,965
	m	9.9	22.7	9.3	0	5.2	4.0	0	8.5	10.5	13.2	8.6
	t	2.8	1.8	3.2	1.0	4.0	3.4	7.2	17.3	13.9	8.6	6.2

n: Number of mosquitoes collected; *m*: MIR=number of positive sample/number of mosquito collected×1,000; *t*: number of mosquitoes collected/traps/day.

We detected avian *Plasmodium* DNA from *Cx. pipiens* gr. consistently every year, although there were annual variations in the abundance of *Cx. pipiens* gr. and the prevalence of avian *Plasmodium* parasites in the vector population (Table 1). A significant positive correlation (r=0.93, $P<0.001$) was found in the relationship between vector abundance and the prevalence of avian *Plasmodium* in vectors (Fig. 3). The slope of the regression line was 0.0285, indicating an average annual infection rate of 2.85% in *Cx. pipiens* gr. throughout the whole study period. The x-intercept was calculated to be 58, indicating a threshold value of vector abundance below which no avian *Plasmodium* parasites were found.

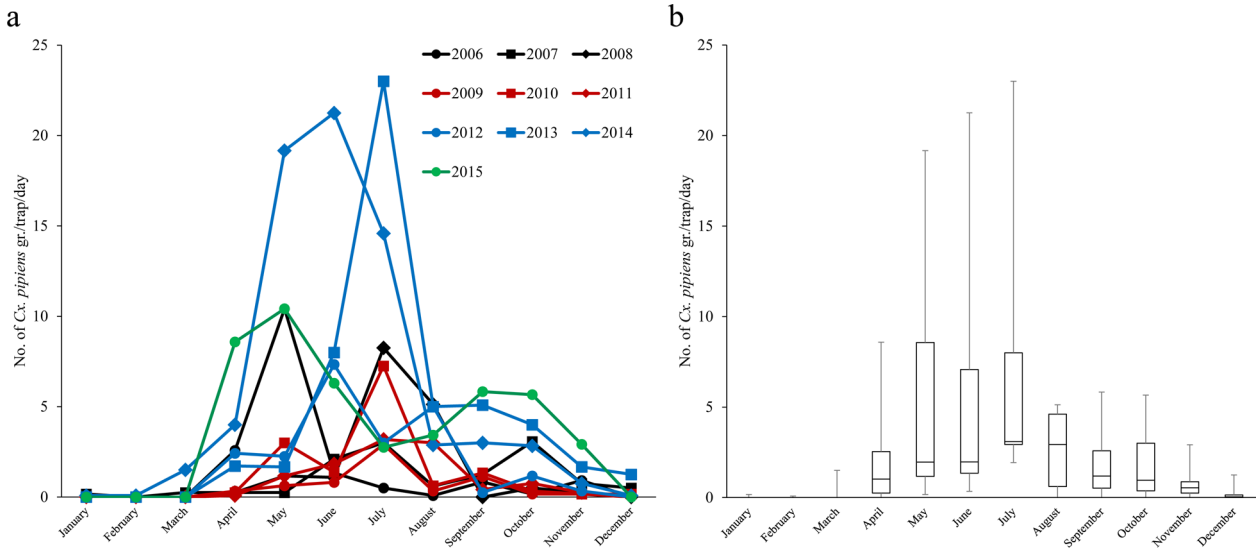

Fig. 1. Annual variations in seasonal prevalence of *Cx. pipiens* group observed at Kanagawa, Japan, from 2006 to 2015. The number of *Cx. pipiens* gr./trap/day is shown for each month, by year (**a**) and overall (**b**).

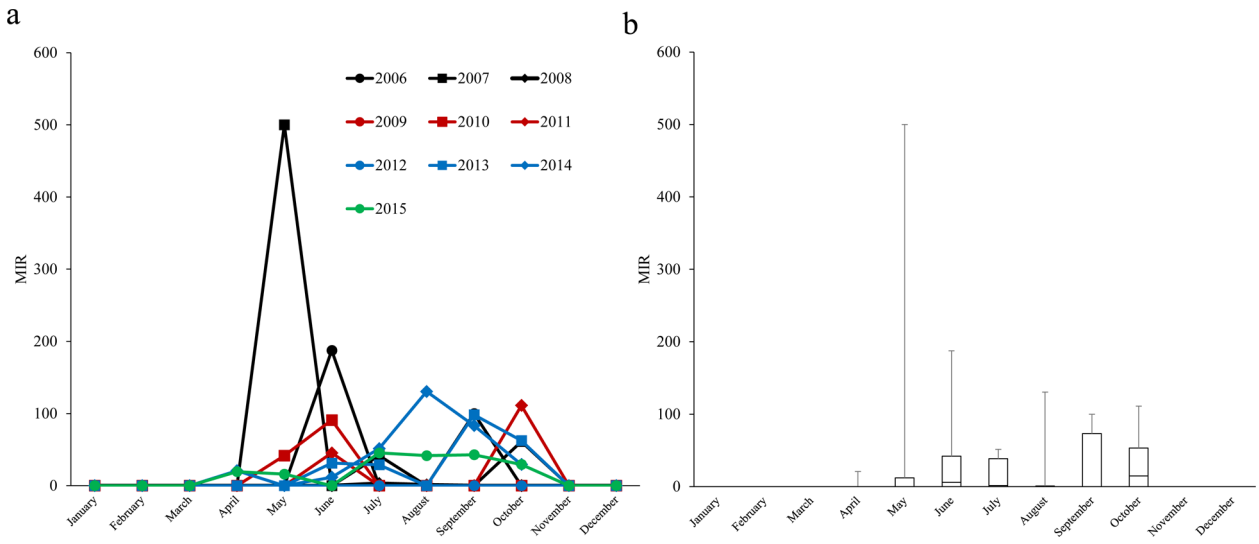

Fig. 2. Annual variations in avian *Plasmodium* in mosquitoes observed at Kanagawa, Japan, from 2006 to 2015. Minimum infection rate (MIR) is shown for each month, by year (**a**) and overall (**b**).

The following seven known avian *Plasmodium* lineages were detected in this study: CXPIP09, GRW04, PADOM02, SGS1, SPMAG10, SYCON02, and ZOCAP03 (Table 2). Of these, the dominant lineages were PADOM02, CXPIP09, SGS1 in order of prevalence at our study site and were detected from both head–thorax and abdomen samples of *Cx. pipiens* gr. The lineages GRW04, SPMAG10, and ZOCAP03 were detected from only abdomen samples of *Cx. pipiens* gr, while SYCON02 was detected from only head–thorax samples of *Cx. pipiens* gr. CXPIP09 and SGS1 were also detected from abdomen of *Ae. albopictus*. PADOM02 was detected from abdomen of *Ae. albopictus* and *Tr. bambusa.* This is the first report of avian *Plasmodium* DNA being detected in *Tr. bambusa*.

Annual changes in the avian *Plasmodium* parasite community composition are presented in Fig. 4. In this study, CXPIP09 and PADOM02 were detected in 6 out of the 10 years examined. SGS1 became prevalent in recent years, between 2013 to 2015. Although the most prevalent lineages changed between years, CXPIP09, PADOM02, and SGS1 comprised 90% of all lineages detected in this study (Table 2). The remaining four lineages were detected only one or two times.

The seasonal occurrence of genetic lineages of avian *Plasmodium* parasites in *Cx. pipiens* gr. is shown in Fig. 5. The seasonal occurrence of the three dominant lineages showed different patterns. CXPIP09 had a large peak in July and a small peak in September. SGS1 had a large peak in October and a smaller peak in April. The PADOM02 lineage had a larger peak in September to October.

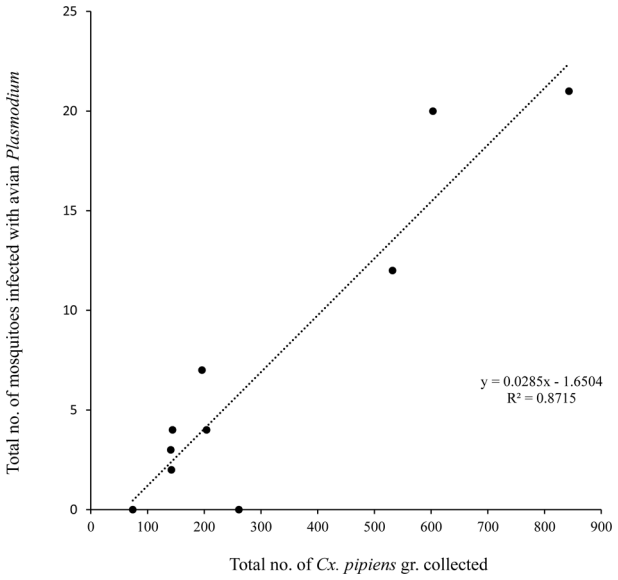

Fig. 3. Correlation between mosquito vector abundance and the number of mosquitoes with avian *Plasmodium* parasites. Each dot represents a year within the study. The line shows the regression line.

Table 2. Summary of avian *Plasmodium* lineages detected by PCR in all mosquitoes by body segment

Genetic lineage	Number detected in			Total	%
	Head-thorax	Abdomen	Head-thorax & abdomen		
CXPIP09	3	20	1	24	33.3
PADOM02	1	20	4	25	34.7
SGS1	0	17	2	19	26.4
GRW04	0	1	0	1	1.4
SPMAG10	0	1	0	1	1.4
SYCON02	1	0	0	1	1.4
ZOCAP03	0	1	0	1	1.4
Total	5	60	7	72	100

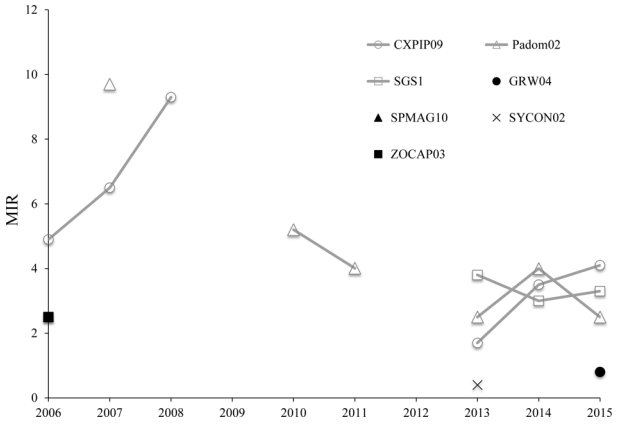

Fig. 4. Annual changes in the avian *Plasmodium* parasite community detected from mosquitoes during 2006 to 2015 in Kanagawa, Japan.

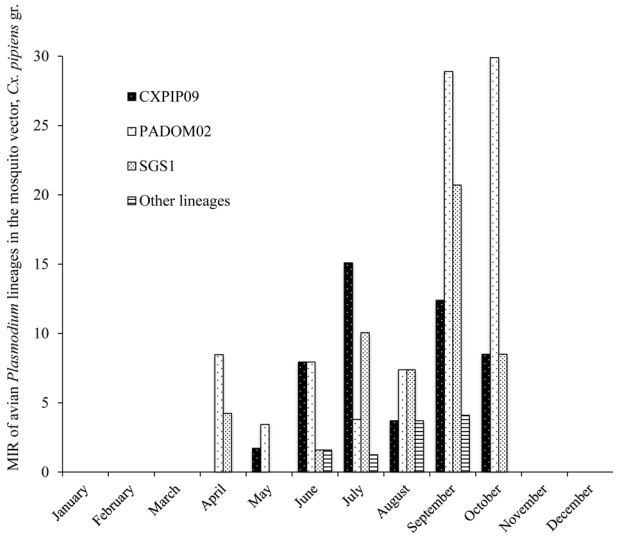

Fig. 5. Seasonal minimum infection rate (MIR) of avian *Plasmodium* lineages in mosquito vector, *Cx. pipiens* group.

Seasonal changes in the total number of *Cx. pipiens* gr. infected with avian *Plasmodium* parasites had two peaks, the first in July and the second in September, indicating a high risk of avian *Plasmodium* transmission during these months.

DISCUSSION

Transmission dynamics and persistence of avian Plasmodium parasites

The present study showed that *Cx. pipiens* gr. is the primary vector species of avian *Plasmodium* parasites at our study site. *Cx. pipiens* gr. and *Ae. albopictus* were the dominant mosquito species, comprising 35.0% and 57.7% of the mosquito community, respectively. However, almost all avian *Plasmodium* lineages (96.1%) were detected from only *Cx. pipiens* gr. in this study, as seen in previous entomological studies of avian *Plasmodium* parasites in Japan [9, 23, 34].

The analysis of the annual variation in seasonal prevalence of *Cx. pipiens* gr. in our study site found that these mosquitoes appeared in April, with a peak density from May to July, then decreasing until October to December. This seasonal prevalence is quite similar

to that observed in Tokyo between 2003 and 2013 [37], suggesting that this observed seasonal prevalence of *Cx. pipiens* gr. could be common across eastern Japan. *Cx. pipiens* gr. in Japan can be classified *into Cx. pipiens* form *molestus* and a subspecies, *Cx. pipiens pallens*. A previous study reported that *Cx. pipiens* form *molestus* account for the high rates in April, May and October, and *Cx. pipiens pallens* account for the high rates from June to September in Tokyo [23]. This suggests that both *Cx. pipiens pallens* and *Cx. pipiens form molestus* were involved in avian malaria transmission at this study site. Compared to the relatively stable seasonal prevalence of *Cx. pipien*s gr., the appearance of avian *Plasmodium* parasites in the vector population showed a larger annual variation, varying from year to year between April to July. However, the peak of MIR was quite consistent, usually being observed between June to July and September to October. The appearance of avian *Plasmodium* parasites in the vector population principally depends on the arrival of infected migrating birds or a recrudescence of chronic infections in resident birds [37]. The results of our study indicated that the density of the vector population in the study site is usually high enough to begin the transmission of avian *Plasmodium* parasites when infected birds appear between April to July, and an annual MIR of 8.6 was achieved every year in the vector population, as suggested in Table 1. No lineages were detected from November to March, presumably because only a small number of mosquitoes were caught. In addition, since *Cx. pipiens* gr. overwinter as adults and infected *Cx. pipiens* gr. was collected in April, it is possible that the overwintered *Cx. pipiens* gr. cause new infections. Since feeding behaviors are regulated by temperature, investigating the timing of blood feeding behaviors may help to confirm whether infections may be carried over to the following year by overwintering mosquitoes.

We also detected avian *Plasmodium* lineages from *Ae. albopictus* and *Tr. bambusa* in 2015. Although a previous study already reported the detection of avian *Plasmodium* spp. in *Ae. albopictus* [7], this is the first report of the detection from *Tr. bambusa*. We only detected avian *Plasmodium* DNA from the abdomen of this mosquito species, so it remains uncertain whether this species is competent for avian *Plasmodium* parasites at present. *Tr. bambusa* is known as an ornithophilic mosquito [29] and is widely distributed throughout Japan [27]. Therefore, this species is likely to have frequent direct contact with infected birds, so it is important to investigate the vector competence of *Tr. bambusa.*

Occurrence of dominant and occasional genetic lineages of avian Plasmodium

During the ten years of our study, we detected 7 genetic lineages of avian *Plasmodium* parasites. Three of these (PADOM02, CXPIP09, and SGS1) were detected frequently and are referred to as "dominant lineages", while the other four genetic lineages detected only once or twice are referred to as "occasional lineages" for the purpose of this study. SGS1 is distributed worldwide [14], whereas CXPIP09 has only been found in mosquitoes in Japan to date. Sporozoites of both genetic lineages was detected from *Cx. pipiens* gr., suggesting that *Cx. pipiens* gr. have competence of SGS1 and CXPIP09 [22]. PADOM02 has only been detected in mosquitoes in Japan and Switzerland [36]. However, sporozoites of PADOM02 was detected from experimentally-infected *Cx. pipiens* gr. [30], suggesting that *Cx. pipiens* gr. have competence for PADOM02.

SGS1 became prevalent in recent years between 2013 to 2015. Since the primer pair for PCR detection was changed in 2013, we could not distinguish whether SGS1 become more prevalent in the study area or whether SGS1 was just detected by the new primers. Different seasonal prevalence was distinguished among the dominant lineages in this study. CXPIP09 was detected from May to October, with two peaks in July and September. SGS1 was detected from April to October, with the peak of detection in September plus a smaller peak in July. PADOM02 could be detected from April to October with a peak in September and October. The different seasonal prevalence among dominant lineages may be partly because of seasonal changes in the availability of host birds for the dominant lineages of avian *Plasmodium* parasites.

Among the four occasional lineages of avian *Plasmodium* parasites found in this study, GRW04 has previously been detected in *Cx. pipiens* gr. in Japan [8, 10, 21, 22]. GRW04 has also been detected in Eurasian tree sparrows (*Passer montanus*) [7, 21]. Eurasian tree sparrows were often observed in our study site, suggesting they could be possible reservoirs of GRW04 there. The remaining three occasional lineages SPMAG10, SYCON02, and ZOCAP03 found in this study have been detected in Magellanic penguin (*Spheniscus magellanicus*) in Brazil [40], Spectacled warbler (*Sylvia conspicillata*) in Spain [16], and Thorn-tailed rayadito (*Aphrastura spinicauda*), White-crested elaenia (*Elaenia albiceps*) and Rufous-collared sparrow (*Zonotrichia capensis*) in Chile [28]. This is the first time in the world that these lineages have been detected in mosquitoes. Since SYCON02 was detected from the head-thorax, *Cx. pipiens* gr. may have vector competence for this lineage. The host birds of these avian *Plasmodium* lineages in our study site are unknown at present. Our study did not examine avian malaria prevalence in birds, and no field studies have been carried out to investigate bird–mosquito contact in this study site. Additional field studies focusing on these aspects will be required to understand the ecological background of avian malaria persistence in wild bird communities.

We have investigated the occurrence of mosquitoes and the prevalence of avian malaria parasites in these mosquitoes over a 10-year period in the same study site. Three dominant and four occasional avian *Plasmodium* lineages were detected from *Cx. pipiens* gr. mosquitoes. The dominant lineages were detected from both head–thorax and abdomen samples of *Cx. pipiens* gr., and a previous study reported that sporozoites of these three lineages was detected from *Cx. pipiens* gr. [22]. This indicated that this mosquito species may be a competent vector of avian malaria for these lineages, although further studies are necessary. The peak of avian *Plasmodium* occurrence in the vector population was quite consistent, usually being observed in June to July and September to October. Our results indicated that the density of the vector population at the study site is usually high enough to begin the transmission of avian *Plasmodium* parasites when infected birds appear between April to October.

CONFLICT OF INTEREST. The authors declare that they have no competing interests.

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