Detection of rickettsial DNA in ticks and wild boars in Kyoto City, Japan

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ABSTRACT.   The tick is a well-known vector for arthropod-borne pathogens, such as tick-borne encephalitis, Lyme disease, Japanese spotted fever and severe fever with thrombocytopenia syndrome. It is therefore important to know the tick population and distribution in our environment and wild animals in order to prevent tick-borne diseases. Here, we report the results of tick surveillance from May to September 2011 at 14 geographical points and in 5 wild boars in Kyoto City, Kyoto prefecture, Japan. We collected 3,198 ticks comprising 5 tick species, *Haemaphysalis* (*H.*) *longicornis*, *H. flava*, *H. kitaokai*, *Amblyomma testudinarium* and *Dermacentor taiwanensis*. Interestingly, the proportion of tick species varied according to geographical region within the city. The ticks collected in the city were reported as potential vectors of pathogens, such as rickettsiosis. We detected rickettsial DNA by PCR in 71.1% of 201 ticks investigated. The ticks that carried rickettsiae were distributed across the whole the city. The sequences of PCR-amplified DNA fragments were determined and showed similarities to spotted fever group rickettsiae. Although their pathogenicity for animals including humans is still unclear, it is important to stay alert and pay attention to tick-borne diseases in order to ensure the safety of the citizens of the city as well as that of visitors.
KEY WORDS: Kyoto City, rickettsia, tick distribution

Arthropods transmit zoonotic pathogens to animals including humans [12, 22]. In Japan, tick-borne encephalitis [29], Lyme disease [17], Japanese spotted fever [21] and other tick-borne diseases have been classically reported. Additionally, some cases of severe fever with thrombocytopenia syndrome (SFTS), whose etiological agents were potentially transmitted by ticks, were reported in early 2013 in the western region of Japan [28]. Ticks might also harbor unknown pathogens that could cause emerging infections in animals and humans. Therefore, it is important to study tick ecology and to survey the vector-borne pathogens in ticks.

As Kyoto City is surrounded by mountains, whose slopes approach areas of human habitation, residents in the city have many opportunities for contact with the arthropods which may harbor zoonotic pathogens. In addition, as Kyoto City is a popular destination for tourists from all over the world, many foreign visitors come to the city all year round. These visitors, possibly infected by certain pathogens from other regions, might accidentally import pathogens to the city from their native environment. Subsequently, other visitors in contact with these infected visitors may export the pathogen back to their place of origin. For instance, Chickungunya virus in Italy [3] and Sever acute respiratory syndrome virus in Hong Kong [30] are considered to have been imported from other countries by infected travelers. In another scenario, visitors to Kyoto City may be bitten by domestic ticks, harboring a zoonotic pathogen and transfer that pathogen back to their place of origin. To prevent an epidemic of infection caused by these pathogens, we need to pay attention to outbreaks of tick-borne diseases in the city and survey tick prevalence as well as the pathogens they harbor.

Genus *Rickettsia* consists of obligate intracellular, gram negative bacteria. The tick has been identified as the vector of these pathogens. The spotted fever group of rickettsiae is the causative agents of rickettsiosis manifested by rash, fever, headache and eschars. In Japan, over 100 patients with Japanese spotted fever caused by *Rickettsia japonica* are reported every year [23, 24]. The increasing distribution of the disease has raised concerns for public health. Therefore, the prevalence of ticks on the plants and wild boars and their harboring rickettsia were investigated in Kyoto city, Japan.

MATERIALS AND METHODS

Tick surveillance: The tick surveillance was undertaken at 14 fixed points and in 5 wild boars in Kyoto City (Fig. 1A) from May to September 2011, to identify tick populations and tick species in human habitats in Kyoto City. We chose 14 surveillance points located on the mountainsides of the northern (A to E in Fig. 1A and Table 1), western (F to K) and eastern (L to N) parts of Kyoto City. The vegetation at these locations consisted mainly of short grasses. Ticks on the plants were captured by a standard flagging method [26]. Ticks were also collected from 5 wild boars hunted in Yamashina-ku (O) for the harmful wildlife control. Adult and nymph ticks were placed in sample bottles containing

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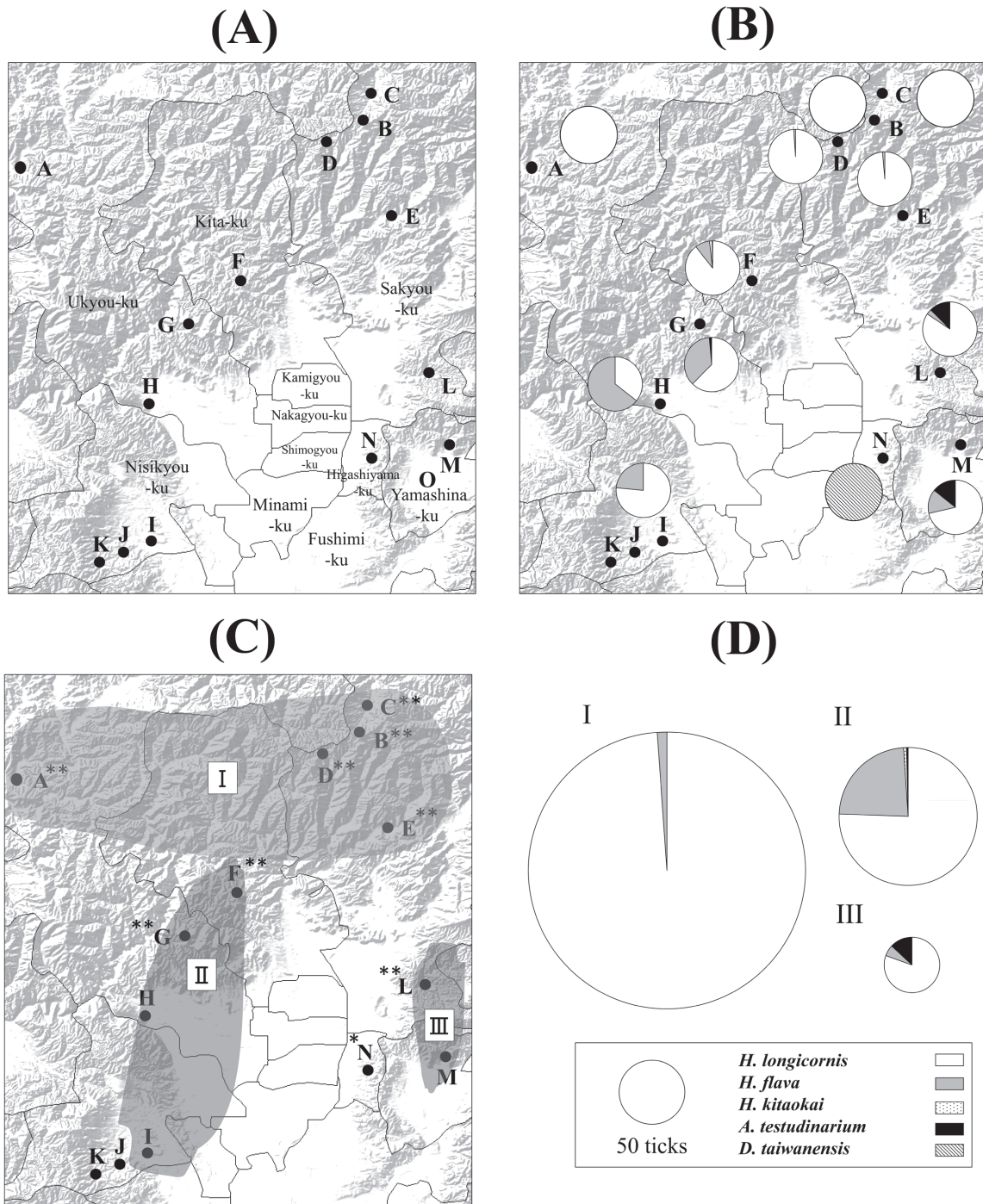
(A)

(B)

(C)

(D)

Fig. 1.   Geographical representation of the tick surveillance points and tick prevalence in Kyoto City, Japan in 2011. We captured the ticks by a flagging method on the mountain slopes around the city, at locations indicated by letters A to N (A). Shaded areas indicate mountain regions. Ticks were captured in the city from May to September 2011. Ticks were also collected from wild boars hunted in Yamashina-ku (point O). The proportions of tick species collected at each surveillance point were plotted on a city map (B). The legend for tick species shown in each pie chart is given in the box in panel D. We classified tick distribution into three general areas, I, II and III, according to the proportion of tick species shown in panel B. The points at which the detection of rickettisal DNA was performed are indicated with an asterisk (*). The points where rickettisal DNA was detected are indicated with a double asterisk (**) (C). We then re-constructed the tick population graphs for each geographical area, I, II and III (D). Tick populations in each area are indicated by the size of the circle. A circle representing a population size of fifty ticks is shown in the box in panel D.

Table 1. Ticks collected in the area around Kyoto City

Plants/ Wild bores	Area of Kyoto City	Collection point (Altitude)	Collection date	Tick species collected	Numbers of ticks collected				Total number
					Male	Female	Nymph	Larva	
Plants	I (North)	A (Keihokuyuzuki) (232 m)	9 Aug	*H. longicornis*	3	6	3	147	159
		B (Hanasetouge) (765 m)	30 Aug	*H. longicornis*	1	1	4	0	6
		C (Hanasebessyo) (496 m)	30 Aug	*H. longicornis*	0	0	12	182	194
		D (Seryoutouge) (428 m)	30 Aug	*H. longicornis*	0	0	1	367	368
				H. flava	0	0	0	4	4
		E (Sizuhara) (274 m)	30 Aug	*H. longicornis*	0	1	3	1,564	1568
				H. flava	0	0	0	22	22
	II (West)	F (Himuro) (380 m)	20 May, 28 Jul	*H. longicornis*	4	1	157	134	296
				H. flava	0	0	15	14	29
				H. kitaokai	2	3	0	0	5
				A. testudinarium	0	0	0	1	1
		G (Sawaike) (370 m)	20 May, 6 Jun, 15 Jul	*H. longicornis*	8	8	72	0	88
				H. flava	5	4	42	0	51
				A. testudinarium	0	0	2	0	2
		H (Arashiyama kouen) (71 m)	25 Aug	*H. longicornis*	0	0	0	26	26
				H. flava	0	0	0	47	47
		I (Haikata) (392 m)	31 Aug	*H. longicornis*	0	0	0	166	166
				H. flava	0	0	0	51	51
		J (Jyurin-ji) (114 m)	31 Aug	*H. longicornis*	0	0	0	0	0
				H. flava	0	0	0	0	0
		K (Yoshimine-dera) (292 m)	31 Aug	*H. longicornis*	0	0	0	0	0
				H. flava	0	0	0	0	0
	III (East)	L (Daimonji-yama) (437 m)	12 Jul	*H. longicornis*	2	9	16	0	27
				H. flava	0	0	1	0	1
				A. testudinarium	0	0	4	0	4
		M (Bisyamonn-dou) (98 m)	26 May	*H. longicornis*	1	1	8	0	10
				H. flava	0	0	2	0	2
				A. testudinarium	0	0	2	0	2
	IV (East)	N (Syougunnzuka) (207 m)	8 Sep	*D. taiwanensis*	0	1	0	0	1
Wild boars		O (Yamashina-ku)	15 Jun	*H. longicornis*	10	9	2	0	21
				H. flava	5	14	0	0	19
				A. testudinarium	25	3	0	0	28
Total number					66	61	346	2725	3198

wet filter paper and taken back to our diagnostic laboratory. They were anesthetized with chloroform. Tick larvae were placed into 70% ethanol. Each tick species was determined by its morphological index [33].

Detection of rickettsial DNA by PCR: A total of 201 ticks collected at 9 locations and from 5 wild boars in Kyoto City from June to September 2011 were selected for the detection of rickettsial DNA by PCR (Table 2). They belonged to 3 genera including 4 species: *Haemaphysalis* (*H.*) *longicornis*, *H. flava*, *Amblyomma* (*A.*) *testudinarium* and *Dermacentor* (*D.*) *taiwanensis* (Table 2). One hundred and ninety four ticks (96.5%) were identified as *H. longicornis*. Twenty four, 30 and 140 ticks were male, female and nymph, respectively. Of these, 21 ticks were captured from 5 wild boars. Two *H. flava* nymphs, 4 *A. testudinarium* nymphs and 1 adult *D.*

taiwanensis female were also examined. To extract DNA from the ticks, collected ticks stored in 70% ethanol were washed with distilled water. These ticks were placed on slide glass and covered with 100 μl of phosphate buffered saline (PBS). After placing cover glasses on top, they were crushed using the head of a tooth pick. The fluid was collected and stored at −80°C until used as a DNA template. A PCR was performed using the genus-specific primer pair of RpCS877, 5′-GGGGGCCTGCTCACGGCGG and RpCS1258, 5′-ATT-GCAAAAAGTACAGTGAACA for the rickettsial citrate synthase (*gltA*) gene [25]. The reaction was carried out using TaKaRa Ex Taq (TAKARA BIO, Otsu, Japan) with a 20 μl reaction mixture containing 2 μl of template DNA, 200 μM each of dNTP and 1 μM each of primer. Following initial denaturation at 95°C for 10 min, 5 cycles of denaturation

Table 2.   Prevalence of ticks investigated for detection of rickettsia

Plants/ Wild boars	Area of Kyoto City	Collection point (Altitude)	Tick species	No. of ticks examined			Total No.	No. of ticks positive for rickettisa[a]	No. of ticks positive for R. japonica[b]
				Male	Female	Nymph			
Plants	I (North)	A (Keihokuyuzuki) (232 m)	H. longicornis	3	6	3	12	8	7
		B (Hanasetouge) (765 m)	H. longicornis	1	1	4	6	4	3
		C (Hanasebessyo) (496 m)	H. longicornis	0	0	12	12	11	11
		D (Seryoutouge) (428 m)	H. longicornis	0	0	1	1	1	1
		E (Sizuhara) (274 m)	H. longicornis	0	1	3	4	4	4
	II (West)	F (Himuro) (380 m)	H. longicornis	3	1	63	67	58	49
		G (Sawaike) (370 m)	H. longicornis	5	3	36	44	32	30
			H. flava	0	0	1	1		
	III (East)	L (Daimonji-yama) (437 m)	H. longicornis	2	9	16	27	18	14
			H. flava	0	0	1	1	0	0
			A. testudinarium	0	0	4	4	2	1
	IV (East)	N (Syougunnzuka) (207 m)	D. taiwanensis	0	1	0	1	0	0
Wild boars		O (Yamashina-ku)	H. longicornis	10	9	2	21	5	5
Total number				24	31	146	201	143	125

a) Number of ticks which were positive in the PCR using at least one primer pair for rickettsial DNA. b) Number of ticks which were positive in the PCR using primer pair, Rj5 and Rj10.

at 94°C for 60 sec, annealing at 53°C for 60 sec and extension at 72°C for 60 sec were performed. Then, 30 cycles of denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec and extension at 72°C for 60 sec were followed. The final extension was performed at 72°C for 7 min. The *Rickettsia* 17-kDa genus-common antigen (17K antigen) gene was also amplified using the primer pair R1; 5′-TCAATTCA-CAACTTGCCATT-3′ and R2; 5′-TTTACAAAATTCTA-AAAACC-3′ [1]. Nested PCR was performed with a primer pair, Rj5; 5′-CGCCATTCTACGTTACTACC-3′ and Rj10; 5′-ATTCTAAAAACCATATACTG-3′ to detect the sequence of *R. japonica* DNA using the 100 times diluted PCR products which were amplified with primer pair of R1 and R2 as a template [9]. The reaction was carried out using TaKaRa Ex Taq with a 20 μl reaction mixture containing 2 μl of template DNA, 200 μM each of dNTP and 1 μM of each primer. After initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 2 min and extension at 70°C for 2 min were carried out. The final extension was performed at 72°C for 7 min. The PCR products were purified using the PCR purification kit (Wizard® SV Gel and PCR and Clean-Up System; Promega, Madison, WI, U.S.A.), and the sequences of amplified products were determined by cycle sequencing.

Phylogenetic analysis: The sequences obtained were evaluated and aligned with each other and those of previously characterized rickettsiae in GenBank by using BLASTn [4]. Multiple alignment analysis was carried out using the pro-

gram ClustalW [6]. Phylogenetic analysis was carried out for the approximately 450 bp of partial sequence of 17K antigen gene using the Genetyx 11.1 software. Phylogenetic trees were constructed by the neighbor joining method.

RESULTS

The results of tick surveillance are shown in Table 1. A total of 3,198 ticks belonging to 5 species of 3 genera were collected from 12 of the 14 sampling points in Kyoto City (Fig. 1A). A breakdown of numbers by percentage shows that 99.2, 6.6, 0.3, 0.2 and 0.03% of the collected ticks from the plants were *H. longicornis* Neumann [13], *H. flava* Neumann [2], *A. testudinarium* Koch [11], *H. kitaokai* Hoogstraal [8] and *D. taiwanensis* Sugimoto [18], respectively (Table 1). The dominant species among the ticks collected in this study were *H. longicornis* and *H. flava* (Fig. 1 and Table 1). The proportions of each growth stage among these ticks were 1.9, 11 and 87.1% for adults, nymphs and larvae, respectively (Table 1). No significant difference in sex among the collected population was observed in this survey (Table 1). Furthermore, the populations of the species collected were not affected by the altitude of the surveillance points (Table 1). Sixty-eight ticks belonging to *H. longicornis*, *H. flava* and *A. testudinarium* were also collected from 5 wild boars (Table 1).

The proportions of tick species found at each surveillance point are shown on a map of Kyoto City (Fig. 1B). Inter-
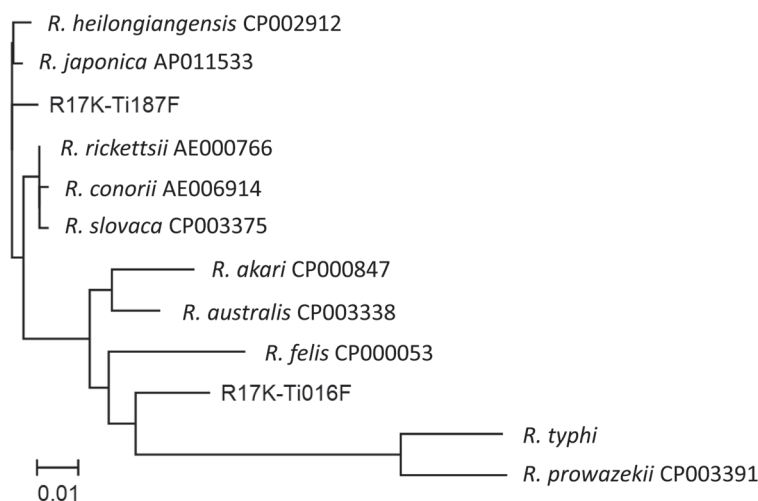
Fig. 2. Phylogenetic tree of SFG rickettsiae isolated from ticks in Kyoto City, Japan derived from sequences of PCR fragments of the 17K antigen gene. The tree was constructed with the neighbor joint method. R17K-Ti187F represents the PCR fragments of the 17K antigen gene obtained in this study from *H. longicornis*. R17K-Ti016F was obtained from *A. testudinarium* in this study.

estingly, the tick population differed markedly according to surveillance point. In the northern mountains (A, B, C, D and E), almost all the ticks captured were *H. longicornis* (Fig. 1B). In the western mountains (F, G, H and I), *H. longicornis* and *H. flava* were the dominant species (Fig. 1B), whereas in the eastern mountains (L and M), *A. testudinarium* was the second common species after *H. longicornis* (Fig. 1B). At the eastern point, N, we captured just one tick of *D. taiwanensis* (Fig. 1B and Table 1).

A total of 201 ticks collected at 9 points in the mountainous area in Kyoto City from July to September 2011 were investigated for the presence of rickettsial genes using primer pairs for *gltA* and 17K antigen genes. Of these, 143 (71.1%) of the ticks appeared to be positive for rickettsial DNA amplified by at least one PCR primer pair (Table 2). Of the PCR- positive ticks, 125 (87.4%) of the samples were positive with the PCR using *R. japonica*-specific primers Rj5 and Rj10. Rickettsial DNA was detected in 141 (72.7%) of the *H. longicornis* consisting of 12 males, 12 females and 117 nymphs. Of these, 1 male, 2 females and 2 nymphs were captured from wild boars. Two *A. testudinarium* nymphs collected from point L also harbored rickettsial DNA. No rickettsial DNA was amplified in *H. flava* and *D. taiwanensis* with the primer pairs (RpCS877 and RpCS1258, R1 and R2, and/or Rj5 and Rj10) used in this study. Rickettsial DNA was detected in the 8 surveillance points A, B, C, D, E, F, G and L (Table 2 and Fig. 1C).

The sequence analysis of selected PCR fragments using the R1 and R2 primer pair (R1/R2 fragment) obtained from *H. longicornis* showed similarity to each other, with 0 to 3 nucleotide sequence differences in 475 bases (99.3–100% homology). The similarity of the R1/R2 fragment of *A. testudinarium* to that of *H. longicornis* was 96.2% (457/475). The R1/R2 fragments were subjected to phylogenetic analysis to confirm the association with previously reported rickettsial sequences (Fig. 2). The R1/R2 fragment from *H. longicornis* (R17K-Ti187F) clustered into the spotted fever group of rickettsia, including *R. japonica*, *R. conorii* and *R. slovaca*, whereas those from *A. testudinarium* were divided into clusters other than SFG rickettsia.

DISCUSSION

The tick species collected in Kyoto City are known to be able to transmit various zoonotic pathogens to humans and animals. For instance, *H. longicornis* is well known to harbor the pathogens of theileriosis [34], babesiosis [7], Japanese spotted fever [14, 21, 27, 31] and many zoonotic viral fevers, including SFTS [20, 35]. *H. flava* is a possible vector of the etiological agents of anaplasmosis [15] and Japanese spotted fever [10]. SFTS viruses [35] and ehrlichial rickettsiae [5] have been detected in *A. testudinarium*, and *R. japonica*, which causes Japanese spotted fever, has been detected in *D. taiwanensis* [10].

According to the map shown in Fig. 1B, the tick distribution in Kyoto City might be classified into at least three geographical areas as shown in Fig. 1C and 1D: I, northern mountain slopes (A, B, C, D and E), II, western mountain slopes (F, G, H and I) and III, eastern mountain slopes (L and M). The vegetation at these points consisted mainly of short grasses. No association between vegetation and the proportion of tick species was observed. Further studies are needed to characterize the distribution of this tick species in the city as the sampling period was limited.

The present study revealed that 71.1% of ticks present near the central city of Kyoto were found to be positive for rickettsial DNA by PCR. Rickettsial DNA was detected at all the 8 surveillance locations where *H. longicornis* was

collected (Table 2 and Fig. 1C). The surveillance points were located in various regions in the city. *H. longicornis*, which was the most abundant tick in this study, and of which 78% were positive for rickettsial DNA, appeared to be the dominant carrier of rickettsial DNA in the area surveyed. *H. longicornis* is well known as a human-biting tick and is considered to be a competent vector of *R. japonica* in Japan [27]. Previously, rickettsial DNA was detected in various amounts in *H. longicornis* obtained from different areas in the north and south of Japan [14, 16]. The detection of *R. japonica* has previously been reported in various areas in Japan [14, 27]. The study on the prevalence of rickettsia in ticks in the Ehime prefecture, where over 70 cases of Japanese spotted fever have been reported during the past decade [24], showed that rickettsial DNA was detected in 12.6% of ticks using the same primer pairs as used in this study [19]. On the other hand, 62.2% of the ticks appeared to be positive for PCR using Rj5 and Rj10 as the primer set for *R. japonica* in the present study. The sequences of the detected DNA segments were analyzed and found to have 98.5% similarity with *R. japonica* YH strain which was isolated from a patient with Japanese spotted fever. The phylogenic tree revealed their sequences clustered with the spotted fever group rickettsiae, including *R.rickettsii* and *R. conorii*, the causative agent of Rocky Mountain spotted fever and Mediterranean spotted fever, respectively. As cases of rickettsiosis have never been reported in the area where we studied, in spite of the high prevalence of rickettsiae in ticks collected, their pathogenicity is still unclear. The sequences of the 17K antigen gene fragment were identical to those of the rickettsia related species, LON-13, which was detected in ticks and considered to be of low pathogenicity [32]. It is important to study the pathogenicity of these rickettsiae for accurate diagnosis. *A. testudinarium* is also a possible vector of rickettsia in this area, although further studies are needed because only a limited number of ticks were tested in this study.

The ticks infesting wild boars were also positive for rickettsia. Unfortunately, only one blood sample was available from the wild boars, and rickettsial DNA was not detected in its blood sample (data not shown). The proportion of ticks obtained from wild boars that harbored rickettsial DNA (5/21, 23.8%) was less than that of ticks sampled in the plants (138/180, 76.7%).

Although there are no reports of Japanese spotted fever in humans in Kyoto City, many ticks that can transmit Japanese spotted fever pathogens were distributed across the whole the city. Therefore, we must stay alert and pay attention to tick-borne diseases to ensure the safety of the citizens of the city as well as that of visitors.

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