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Isolation and phylogenetic analysis of *Bartonella* species from wild carnivores of the suborder Caniformia in Japan

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ABSTRACT

The prevalence of *Bartonella* species was investigated among wild carnivores of the suborder Caniformia, including 15 Japanese badgers (*Meles anakuma*), 8 Japanese martens (*Martes melampus*), 2 Japanese weasels (*Mustela itatsi*), 1 Siberian weasel (*Mustela sibirica*), 171 raccoon dogs (*Nyctereutes procyonoides*), and 977 raccoons (*Procyon lotor*) in Japan. *Bartonella* bacteria were isolated from one Japanese badger (6.7%) and from one Japanese marten (12.5%); however, no *Bartonella* species was found in other representatives of Caniformia. Phylogenetic analysis was based on concatenated sequences of six housekeeping genes (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) and sequence of the 16S–23S internal transcribed spacer region. The sequence analysis indicated that the isolate derived from the Japanese badger (strain JB-15) can represent a novel *Bartonella* species and the isolate from the Japanese marten (strain JM-1) was closely related to *Bartonella washoensis*. This is the first report on isolation of *Bartonella* from badger and marten.

1. Introduction

Bartonella species are gram-negative bacteria that infect erythrocytes of various mammals (Chomel et al., 2009). Of the 24 species and three subspecies of *Bartonella* currently identified, at least twelve species are known to be zoonotic agents (Chomel et al., 2006; Raoult et al., 2006).

The order Carnivora consists of two suborders, namely Caniformia and Feliformia. In Japan, the Japanese badger (*Meles anakuma*), Japanese marten (*Martes melampus*), Japanese weasel (*Mustela itatsi*), raccoon dog (*Nyctereutes procyonoides*), and Siberian weasel (*Mustela sibirica*) are known to be native species of the suborder Caniformia. In contrast, the raccoon (*Procyon lotor*) was imported to Japan from North and Central Americas as a pet, but later the feral populations have been notably spread throughout Japan (Asano et al., 2003).

In wild carnivores in the USA, the high prevalence of *B. rochalimae* which is known to cause fever, rash, and splenomegaly in humans (Eremeeva et al., 2007) have been documented in 43% of gray foxes (*Urocyon cinereoargenteus*) and 26% of raccoons, respectively (Henn et al., 2007, 2009). The high prevalence of *B. vinsonii* subsp. *berkhoffii*, which causes endocarditis in humans was also reported in 28% of coyotes (*Canis latrans*) captured in the USA (Chang et al., 2000).

Bartonella washoensis is a causative agent of myocarditis and meningitis in humans (Kosoy et al., 2003; Probert

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et al., 2009) and the natural reservoir is considered to be the ground squirrel (*Spermophilus beecheyi*) in North America (Kosoy et al., 2003). The organism was also isolated from endocarditis in a dog in California (Chomel et al., 2003), but not from any wild carnivores in other countries.

The present study was conducted to investigate the prevalence of *Bartonella* bacteria in wild carnivores in Japan and to characterize the isolates by molecular techniques.

2. Material and methods

2.1. Sample collection

During the four-year period 2008–2011, blood samples were collected from 15 Japanese badgers, 8 Japanese martens, 3 Japanese weasels, 1 Siberian weasel, and 171 raccoon dogs in Wakayama Prefecture located in the western part of Japan. Additionally, blood samples were also collected from feral raccoons in Hokkaido ($n = 95$), Chiba ($n = 186$), and Wakayama ($n = 696$) Prefectures. All blood samples of the animals, except the raccoons, were collected from hunted, road-killed, or debilitated individuals in the field. Raccoons were captured by live cage traps and then euthanized following the guidelines for invasive alien species prepared by the Japanese Veterinary Medical Association. The blood samples were collected in EDTA-containing 2 ml blood collection tubes and then sent to the Laboratory of Veterinary Public Health at the Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University. Blood samples were stored at −70 °C until examined.

2.2. Isolation of Bartonella bacteria

Frozen blood samples were thawed at room temperature and 200 μl blood aliquots were placed in sterile 1.5 ml conical tubes. The tubes were centrifuged at 1800 × *g* for 70 min; the supernatant was removed from each tube, and the sediment was mixed with 100 μl of medium 199 supplemented with 1 mM sodium pyruvate solution and 20% volume of fetal bovine serum (Life Technologies, Carlsbad, USA), and 100 μl aliquots of the mixture were plated on heart infusion agar plates (Difco, Sparks Glencoe, MI, USA) containing 5% rabbit blood (Maruyama et al., 2000). The inoculated plates were incubated at 35 °C in a moist atmosphere under 5% CO_2 for up to 4 weeks. Bacterial colonies were tentatively identified as *Bartonella* species based on colony morphology (small, gray or cream-yellow, round colonies), Gram negative staining, and the long culture period (>1 week). Five colonies were picked from each sample and each colony was subcultured using the same conditions as the primary culture.

2.3. PCR amplification and DNA sequence analysis of six housekeeping genes and ITS

The genomic DNA was extracted from each isolate by using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) and submitted for identification as *Bartonella* by genus-specific PCR targeting the *gltA* gene (Inoue et al., 2008). The organisms identified as *Bartonella* were subjected to DNA sequencing of other five housekeeping genes (16S rRNA, *ftsZ*, *groEL*, *ribC*, and *rpoB*), as described previously (Inoue et al., 2011). Eight strains of *B. washoensis* (Sb944nv, AM2-1, AR2-2, CJ22-1, DR1-1, ER14-3, RJ21-1, and SR22-1) derived from squirrels were added to the phylogenetic analysis based on concatenated sequences of six housekeeping genes because of the considerable genetic variation between these strains (Inoue et al., 2011). We also applied the analysis of the 16S–23S internal transcribed spacer region (ITS) for further genetic characterization of the isolates (Houpikian and Raoult, 2001). The PCR products were purified by using the Spin Column PCR product purification kit (Bio Basic, Markham, Ontario, Canada), and then sequenced directly using the Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA). A band of expected size of ITS was obtained from gel and purified by using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sub-cloned with the plasmid pGEM-T Easy vector system (Promega, Madison, WI, USA). The sequence reactions were performed by using the BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed by the same procedure as for six housekeeping genes and ITS. The sequence alignments obtained were compared with genomic sequences of prokaryotes registered in the GenBank/EMBL/DDBJ database using the BLAST program to confirm *Bartonella* species.

2.4. Phylogenetic analysis

The DNA sequences of the isolates were imported into the Lasergene sequence analysis software (DNASTAR, Madison, WI, USA) to obtain consensus sequences, and then aligned with those of type strains of known *Bartonella* species by using the CLUSTAL W program (Thompson et al., 1994). Neighbor-joining trees were constructed based on analysis of concatenated sequences for six genes (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*) with the Jukes–Cantor parameters method and the sequence of ITS with Kimura's 2-parameter distance method in MEGA 4.0.2 (Jukes and Cantor, 1969; Kimura, 1980; Tamura et al., 2007). Support for nodes in the trees was assessed by bootstrapping with 1000 replicates.

The sequence homologies of *gltA* and ITS between the isolates and the closest species were calculated by using GENETYX-win software, version 9 (Genetyx Corp., Tokyo, Japan).

Table 1
GenBank accession numbers for six genes and one internal spacer region of *Bartonella* isolates from Japanese badger (JB-15) and marten (JM-1).

Registered genes or region	Accession numbers for	
	JB-15	JM-1
16S rRNA	AB673447	AB611850
ftsZ	AB674230	AB611851
gltA	AB674231	AB611852
groEL	AB674232	AB611853
ribC	AB674233	AB611854
rpoB	AB674234	AB611855
ITS	AB674235	AB674236

3. Results

Bartonella bacteria were isolated from blood of one of the 15 Japanese badgers (6.7%) and one of the eight martens (12.5%). No isolates were obtained from tested Japanese weasels, raccoon dogs, Siberian weasel, or raccoons.

Strains JB-15 and JM-1, from the badger and the marten, respectively, were used as representatives for further investigation because five subcultures derived from both

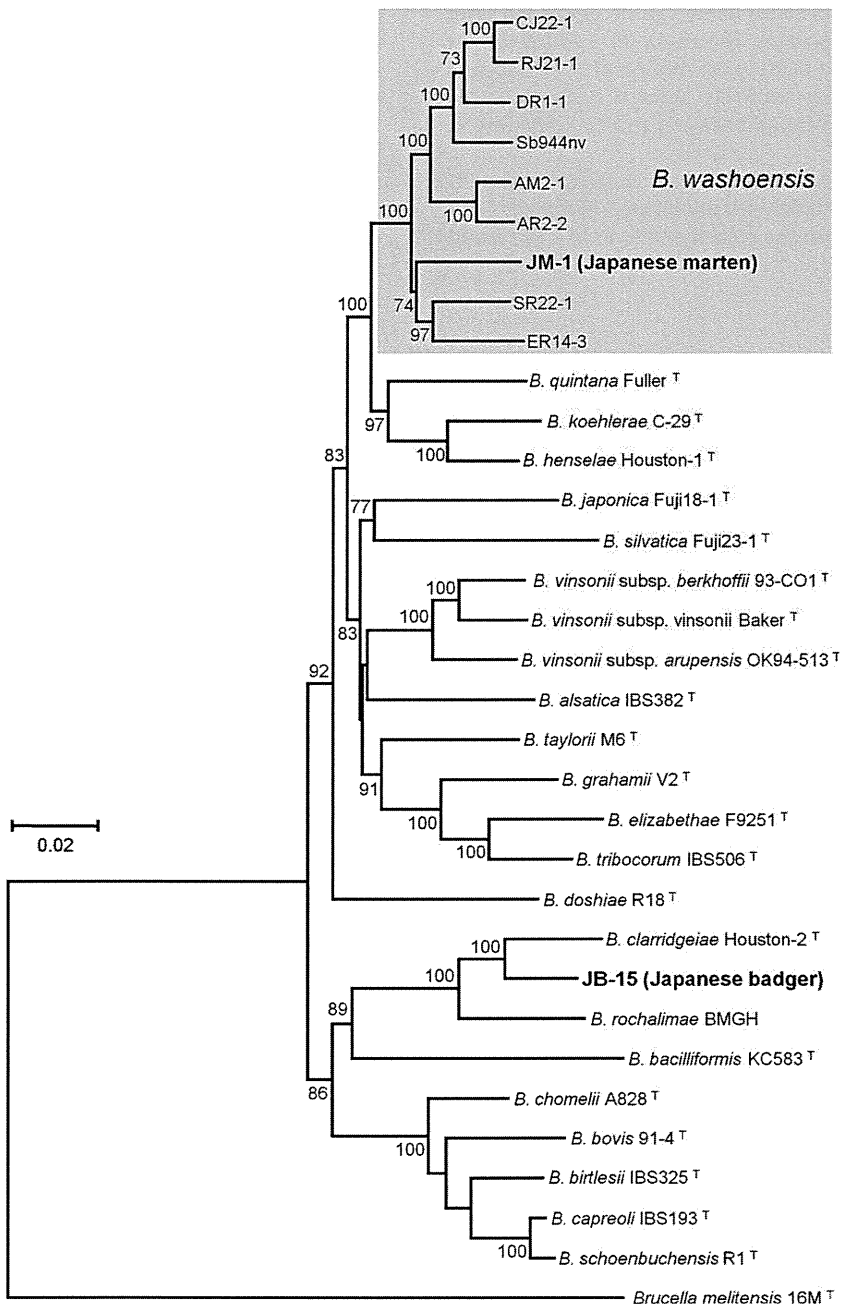

Fig. 1. Phylogenetic tree of *Bartonella* isolates (JB-15 and JM-1) and type strains of known *Bartonella* based on the concatenated sequences of six housekeeping genes (16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC*, and *rpoB*). The tree was constructed by using the neighbor-joining method with the Jukes–Cantor parameters model. Strain JB-15, JM-1, *B. rochalimae* (strain BMGH), *B. washoensis* (strains Sb944nv, AM2-1, AR2-2, CJ22-1, DR1-1, ER14-3, RJ21-1, and SR22-1) and the type strains of 21 *Bartonella* species including 3 subspecies, namely, *B. alsatica* IBS382^T, *B. bacilliformis* KC583^T, *B. bovis* 91-4^T, *B. birtlesii* IBS325^T, *B. capreoli* IBS193^T, *B. chomelii* A828^T, *B. clarridgeiae* Houston-2^T, *B. doshiae* R18^T, *B. elizabethae* F9251^T, *B. grahamii* V2^T, *B. henselae* Houston-1^T, *B. japonica* Fuji 18-1^T, *B. koehlerae* C-29^T, *B. quintana* Fuller^T, *B. schoenbuchensis* R1^T, *B. silvatica* Fuji 23-1^T, *B. taylorii* M6^T, *B. tribocorum* IBS506^T, *B. vinsonii* subsp. *arupensis* OK94-513^T, *B. vinsonii* subsp. *berkhoffii* 93-CO1^T and *B. vinsonii* subsp. *vinsonii* Baker^T were included in the phylogenetic analysis. The tree was rooted by use of *Brucella melitensis* strain 16M ^{T} as an out-group. Bootstrap values (percentages of 1000 replicates) with over 70% confidence are indicated at the tree nodes. The bar indicates 0.02 estimated nucleotide substitutions per site.

animals were genetically identical for each of the six genes. All the DNA sequences were submitted to the GenBank and accession numbers are provided in the Table 1.

A phylogenetic analysis based on the concatenated sequences of six genes suggested that the strain JB-15 isolated from the badger is distinct from all known *Bartonella* species and more likely can be described later as a novel *Bartonella* species. The strain JM-1 isolated from the marten formed a clade with strains of *B. washoensis* isolated from diverse squirrels though a considerable genetic variation was found between strains belonging to this cluster (Fig. 1).

In the phylogenetic tree based on ITS analysis, strain JB-15 formed a unique clade with uncultured *Bartonella* DNA clone T8 detected from the spleen and liver of a European badger (Fig. 2). BLAST searching has also indicated that the sequence of *gltA* from strain JB-15 was identical to that from the DNA clone 01011510020 from a European badger (Gerrikagoitia et al., 2011). The ITS sequence of strain JB-15 showed 96.7% similarity with uncultured *Bartonella* DNA clone T8 (Table 2).

Strain JM-1 formed a clade with *B. washoensis* strains isolated from squirrels based on ITS analysis (Fig. 3). The *gltA* sequence similarity between strain JM-1 and reported strains of *B. washoensis* ranged from 93.2% to 97.7% (Table 3).

4. Discussion

This is the first isolation of *Bartonella* bacteria from the badger and marten, and first detection from Asia. The

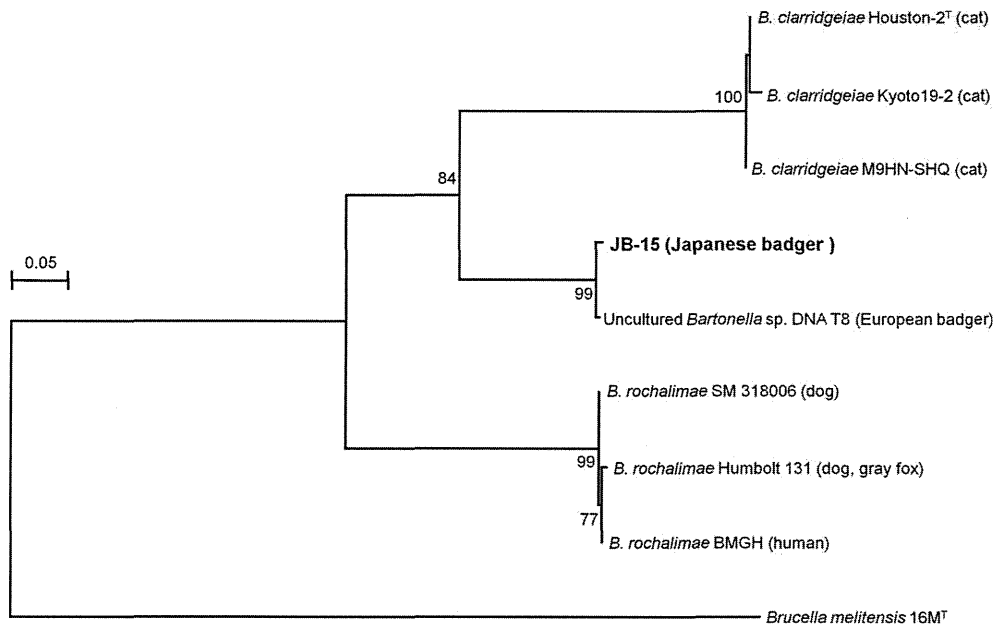

Fig. 2. Phylogenetic tree of *Bartonella* isolate (JB-15) and its related *Bartonella* strains based on the DNA sequence of ITS. The tree was constructed by using the neighbor-joining method with Kimura's 2-parameters model. Strain JB-15, *B. clarridgeiae* strains and *B. rochalimae* strains are included in the tree. Host animals of the *Bartonella* strains are shown in parentheses. The tree was rooted by using *Brucella melitensis* strain 16M ^{T} as an out-group. Bootstrap values (percentages of 1000 replicates) with over 70% confidence are indicated at the tree nodes. The bar indicates 0.05 estimated nucleotide substitutions per site.

Table 2
Sequence similarities between strain JB-15 and some closest strains of *Bartonella* in the *gltA* and ITS.

Strain	Host	% of sequence similarities to JB-15/GenBank accession number	
		gltA (312 bp)	ITS (437 bp)
Uncultured *Bartonella* sp.			
01011510020	European badger	100/GU570947	NA
T8	European badger	NA	96.7/EU098132
B. clarridgeiae			
Houston-2	Cat	96.5/U84386	65.6/AF312497
Kyoto19-2	Cat	96.5/AB674237	65.2/AB674239
M9HN-SHQ	Cat	96.3/EU770616	68.6/EU589237
B. rochalimae			
BMGH	Human	96.5/DQ683195	60.7/DQ683199
Humboldt 131	Dog, Gray fox	96.3*/DQ676484	61.3/DQ676487
SM 318006	Dog	95.9*/DQ676488	60.5/DQ676491

NA, the sequence was not available in GenBank.
* The sequence length compared was 270 bp.

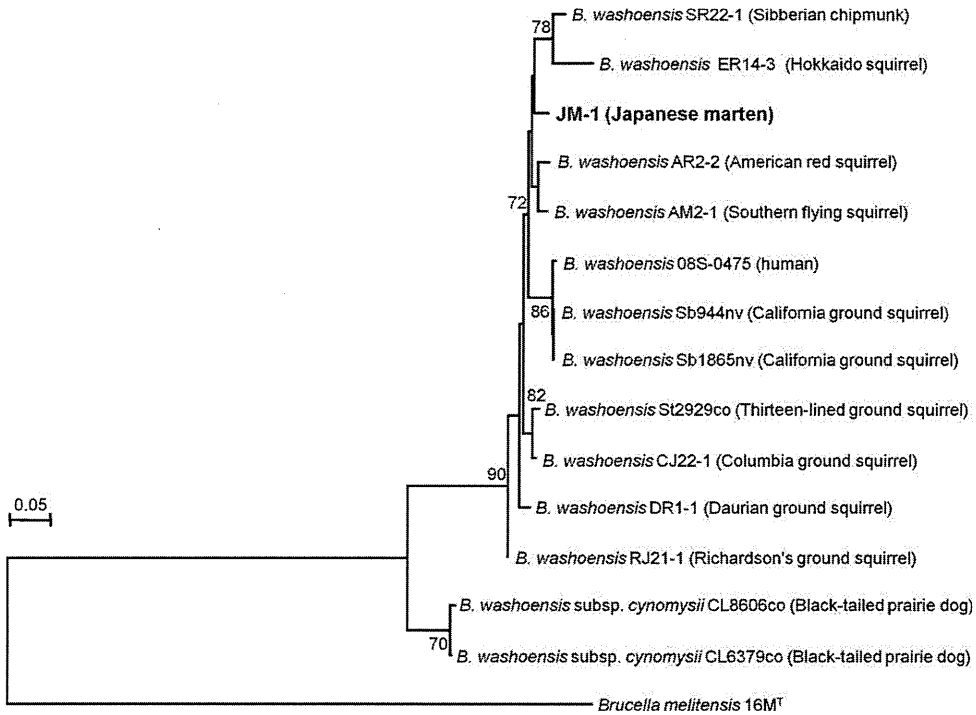

Fig. 3. Phylogenetic tree of *Bartonella* isolate (JM-1) and its related *Bartonella* strains based on the DNA sequence of ITS. The tree based on the DNA sequence of ITS was constructed by using neighbor-joining method with Kimura's 2-parameters model. Strain JM-1 and *B. washoensis* strains are included in the tree. Host animals of the *Bartonella* strains are shown in parentheses. The tree was rooted by use of *Brucella melitensis* strain 16M ^{T} as an out-group. Bootstrap values (percentages of 1000 replicates) with over 70% confidence are indicated at the tree nodes. The bar indicates 0.05 estimated nucleotide substitutions per site.

Table 3
Sequence similarities between strain JM-1 and some closest strains of *Bartonella* in the *gltA* and ITS.

Strain	Host	% of sequence similarities to JM-1/GenBank accession number	
		gltA (312 bp)	ITS (449 bp)
B. washoensis			
AR2-2	American red squirrel	95.5/AB519076	89.0/AB674246
AM2-1	Southern flying squirrel	96.8/AB444972	90.7/AB674247
CJ22-1	Columbia ground squirrel	97.4/AB444956	93.4/AB674248
DR1-1	Daurian ground squirrel	97.1/AB444962	91.5/AB674249
ER14-3	Hokkaido squirrel	93.2/AB444974	86.9/AB674250
RJ21-1	Richardson's ground squirrel	96.5/AB444959	92.6/AB674251
SR22-1	Sibberian chipmunk	97.4/AB444968	93.6/AB674252
Sb944nv	California ground squirrel	97.1/AF470616	88.2/AB674253
Sb1865nv	California ground squirrel	95.8/AB674240	88.2/AB674254
St2929co	Thirteen-lined ground squirrel	97.7*/AB674241	91.5/AB674255
08S-0875	Human	97.1/FJ719016	87.1/AB674256
B. washoensis subsp. *cynomysii*			
CL8606co	Black-tailed prairie dog	96.7/DQ897367	88.4†/AB674257
CL6379co	Black-tailed prairie dog	96.5/AY589564	95.2†/AB674258

* The sequence length compared is 306 bp.
† The sequence length compared is 293 bp.

phylogenetic analysis based on the concatenated sequences of six housekeeping genes showed that the strain JB-15 from the Japanese badger appears to be different from other known *Bartonella* species. On the other hand, strain JM-1 from the Japanese marten is relatively close to *B. washoensis*. In our previous study, the considerable genetic variation has been found among the isolates of squirrels (Inoue et al., 2011). In the present study, strain JM-1 formed a large clade with *B. washoensis* strains, suggesting that the strain may belong to *B. washoensis*.

Phylogenetic analysis of ITS is recognized to be an useful tool to identify *Bartonella* species (Houpikian and Raoult, 2001). Therefore, in addition to six housekeeping

genes, we utilized the ITS region for further genetic characterization of the *Bartonella* species isolated from wild Caniformia in Japan. Strain JB-15 was distinct from other known *Bartonella* species according to the phylogenetic analysis of ITS. It was also found that the sequence of ITS from strain JB-15 was closest (96.7%) to that of uncultured *Bartonella* DNA from a European badger (*Meles meles*) captured in northern Spain (Gerrikagoitia et al., 2011). The sequence of *gltA* from strain JB-15 was identical to the DNA detected in the European badger. The Japanese badger is taxonomically related to the European badger (Kurose et al., 2001). Interestingly, badgers in both Asia and Europe were infected with similar *Bartonella* species. More samples are needed to investigate whether badgers in other countries harbor similar *Bartonella* species.

The analysis based on *gltA* supported the similarity between strain JM-1 and previously reported strains of *B. washoensis* (AM2-1, CJ22-1, DR1-1, RJ21-1, SR22-1, Sb944nv, St2929co, 08S-0875, CL8606co, and CL6379co); the sequence identity was above the cut-off value (>96.0%) accepted for differentiation of *Bartonella* species (La Scola et al., 2003). In addition, the sequence of ITS from strain JM-1 also demonstrated the closeness to *B. washoensis* strains obtained from squirrels and the strain formed a clade with *B. washoensis* strains in the phylogenetic analysis of ITS. Generally, *Bartonella* species are recognized as host-specific parasitic bacteria; however, evidence that the *Bartonella* species could occasionally jump from the natural reservoir to other mammals has been documented (Bai et al., 2007). Since only one of the eight martens was found to be bacteremic in the present study, more samples are necessary to show the possibility that martens are the natural reservoir or accidental host of *B. washoensis*.

Although raccoons in the USA frequently harbor *B. rochalimae* (11/42; 26.2%), no *Bartonella* was isolated from feral raccoons in Japan despite testing 977 samples. It has been reported that *Pulex* fleas carrying DNA of *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae* infest wild carnivores, suggesting that the fleas could be a potential vector for transmission of *Bartonella* agents among wild carnivores in the USA (Gabriel et al., 2009). We have no information about infestation of Japanese carnivores with *Pulex*. Another possible explanation for the absence of *Bartonella* in raccoons can be a historical situation when non-bacteremic animals were originally introduced to Japan.

No *Bartonella* was isolated from Japanese weasels or Siberian weasel, but the sample size was small for making any conclusion. Although *Bartonella* were not isolated from raccoon dogs, *Bartonella* DNA was detected in 11 of 171 animals (6.4%) by PCR amplification of *gltA* and *rpoB* genes and all of the amplicons were found to be closest to *B. rochalimae* by the sequence analysis of both genes (data not shown). It is possible that culturing of *Bartonella* from raccoon dogs require different culturing conditions. For example, we know that temperature at 28 °C without CO_2 is recommended for culturing *B. bacilliformis* (Ellis et al., 1999).

More than 70% of emerging infections have been thought to come from wildlife (Kuiken et al., 2005). Increased outdoor activities might result in higher risk of exposure to various types of zoonoses (Boulouis et al., 2005; Breitschwerdt et al., 2007). Previous studies have shown that wild carnivores may play an important role as potential reservoirs for zoonotic *Bartonella* (Breitschwerdt et al., 2005; Henn et al., 2007). Comprehensive surveillance should be done to determine a prevalence and distribution of *Bartonella* species in wildlife in Japan and to clarify their role as potential human pathogens.

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Prevalence and genetic diversity of *Bartonella* species in sika deer (*Cervus nippon*) in Japan

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ABSTRACT

We report the first description of *Bartonella* prevalence and genetic diversity in 64 Honshu sika deer (*Cervus nippon centralis*) and 18 Yezo sika deer (*Cervus nippon yesoensis*) in Japan. Overall, *Bartonella* bacteremia prevalence was 41.5% (34/82). The prevalence in wild deer parasitized with ticks and deer keds was 61.8% (34/55), whereas no isolates were detected in captive deer (0/27) free of ectoparasites. The isolates belonged to 11 genogroups based on a combination of the *gltA* and *rpoB* gene sequences. Phylogenetic analysis of concatenated sequences of the *ftsZ*, *gltA*, *ribC*, and *rpoB* genes of 11 representative isolates showed that Japanese sika deer harbor three *Bartonella* species, including *B. capreoli* and two novel *Bartonella* species. All Yezo deer's isolates were identical to *B. capreoli* B28980 strain isolated from an elk in the USA, based on the sequences of the *ftsZ*, *gltA*, and *rpoB* genes. In contrast, the isolates from Honshu deer showed a higher genetic diversity.

1. Introduction

Bartonella species are Gram-negative bacteria that infect the erythrocytes of various mammals, and are putatively transmitted by blood-feeding arthropods [1–3]. Various mammalian species, such as canines, felines, rodents, and ruminants are the reservoir hosts of *Bartonella*, and 11 of the 26 *Bartonella* species or subspecies are recognized to be zoonotic agents [4–6].

Several epidemiological studies of *Bartonella* in domestic and wild ruminants reported that roe deer (*Capreolus capreolus*) in France [7] and elk (*Cervus elaphus*) in Wyoming, USA [8], and roe deer in Germany [9] and cows in France [10] harbor *B. capreoli* and *B. schoenbuchensis*, respectively. Mule deer (*Odocoileus hemionus*) and elk in the USA have also been found to carry *Bartonella bovis* [11], which was first isolated from a cow in France [7]. Domestic cattle in France [12] and New Caledonia [13] have been shown to harbor *Bartonella chomelii*, which is thought to have been brought with cattle from France to New Caledonia. Recently, sheep were identified as natural hosts of candidatus *Bartonella melophagi* in the USA [14], which is a species pathogenic for humans [15]. *B. bovis* was reported

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to cause endocarditis in cattle [16], while the pathogenicity of other ruminant-associated *Bartonella* species except candidatus *B. melophagi* for humans and animals is still unknown.

B. schoenbuchensis was isolated from European deer keds (*Lipoptena cervi*) in Germany [3]. In addition, the DNA of *B. chomelii* and *B. schoenbuchensis* was detected from European deer keds in France [17], and the latter was also found in both European [18] and neotropical deer keds (*Lipoptena mazamae*) in the USA [19]. In contrast, viable *Bartonella* species were not found in ticks collected from ruminants though the DNA of *B. capreoli* and *B. schoenbuchensis* was detected from sheep tick (*Ixodes ricinus*) in Poland [20] and deer tick (*Ixodes scapularis*) in the USA [18], respectively. Since several *Bartonella* species were isolated from deer keds collected from wild deer, these ectoparasites have been thought to be a possible vector in deer. On the other hand, ticks are less likely to be another vector.

Recently, the population of Japanese sika deer (*Cervus nippon*) has been significantly expanding throughout Japan [21], and animals are now sharing human environment. Furthermore, wild deer are recognized to be infectious sources of zoonoses, such as hepatitis E, salmonellosis, and brucellosis [22,23]. Therefore, it is important to investigate the prevalence of *Bartonella* species in deer and determine which species are present from the standpoint of public health.

The aim of the present study was to investigate the prevalence of *Bartonella* species among wild and captive sika deer in Japan and to characterize the isolates by molecular biological techniques.

2. Materials and methods

2.1. Sample collection

During November 2008 to October 2010, blood samples (2–7 ml) were collected from 64 Honshu deer (*C. nippon centralis*) and 18 Yezo deer (*C. nippon yesoensis*) in Japan. Thirty-seven Honshu deer and 18 Yezo deer were free-ranging wild deer, and were hunted in Wakayama, Nara, and Hokkaido Prefectures. A total of 27 Honshu deer were captive and raised in rearing facilities in Aichi and Miyagi Prefectures. Most of the free-ranging wild deer were infested with deer keds (*Lipoptena fortisetosa*) and/or *Haemaphysalis* and *Ixodes* ticks, while no ectoparasites were detected on the captive deer, as most of these deer ($n = 23$) had been treated with ivermectin and four of them were raised as breeding animals.

The blood samples of wild deer were immediately collected from the heart of carcass and those of captive deer were collected from cervical vein of anesthetized individuals. All blood samples were transferred into EDTA-containing collection tubes and sent to the Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University. These samples were stored at −70 °C until examined.

2.2. Isolation of bacterial strains

Frozen blood samples were thawed at room temperature, and 200 μl blood aliquots were separated into 1.5 ml conical tubes. The tubes were centrifuged at 1800 × *g* for 70 min. After centrifugation, the supernatants were removed from the tubes, and each sediment was mixed with 100 μl of medium 199 supplemented with sodium pyruvate solution and fetal bovine serum (Life Technologies, Carlsbad, CA, USA). Aliquots (100 μl) of the mixture were plated on heart infusion agar plates (Difco, Sparks Glencoe, MI, USA) containing 5% rabbit blood [7,12,24]. The inoculated plates were incubated at 35 °C in a moist atmosphere under 5% CO_2 for up to 4 weeks. Bacterial colonies were tentatively identified as *Bartonella* based on colony morphology (small, gray or cream-yellow, round colonies), and subsequently 5 colonies were picked up from each sample, and sub-cultured on a fresh blood agar plate using the same conditions as the primary culture.

2.3. PCR amplification and DNA sequencing

Genomic DNA was extracted from whole bacterial cells by using a commercial kit, Instagene matrix (Bio-Rad, Hercules, CA, USA) and subjected to genus-specific PCR targeting the citrate synthase gene (*gltA*) and RNA polymerase beta-subunit-encoding gene (*rpoB*) to identify *Bartonella*. The PCR was performed with 20 μl mixtures containing 20 ng of DNA, 200 μM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 1 pmol of each primer. Positive and negative controls were prepared by extracting DNA from *Bartonella doshiae* R-18^T and double-distilled water, respectively. Primers used for the amplification of *gltA* [25] and *rpoB* [26] have been previously described. The products were analyzed by electrophoresis on 2% agarose gels, and the target bands were detected by staining gels with ethidium bromide and viewing under UV light.

The PCR products were purified by using the Spin Column PCR product purification kit (Bio Basic, Ontario, Canada), and then sequenced directly by using the BigDye Terminator Cycle Sequencing Ready Reaction kit and a Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer's instructions. The sequence alignments obtained in this study were compared with those of other known *Bartonella* species deposited in the GenBank/EMBL/DDBJ database by using the BLAST program. The Clustal W program within GENETYX-win software, version 9 (Genetyx Corp., Tokyo, Japan) was used to compare homologous *gltA* and *rpoB* sequences to identify genetic variants. The combination of the *gltA* and *rpoB* gene sequences gave a total of 11 genogroups, and a representative isolate from each of the 11 genogroups was used for additional PCR and sequence analysis of the cell division protein gene (*ftsZ*) and the riboflavin synthase gene (*ribC*). Primers used for the amplification of *ftsZ* [27] and *ribC* [28] have been previously described. The sequences of *ftsZ*, *gltA*, *ribC*, and *rpoB* from the representative isolates have been submitted to GenBank/EMBL/DDBJ and assigned accession numbers.

Table 1
Prevalence of *Bartonella* bacteria in Japanese sika deer and the state of ectoparasite infestation.

Habitat of deer	Prefecture	Subspecies of deer	Ectoparasite infestation[a]	No. of deer examined	No. of positive deer (%)
Wild	Hokkaido	Yezo deer	+	18	9(50.0)
	Nara	Honshu deer	+	16	12(75.0)
	Wakayama	Honshu deer	+	21	13(61.9)
		Subtotal		55	34(61.8)[†]
Captive	Aichi	Honshu deer	–	23	0
	Miyagi	Honshu deer	–	4	0
		Subtotal		27	0
		Total		82	34(41.5)[†]

[a] Ectoparasites consisted of deer keds and/or *Haemaphysalis* and *Ixodes* ticks.
[†] $p < 0.001$, versus prevalence of the captive deer.

2.4. Construction of a phylogenetic tree based on *ftsZ*, *gltA*, *ribC*, and *rpoB*

A phylogenetic tree was constructed based on the concatenated sequences of *ftsZ*, *gltA*, *ribC*, and *rpoB* by using the neighbor-joining method with the Jukes–Cantor parameter method within MEGA 4.0.2 software [29,30]. Eleven representative isolates from Japanese sika deer examined, the ruminant-associated *Bartonella* species (*B. bovis* 91-4^T, *B. capreoli* IBS193^T, *B. capreoli* B28980, *B. chomelii* A828^T, *B. schoenbuchensis* R1^T, and candidatus *B. melophagi* K-2C), and other known *Bartonella* species were included in the analysis. Bootstrap analysis was performed with 1000 trials of bootstrap data (bootstrap values lower than 70% are not shown in the tree).

2.5. Statistical analysis

Statistical analysis was performed using the statistical package StatMate III for Windows, Release 2005 (ATMS, Corp., Tokyo, Japan). Chi-square tests were used to examine the statistical significance; $p < 0.05$ was considered as significant.

3. Results

3.1. Prevalence of Bartonella bacteremia

Various numbers of colonies, ranging from 20 to more than 500, appeared on the agar plates when *Bartonella* bacteria were detected. The overall prevalence of *Bartonella* bacteremia in Japanese sika deer was 41.5% (34/82). *Bartonella* bacteria were isolated from 61.8% (34/55) of the wild deer, whereas no isolates were obtained from the captive deer (0/27). Many ectoparasites such as deer keds and/or ticks were detected throughout the body of wild deer only and not in captive deer. *Bartonella* prevalence in wild deer was 50.0% (9/18) in Hokkaido, 75.0% (12/16) in Nara, and 61.9% (13/21) in Wakayama Prefectures; no significant difference was observed among the three prefectures (Table 1).

3.2. Phylogenetic analysis and genetic diversity of Bartonella isolates

Based on a phylogenetic analysis of the concatenated sequences of *ftsZ*, *gltA*, *ribC*, and *rpoB* genes, the 11 representative isolates formed three clusters designated lineages A, B, and C. Strains Honshu-8.1, Honshu-18.2, and Yezo-25.1 were grouped in lineage A with *B. capreoli* IBS193^T isolated from a roe deer in France and *B. capreoli* B28980 isolated from an elk in the USA. The strains in lineages B and C were distinct from other known *Bartonella* species (Fig. 1).

The sequences of *ftsZ*, *gltA* and *rpoB* genes from strain Yezo-25.1 were identical to those of *B. capreoli* B28980. Strains Honshu-8.1 and Honshu-18.2 in lineage A showed the highest similarities with *B. capreoli* IBS193^T or B28980 for *gltA* (99.4%) and *rpoB* (98.4–98.9%) genes. In contrast, the sequence similarities for *gltA* and *rpoB* between the strains in lineage B (Honshu-9.1, Honshu-9.3, Honshu-11.1, Honshu-12.1, Honshu-18.5, Honshu-53.5, and Honshu-58.5) and the ruminant-associated *Bartonella* species ranged from 93.6 to 94.6% and 96.5 to 97.6%, respectively. Comparing the sequences of *gltA* and *rpoB* between Honshu-16.1 and the ruminant-associated *Bartonella* species, the similarities were 93.6% and 95.3%, respectively (Table 2).

The sequence similarities for *gltA* and *rpoB* genes among seven strains in lineage B ranged from 98.1 to 100% and 97.1 to 100%, respectively. Strain Honshu-16.1 in lineage C showed the lowest similarities with the strains in lineage B for both *gltA* (93.9–94.6%) and *rpoB* (94.5–95.0%) genes (Table 3).

Honshu deer harbored three *Bartonella* species designated as lineages A (*B. capreoli*), B, and C. Lineage B was dominant (20/37; 54.1%) in Honshu deer. In addition, 8 (21.6%) of the Honshu deer were co-infected with *B. capreoli* and the strains belonging to lineage B. In contrast, Yezo deer harbored only strains belonging to lineage A (Table 4).

4. Discussion

The present study provides the first evidence of *Bartonella* species infecting 61.8% (34/55) of wild Japanese

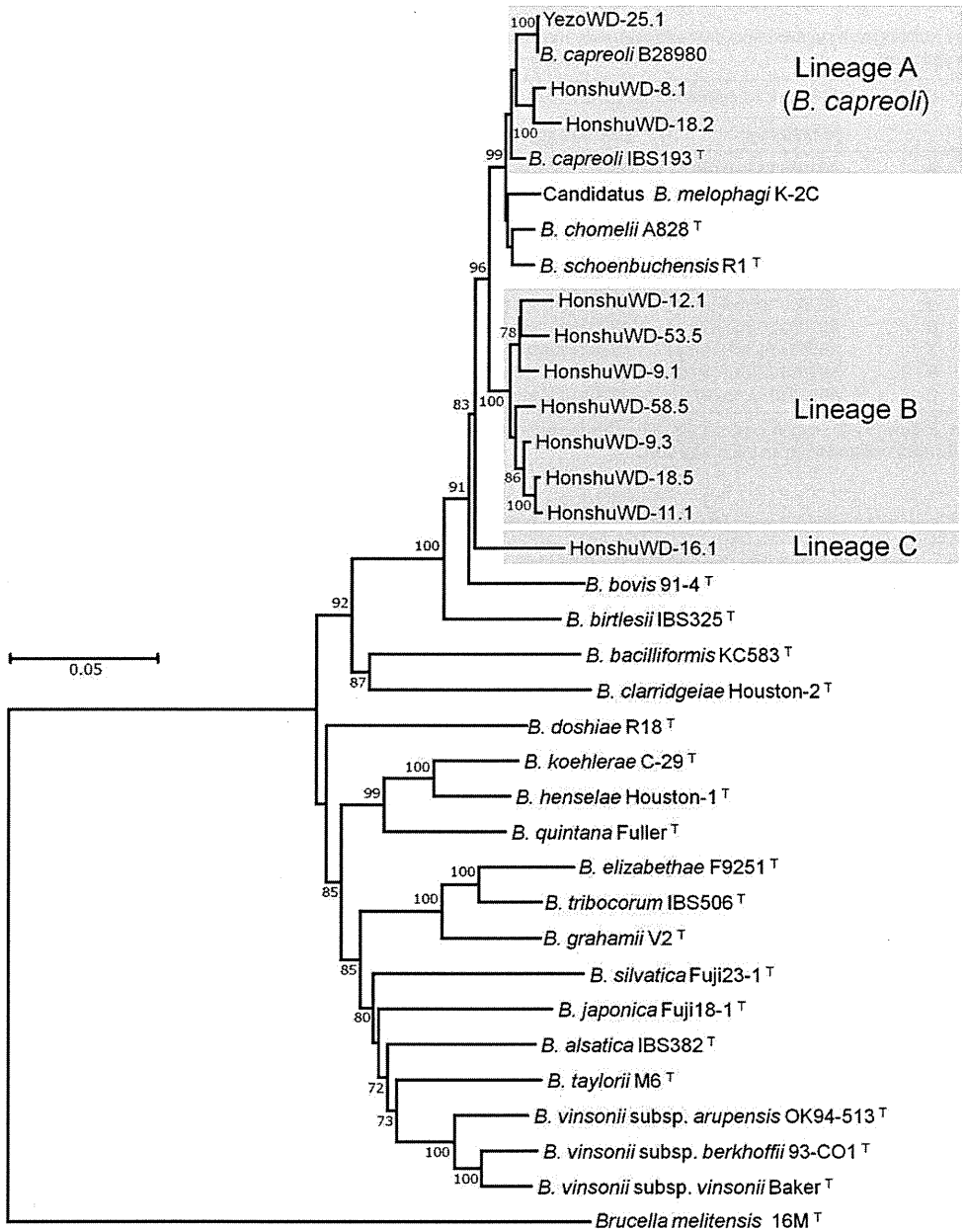

Fig. 1. Phylogenetic tree of *Bartonella* isolates from Japanese sika deer and other known *Bartonella* species based on the concatenated sequences of 4 housekeeping genes (*ftsZ*, *gltA*, *ribC*, and *rpoB*). The tree was constructed by using neighbor-joining method with Jukes–Cantor parameter model. Eleven representative *Bartonella* isolates (Honshu-8.1, -9.1, -9.3, -11.1, -12.1, -16.1, -18.2, -18.5, -53.5, -58.5, and Yezo-25.1) from Japanese sika deer, the ruminant-associated *Bartonella* species (*B. bovis* 91-4^T, *B. capreoli* IBS193^T, *B. capreoli* B28980, *B. chomelii* A828^T, *B. schoenbuchensis* R1^T, and candidatus *B. melophagi* K-2C), and other known *Bartonella* species were included in the tree. The tree is rooted by use of *Brucella melitensis* strain 16M^T as an out-group. Bootstrap values (percentages of 1000 replicates) with over 70% confidence are indicated at the tree nodes. The bar indicates 0.05 estimated nucleotide substitutions per site.

sika deer. Similarly, high prevalence of *B. bovis* and *B. schoenbuchensis* has been reported in 90% (38/42) of mule deer in CA, USA [11] and 80% (4/5) of roe deer in Germany [9]. *Bartonella capreoli* was also isolated from roe deer in France [7]. *Bartonella* DNA has also been detected in 39% (23/59) of the roe deer and 35% (7/20) of the red deer in Poland [31]. Thus, a high prevalence of *Bartonella* infection was observed in various species of deer in other countries.

In the present study, *Bartonella* bacteria were found only in wild deer, but not in captive deer. Although a number of ectoparasites including deer keds and *Haemaphysalis* and *Ixodes* ticks were found on the wild deer, no ectoparasites were detected on the captive deer, as most of the animals had been treated with ivermectin. DNA from several *Bartonella* species has been detected in both deer keds and ticks collected from wild deer in the USA [18,19] and in European

Table 2
Genogroups of the isolates from Japanese sika deer and sequence similarities between the isolates and ruminant-associated closest *Bartonella* species based on *gltA*, *ftsZ*, *ribC*, and *rpoB*.

Strain	Lineage	GenBank accession numbers/ruminant-associated closest *Bartonella* species (sequence similarity, %)			
		ftsZ (788 bp)	*gltA* (312 bp)	*ribC* (557 bp)	*rpoB* (825 bp)
Honshu-8.1	A	AB703114/cap (99.1)	AB703124/cap (99.4)	AB703132/cho (96.8)	AB703142/B28980 (98.9)
Honshu-18.2	A	AB703115/cap (97.6)	Identical to Honshu-8.1	AB703133/B28980 (97.5)	AB703143/B28980 (98.4)
Yezo-25.1	A	Identical to B28980	Identical to B28980	AB703134/B28980 (99.8)	Identical to B28980
Honshu-9.1	B	AB703116/mel (98.6)	AB703125/cho, mel, sch (93.6)	AB703135/cho (98.6)	AB703144/sch (97.1)
Honshu-9.3	B	AB703117/mel (98.9)	AB703126/bov, cho, mel, sch (94.6)	AB703136/cho (97.8)	AB703145/sch (97.6)
Honshu-11.1	B	AB703118/mel (97.7)	AB703127/bov, cho, mel, sch (94.2)	AB703137/cho (97.5)	Identical to Honshu-9.3
Honshu-12.1	B	AB703119/mel (98.2)	AB703128/bov (94.6)	AB703138/cho (96.8)	AB703146/sch (96.5)
Honshu-18.5	B	AB703120/mel (98.1)	AB703129/bov (94.2)	AB703139/cho (97.7)	AB703147/sch (97.2)
Honshu-53.5	B	AB703121/mel (98.4)	AB703130/cho (94.2)	AB703140/cho (96.8)	AB703148/sch (97.1)
Honshu-58.5	B	AB703122/cap (99.0)	Identical to Honshu-9.1	Identical to Honshu-8.1	Identical to Honshu-9.3
Honshu-16.1	C	AB703123/cap (97.1)	AB703131/bov, cap (93.6)	AB703141/B28980 (94.8)	AB703149/cho (95.3)

Abbreviations: bov, *B. bovis* 91-4^T; cap, *B. capreoli* IBS193^T; cho, *B. chomelii* A828^T; mel, Candidatus *B. melophagi* K-2C; sch, *B. schoenbuchensis* R1^T; B28980, *B. capreoli* B28980 strain isolated from an elk in the USA.

Table 3
Sequence similarities of *gltA* and *rpoB* among the isolates in lineages B and C.

Isolates/lineages	Sequence similarities (%) of *gltA* on the lower left and *rpoB* on the upper right							
	Honsyu-9.1	Honsyu-9.3	Honsyu-11.1	Honsyu-12.1	Honsyu-18.5	Honsyu-53.5	Honsyu-58.5	Honsyu-16.1
Honshu-9.1/B	–	98.1	98.1	97.9	97.9	99.8	98.1	94.5
Honshu-9.3/B	99.0	–	100	97.2	99.9	98.1	100	95.0
Honshu-11.1/B	98.7	99.0	–	97.2	99.9	98.1	100	95.0
Honshu-12.1/B	99.0	99.4	99.7	–	97.1	97.6	97.2	94.7
Honshu-18.5/B	98.7	99.0	99.4	99.7	–	97.9	99.9	94.9
Honshu-53.5/B	98.7	98.4	98.1	98.4	98.1	–	98.1	94.5
Honshu-58.5/B	100	99.0	98.7	99.0	98.7	98.7	–	95.0
Honshu-16.1/C	94.6	94.2	94.6	94.9	94.6	93.9	94.6	–

countries [3,17,20]. In contrast, isolation of *Bartonella* was confirmed only in deer keds infesting wild deer in Germany [3]. Such findings strongly suggest that deer keds may be potential vectors responsible for the transmission of *Bartonella* species among sika deer. Future studies are needed to investigate *Bartonella* prevalence of deer keds collected from sika deer.

The strains in lineage A, identified as *B. capreoli*, were prevalent in both Honshu and Yezo deer, suggesting that this *Bartonella* species is common in wild deer throughout Japan. Bai et al. [8] have suggested that *B. capreoli* may be widely distributed among deer of the genus *Cervus*. Thus, *B. capreoli* may be a common species in *Cervus* deer around the world, including Japan.

La Scola et al. [32] reported that *gltA* and *rpoB* were the most appropriate genes for discriminating species of the genus *Bartonella* and proposed that gene sequence similarities <96.0% in *gltA* and <95.4% in *rpoB* between *Bartonella* species could be used as cut-off values for the designation of a novel *Bartonella* species. The strains in lineage B could be clearly differentiated by the sequence similarities (93.6–94.6%) in the *gltA* gene by the cut-off value. On the other hand, the sequence similarity (96.5–97.6%) in the *rpoB* gene of the lineage was above the cut-off value. A similar phenomenon was observed in designating novel *Bartonella* species isolated from wild rodents [33]. Although most of the strains in lineage B were relatively close to *B. melophagi* for the *ftsZ* gene and *B. chomelii* for the *ribC* gene, the discriminatory power with both of the genes have been reported to be inadequate for identification of *Bartonella* species [8,32]. However, in the phylogenetic analysis based on the concatenated sequences, the strains in lineage B formed a distinct cluster from other known *Bartonella* species, supporting the view that lineage B is a novel *Bartonella* species. Strain Honshu-16.1 in lineage C was confirmed to be a novel *Bartonella* species by the sequence similarities in both *gltA* (93.6%) and *rpoB* (95.3%) genes. Furthermore, the strain was distinct from other known *Bartonella* species based on the phylogenetic analysis of concatenated sequences. Based on the sequence similarities in the *gltA* (93.9–94.6%) and *rpoB* (94.5–95.0%) genes between the strains in lineages B and

Table 4
Prevalence of *Bartonella* lineages in wild Japanese sika deer.

Subspecies of deer	No. of deer examined	No. of positive deer (%)			
		Lineage A	Lineage B	Lineage C	Lineages A and B
Honshu deer	37	12(32.4)	20(54.1)	1(2.7)	8(21.6)
Yezo deer	18	9(50.0)	0	0	0
Total	55	21(38.2)	20(36.4)	1(1.8)	8(14.5)

C, both lineages belonged to different species. From these results, Japanese sika deer harbor three different *Bartonella* species, including *B. capreoli* and two novel species.

Interestingly, the sequences of *ftsZ*, *gltA*, and *rpoB* genes of strain Yezo-25.1 in lineage A were identical to those from *B. capreoli* B28980, which was obtained from an elk in the USA. Japanese sika deer and elk, otherwise known as wapiti or American red deer, were estimated to have diverged into different species 0.57 million years ago [34]. Although this appears to be sufficient time for mutations to occur within the housekeeping genes of *Bartonella* species, no sequence differences were observed in the three genes examined. Since the reason for the accordance between both strains is unclear in our study, more strains derived from Yezo deer and North American elk need to be analyzed to resolve the relationships of *Bartonella* species in deer from Japan and North America.

Honshu deer harbored a higher genetic diversity of *Bartonella* species in comparison with Yezo deer. Japanese sika deer are divided into two genetically distinct groups based on the mitochondrial D-loop region sequences: the Northern and Southern groups. Both deer groups have been estimated to have migrated separately to the Japanese archipelago during the Middle to Late Pleistocene across land bridges from the Asian continent [35]. Nara and Wakayama Prefectures are located at the boundary region of both groups, and some genetic crossing has been suggested to have occurred in this region [36]. Thus, the genetic crossing between Southern and Northern groups might have contributed to the genetic diversity of *Bartonella* species in Honshu deer.

In conclusion, we confirmed that Japanese sika deer harbor *B. capreoli* and two novel *Bartonella* species, with high percentages of infections. The present study also suggested that ectoparasites such as deer ked may be the potential vector of *Bartonella* in Japanese sika deer. Further studies are necessary to explain the role of the ectoparasites in the transmission of *Bartonella* and to determine the potential threat of *Bartonella* species from deer to humans, as reported for *B. melophagi* from sheep to humans [15].

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特集

人と動物の共通感染症最前線 9

ベトナム・メコンデルタに生息するヤモリにおけるサルモネラの疫学

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要　約

東南アジアなどの熱帯や亜熱帯地方に高密度に生息するヤモリにおけるサルモネラ保有の実態を明らかにする目的で，ベトナム・メコンデルタにおいて野生のヤモリを捕獲し，その保有状況を調べた。その結果，*Salmonella* 属菌はヤモリ 646 検体中 95 検体（14.7%）から分離された。*Salmonella* 属菌の分離率は，季節，捕獲地域およびヤモリの種類により有意な差は認められなかった。また，ヤモリから分離された *Salmonella* の血清型は，本地域の人の *Salmonella* 感染患者から分離されたものと共通するものが多かった。これらのことから，ヤモリは自然界における *Salmonella* 属菌のレゼルボアであり，ベトナム・メコンデルタでは人の *Salmonella* 感染症の感染源となっている可能性が高いことが示された。

はじめに

爬虫類は *Salmonella* 属菌を比較的高率に保有していることが報告されており，ヤモリからも *Salmonella* 属菌を分離した報告がみられる。ヤモリは東南アジアなどの熱帯・亜熱帯では高い密度で人の生活環境に生息しており，人のサルモネラ症の感染源になっている可能性が考えられるが，ヤモリにおける *Salmonella* 属菌の生態について詳細に検討した報告はみられない。また，近年，東南アジアからペットとしてヤモリが多数輸入されているが，現在，我が国では，「感染症の予防及び感染症の患者に対する医療に関する法律」（感染症法）において，爬虫類は輸入届け出の対象となっておらず，「絶滅のおそれのある野生動植物の種の国際取引に関する条約」（ワシントン条約）や「特定外来生物による生態系等に係る被害の防止に関する法律」（外来生物法）に基づき，一部の爬虫類については輸入が規制されているものの，ほとんどのものは制限されることなく輸入されている。また，「動物の愛護及び管理に関する法律」（動物愛護法）で危険動物としてカミツキガメ，毒ヘビ，ワニなどの飼育が制限されているが，それ以外は特に規制がないのが現状である。しかし，近年，我が国では爬虫類に起因する人の *Salmonella* 症が散発している。このような背景のもと，本研究ではベトナム・メコンデルタにおいて野生のヤモリにおける *Salmonella* 属菌の保有状況や血清型などについて調査し，*Salmonella* の生態に果たすヤモリの役割について疫学的に検討した。

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ここのところ年に数回，ベトナムに調査で出かけていますが，街中のヤモリの数が以前に比べ減ったような気がします。これは経済発展による都市化の影響なのか，はたまた農薬などの化学物質の多用によるものなのか不明ですが，個人的には興味のわくところです。

材料と方法

供試検体として，2008 ～ 2010 年にベトナム南部に位置するメコンデルタの Can Tho 市，Kien Giang 省および Ca Mau 省で，定期的に野生のヤモリを捕獲して腸管内容物を採取し，定法によりサルモネラの分離を行った。また，分離されたサルモネラについては血清型別を行った。

結果と考察

ベトナム・メコンデルタで捕獲した野生ヤモリ 646 検体中 95 検体（14.7%）から *Salmonella* 属菌が分離された。この姿を季節別にみると，雨季（5 ～ 11 月）では 343 検

表1　季節別・地域別にみたヤモリからの *Salmonella* 分離状況

地域	乾季	雨季
Kien Giang	9/62 (14.5)	10/60 (16.6)
Ca Mau	6/55 (10.9)	7/53 (13.2)
Can Tho	32/186 (17.2)	31/230 (13.5)
計	47/303 (15.5)	48/343 (14.0)

体中 48 検体（14.0%），乾季（12 ～ 4 月）では 303 検体中 47 検体（15.5%）から *Salmonella* 属菌が分離されたが，季節間では有意差は認められなかった。

また，種別や地域別にみても，分離率に有意な差は認められなかった（表 1）。ベトナム・メコンデルタに生息する野生ヤモリから *Salmonella* 属菌は高率に分離され，季節，捕獲地域およびヤモリの種類に関わらず分離されたことから，本地域ではヤモリは *Salmonella* 属菌の重要なレゼルボアになっているものと思われた。

Salmonella 陽性のヤモリから分離された *Salmonella*95 株中 73 株は市販免疫血清で 10 種類の血清型に型別された。*S.* Weltevreden が 39 株で最も多く，次いで *S.* Lexington が 11 株，*S.* Newport が 9 株，*S.* Brunei が 7 株，*S.* Vejle が 2 株の順であった。*S.* Weltevreden は調査した 3 地域いずれにおいても最も高頻度に分離された。*S.* Weltevreden, *S.* Newport, *S.* Bovismobificans および *S.* Agona は，本地域の人の下痢患者からも分離されている血清型であり，分離された血清型は人とヤモリでは比較的共通していた。特に *S.* Weltevreden はベトナムを含む東南アジアでは人の *Salmonella* 感染患者から高頻度に検出される主要な血清型として知られており，家畜，食品，環境などからの広く分離されるが，その自然界におけるレゼルボアは明らかになっていない。今回，*S.* Weltevreden はメコンデルタの 100km 以上離れた 3 地域において，ヤモリからいずれも高頻度に分離された。ヤモリはベトナムを含む東南アジアでは，人の生活環境周辺に高密度で生息しており，しばしば人の生活環境はヤモリの糞便に汚染されている。これらのことから，ヤモリは少なくともベトナム・メコンデルタにおいては，人の *Salmonella* 感染症，特に *S.* Weltevreden の主たる感染源になっている可能性が高いものと思われる。

ヤモリにおける *Salmonella* の生態を解明する一助として，ヤモリにおける *Salmonella* の感染経路を明らかにする目的で，妊娠した母ヤモリの体内の卵における *Salmonella* の汚染状況を検討したところ，母ヤモリが腸管内に *Salmonella* を保有していた場合でも，体内の卵からは *Salmonella* は全く検出されなかった。ヘビにおいても同様の現象が観察されており，母ヘビが *Salmonella* を保有していても体内の卵からは *Salmonella* は分離されないが，しかし産卵・孵化した子ヘビからは母ヘビが *Salmonella* を保菌していた場合は，同じタイプの *Salmonella* が高率に分離されることから，ヘビでは母子間で in egg ではなく，on egg で感染が起こっているものと推察されている。ヤモリでもヘビと同様に母子間で on egg 感染が起こっているものと推察されるが，今後さらなる検討が必要である。

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Short communication

A novel relapsing fever *Borrelia* sp. infects the salivary glands of the molted hard tick, *Amblyomma geoemydae*

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ABSTRACT

A novel relapsing fever *Borrelia* sp. was found in *Amblyomma geoemydae* in Japan. The novel *Borrelia* sp. was phylogenetically related to the hard (ixodid) tick-borne relapsing fever *Borrelia* spp. *Borrelia miyamotoi* and *B. lonestari*. The novel relapsing fever *Borrelia* sp. was detected in 39 *A. geoemydae* (39/274: 14.2%), of which 14 (14/274: 5.1%) were co-infected with the novel relapsing fever *Borrelia* sp. and *Borrelia* sp. tAG, one of the reptile-associated borreliae. Transstadial transmission of the novel relapsing fever *Borrelia* sp. occurred in the tick midgut and the salivary glands, although *Borrelia* sp. tAG was only detected in the tick midgut. The difference of the borrelial niche in molted ticks might be associated with borrelial characterization.

Introduction

The spirochete genus *Borrelia* is comprised of arthropod-borne infectious agents classified into 3 major groups by phylogenetic analysis: the Lyme disease (LD) *Borrelia* spp., the reptile-associated (REP) *Borrelia* spp., and the relapsing fever (RF) *Borrelia* spp. (Parola and Raoult, 2001; Takano et al., 2010, 2011). The LD *Borrelia* spp. include 3 LD agents, *B. burgdorferi*, *B. garinii*, and *B. afzelii*, which are transmitted by hard (ixodid) ticks of the genus *Ixodes* (Parola and Raoult, 2001). The REP *Borrelia* spp. have been recently discovered in reptiles and their associated hard ticks, genera *Amblyomma* and *Hyalomma* (Takano et al., 2010). The RF *Borrelia* spp. are transmitted by ticks and human body lice. Most tick-borne RF *Borrelia* spp. are transmitted by soft (argasid) ticks excluding 3 species of RF *Borrelia* spp., *B. miyamotoi*, *B. lonestari*, and *B. theileri*. These *Borrelia* spp. are found in hard ticks: in ticks of the genus *Ixodes*, in *Amblyomma americanum*, and in ticks of the genus *Rhipicephalus*, respectively (Barbour, 2005; Steere et al., 2005). In this study, we found a novel *Borrelia* sp. in *Amblyomma geoemydae* which clusters with hard tick-borne RF *Borrelia* spp. based on phylogenetic analysis, and we investigated the transstadial transmission of the borrelial organism in molted ticks.

Materials and methods

For this study, we examined 274 *A. geoemydae* ticks collected from Ryukyu yellow-margined box turtles (*Cuora flavomarginata evelynae*) and Asian yellow pond turtles (*Mauremys mutica kami*) found on Iriomote-jima Island, Okinawa prefecture, Japan. Of these 274 ticks, 173 ticks (124 larvae, 29 nymphs, 4 males, and 16 females) were analyzed without incubation as blood-feeding ticks. Another 101 ticks (87 larvae, 14 nymphs) were allowed to molt to 87 nymphs, 6 males, and 8 females in our laboratory. Molted ticks were obtained by incubating engorged larvae and nymphs at 24 °C. After molting, the ticks were incubated at 24 °C for an additional month (Takano et al., 2011). All ticks were subjected to DNA preparation of the whole body or in part (salivary glands and midgut) after dissection. The total DNA of ticks was prepared as previously described (Takano et al., 2011). For borrelial DNA detection, conventional PCRs for the flagellin gene (*flaB*) and the glycerophosphoryl diester phosphodiesterase gene (*glpQ*) were performed as previously described (Takano et al., 2011). PCR products were purified by using ExoSAP-IT (GE Healthcare UK Ltd.,

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Table 1
Number of *Borrelia* spp.-positive ticks.

Stage	Number of ticks examined	*Borrelia* spp.-positive ticks	Infection		
			Borrelia sp. AGRF	Co-infection	*Borrelia* sp. tAG
Blood-feeding					
Larva	124	48	15(12.1%)	10(8.1%)	23(18.5%)
Nymph	29	15	2(6.9%)	3(10.3%)	10(34.5%)
Male	4	3	0(0%)	1(25%)	2(50%)
Female	16	10	3(18.8%)	0(0%)	7(43.8%)
Subtotal	173	76	20(11.6%)	14(8%)	42(24.3%)
Molted					
Nymph	87	29	3(3.4%)	0(0%)	26(29.9%)
Male	6	2	0(0%)	0(0%)	2(33.3%)
Female	8	4	2(25%)	0(0%)	2(25%)
Subtotal	101	35	5(5%)	0(0%)	30(29.7%)
Total	274	111	25(9.1%)	14(5.1%)	72(26.3%)

UK) and were directly sequenced (ABI Prism 3130xl Genetic Analyzer; Life Technologies Corporation, USA). Sequence data were analyzed using MEGA4 software (http://www.megasoftware.net) (Tamura et al., 2007). The construction of phylogenetic tree and bootstrap tests were carried out according to the protocol previously described (Takano et al., 2011). In this study, 76 feeding ticks (76/173, 43.9%) and 35 molted ticks (35/101, 34.7%) were borrelial DNA-positive by PCR for both genes (Table 1). From sequencing analysis of *flaB* amplicons, these borreliae were classified into 2 phylogenetic groups, REP *Borrelia* spp. and RF *Borrelia* spp. One group was clustered with a REP *Borrelia* sp., *Borrelia* sp. tAG, as previously described (Takano et al., 2011). The other group was an unknown *Borrelia* sp. (preliminarily designated as *Borrelia* sp. AGRF in this study), and it clustered with hard tick-borne RF *Borrelia* spp. (Figs. S1, S2, and S3). To characterize *Borrelia* sp. AGRF, partial *flaB* sequence (397 bp), 16S rRNA gene (*16S rDNA*) (1490 bp), and *glpQ* (1014 bp) were determined according to previously described methods (Takano et al., 2011), and representative sequences of these genes were deposited in GenBank as AB529429, AB529435, and AB529433, respectively.

It was thought that 2 different *Borrelia* spp. were occasionally co-infected within a single tick. To determine mixed infections with these borreliae, we established a *Borrelia* sp. AGRF detection system based on quantitative real-time PCR (qPCR) technology and performed qPCR on ticks that were positive for borrelial DNAs. From sequence analysis of the *flaB* gene of *Borrelia* sp. AGRF, the *Borrelia* sp. was expected to be clonal because nucleic acid substitution rate was less than 0.5%. Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) was used to design oligonucleotide primers and a TaqMan probe, sequence-specific for the *Borrelia* sp. AGRF *flaB*. Forward and reverse primers were MGB_92F (5′-GCTGGAGCACAAGCTTCATG-3′) and MGB_242R (5′-CCTGTTGTGCCCCTTCTTGA-3′), respectively. The dye-labeled probe is MGB_P174 (VIC-CTAATGTTGCAAATCTCTTT-3′) with the 3′ end modified with a minor groove-binding protein (Applied Biosystems). The qPCR was performed using Premix Ex TaqTM (Perfect Real Time) (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions and run on an ABI PRISM 7000 system (Life Technologies Corporation, Gaithersburg, MD). For analysis of qPCR, the threshold line was fixed at 0.2 to avoid detection of nonspecific fluorescence. To evaluate a specificity of the qPCR (designated as *flaB*-qPCR in this study), a total of 37 borrelial strains were used: 32 REP *Borrelia* spp. (8 of *Borrelia* sp. tAG, 9 of *B. turcica*, 9 of *Borrelia* sp. GP, 4 of *Borrelia* sp. BF, *Borrelia* sp. TA2, and *Borrelia* sp. Tick98 M), 5 LD *Borrelia* spp. (*B. burgdorferi* B31, *B. garinii* 20047, *B. afzelii* VS461, *B. valaisiana* VS116, and *B. lusitaniae* PotiB2). As a result, none of the REP *Borrelia* spp. and LD *Borrelia* spp. was detected in this assay. To determine the sensitivity of *flaB*-qPCR, an external standard template containing 10^1 to 10^8 copies of the *Borrelia* sp. AGRF *flaB* was included in a run. For establishing the external standard plasmids, a fragment of the *Borrelia* sp. AGRF *flaB* (397 bp) was amplified by PCR using primer set BflaPAD and BflaPDU and cloned as previously described (Takano et al., 2011). As a result, the limit of detection consistently observed was a minimum of 10 plasmid copies (data not shown). In addition to the *Borrelia* sp. AGRF detection system, we performed qPCR to detect the *Borrelia* sp. tAG phosphotransferase system maltose-specific enzyme IICB component gene (*glvC*) (Takano et al., 2011), for *Borrelia* sp. AGRF-positive ticks (Fig. S4). As a result, 14 of 274 ticks (10 larvae, 3 nymphs, and 1 male tick) were found to have mixed infections of these borreliae (Table 1 and Fig. S4).

Results and discussion

In a previous study, we found that *Borrelia* sp. tAG colonized the midgut, but not the salivary glands after tick molting (transstadial transmission occurred in the midgut only (Takano et al., 2011)). On the other hand, it was unknown how the transstadial transmission of *Borrelia* sp. AGRF occurred. To elucidate the transstadial transmission niches of *Borrelia* sp. AGRF in ticks, the presence of *Borrelia* sp. AGRF in one salivary gland and the midgut of molted ticks were investigated. Consequently, we detected DNA fragments of *Borrelia* sp. AGRF from both the midgut and the salivary glands of 5 molted ticks. The results were confirmed by immunofluorescence assay of the second and remaining salivary gland. Confocal imaging analysis suggested that the borrelial cells had entered into the salivary gland of molted ticks (Fig. 1).

Prior to this study, a hard tick-borne RF borrelia, *B. miyamotoi*, was suggested to be a co-infection with LD borreliae in a tick (Barbour et al., 2009; Scoles et al., 2001). In addition to these cases, in this study, we found a novel hard tick-borne RF *Borrelia* sp. in *A. geoemydae* and showed evidence of co-infection of *Borrelia* sp. AGRF and *Borrelia* sp. tAG within ticks. In this study, all co-infected ticks were detected among *Borrelia* sp. AGRF-positive ticks (Fig. S4). We used *flaB*-PCR as a first screening. However, the screening may preferentially detect the dominant *Borrelia* species in tick samples. In this study, all co-infected ticks were observed in the tick group which were *Borrelia* sp. AGRF-positive in the first screening (Fig. S4). This result may be due to the borrelial dominance in ticks, similar to that noted for co-infection of *B. miyamotoi* and *B. burgdorferi* in *Ixodes scapularis* nymphs (Barbour et al., 2009). Moreover, we demonstrated the transstadial transmission niches of the hard tick-borne RF *Borrelia* sp. in the vector tick (Fig. 1). Since *Borrelia* sp. AGRF was detected from the salivary gland and the midgut of molted ticks, the transstadial transmission niches of *Borrelia* sp. tAG and *Borrelia* sp. AGRF were somewhat different in the same tick, *A. geoemydae*. According to some reports, it has been shown that transstadial transmission of RF borreliae (e.g. *B. hermsii*) does not occur only in the midgut, but also in the salivary grand of molted