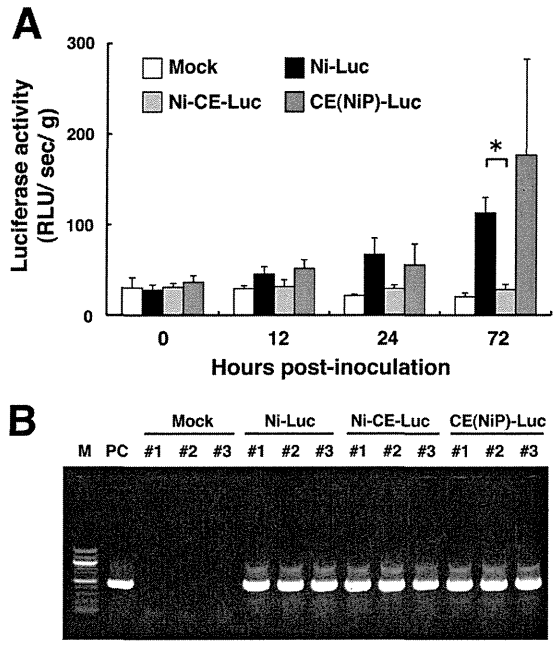

FIG 6 Viral replication in mouse thigh muscle. Mice were intramuscularly inoculated with 10^6 FFU of the Ni-Luc, Ni-CE-Luc, or CE(NiP)-Luc strain. (A) At 0, 12, 24, and 72 hpi, mouse thigh muscles were collected and used for analyses of luciferase activities (calculated as relative light units [RLU])/s/g muscle weight) by the luciferase assay system. The values in the graph are shown as means ± standard errors of the means. *, significant difference ($P < 0.05$). (B) Muscle tissue debris in the lysate prepared at 72 hpi for the above luciferase assay was examined for viral genomic RNAs by RT-nested PCR targeting the N gene region (461 bp). M, marker; #1 to #3, identification numbers of mice; PC, positive control (mouse brain i.c. inoculated with CVS strain).

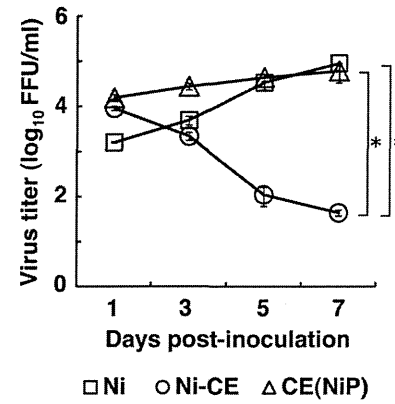

FIG 8 Growth curves of each strain in muscle G-8 cells. G-8 cells were infected with the Ni, Ni-CE, or CE(NiP) strain at an MOI of 1. The viruses in the supernatants were harvested at 1, 3, 5, and 7 dpi and titrated in NA cells by focus assay. All assays were carried out in triplicate, and the values in the graph are shown as means ± standard errors of the means. *, significant difference ($P < 0.05$).

DISCUSSION

Previous studies have demonstrated that G protein plays an important role in neuroinvasiveness of RABV (22–29). On the other hand, there is accumulating evidence that G protein is not the only viral component determining the ability of RABV to cause lethal neurological symptoms after peripheral inoculation (25–29). Although minor contributions of M and L proteins to this viral ability have been reported (25, 27), the importance of viral proteins other than G protein in neuroinvasiveness of RABV remains to be elucidated. In this study, we conducted comparative analysis of the two RABV strains Ni and Ni-CE, which cause lethal and asymptomatic infections, respectively, in mice after i.m. inoculation (Fig. 2). Using chimeric viruses with the respective genes from the Ni strain in the genetic background of the Ni-CE strain, we revealed that the P gene is mainly related to the difference in levels of neuroinvasiveness of the Ni and Ni-CE strains (Fig. 3 and Table 2). To our knowledge, this is the first report that the P gene plays a major role in neuroinvasiveness of RABV.

Since the Ni-CE and CE(NiP) strains are genetically identical except for five amino acid substitutions in the P protein (at positions 56, 58, 66, 81, and 226) (30), it is obvious that one or a combination of these amino acid substitutions determine the levels of neuroinvasiveness of the Ni and Ni-CE strains. Our preliminary experiment revealed that neither of the Ni-CE mutant strains, CE(NiP56-81) and CE(NiP226), in which amino acids at positions 56, 58, 66, and 81 and the amino acid at position 226 in P protein were replaced with those the Ni strain, respectively, caused symptomatic infection in any of the mice after i.m. inoculation (data not shown). This indicates that another combination of amino acid substitutions in P protein is related to the difference

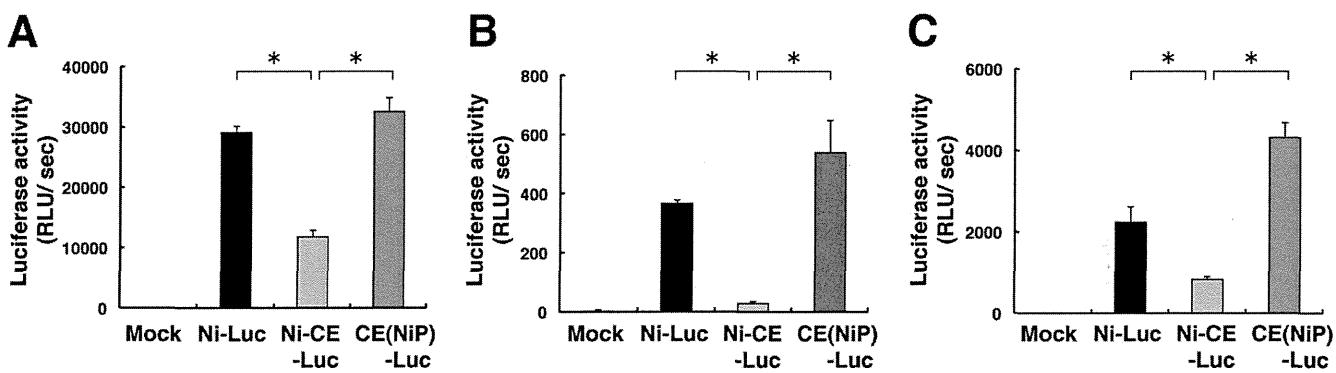

FIG 7 Viral replication in cultured muscle cell lines. (A) Mouse muscle myoblast G-8 cells, (B) C2C12 cells, and (C) human rhabdomyosarcoma A-673 cells were infected with the Ni-Luc, Ni-CE-Luc, or CE(NiP)-Luc strain at an MOI of 1. At 7 dpi, the cells were used for analyses of luciferase activities (calculated as RLU/s) by the luciferase assay system. All assays were carried out in triplicate, and the values in the graph are shown as means ± standard errors of the means. *, significant difference ($P < 0.05$).

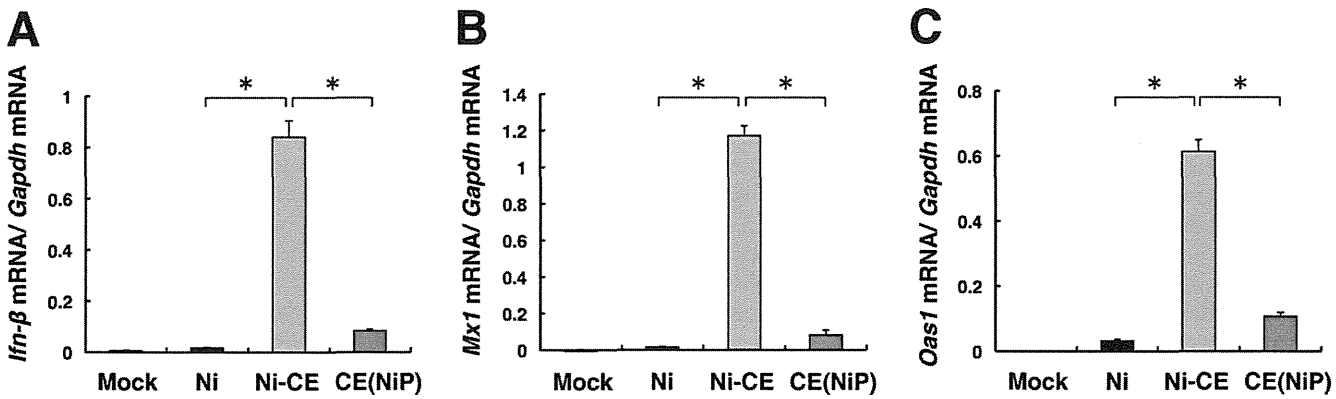

FIG 9 Relative expression levels of IFN-related genes in virus-infected muscle G-8 cells. G-8 cells were infected with the Ni, Ni-CE, or CE(NiP) strain at an MOI of 1 and then lysed at 24 hpi for RNA extraction. The expression levels of the (A) *Ifn*-β, (B) *Mx1*, and (C) *Oas1* genes are indicated as the number of copies of specific mRNA per copy of mouse *Gapdh* mRNA. All assays were carried out in triplicate, and the values in the graph are shown as means ± standard errors of the means. *, significant difference ($P < 0.05$).

in levels of neuroinvasiveness of the Ni and Ni-CE strains. Further experiments with additional Ni-CE mutants will be required to identify the key amino acid residues in P protein.

We revealed by i.m. inoculation of mice with 10^6 FFU of each strain that, in contrast to the Ni and CE(NiP) strains, the Ni-CE strain does not infect peripheral nerves (Fig. 4). This raises the possibility that Ni-CE P protein, but not Ni P protein, has a functional defect that disrupts the ability of the virus to be internalized into and/or to be retrogradely transported in peripheral nerves. However, previous findings strongly suggest that this is not the case. More specifically, it was reported that retrograde axonal transport of RABV takes place in the form of a viral particle, not a viral RNP complex (38). On the other hand, P protein is a component of the viral RNP complex and is theoretically not exposed to the outside of the viral particle through the processes of viral internalization and retrograde axonal transport. Therefore, it is expected that P protein is not able to directly interact with host cellular machineries, which play important roles in these processes.

To experimentally check the above theory, we grew primary cultured neurons by using a microfluidic culture platform, which allows us to fluidically separate their cell body and axon terminal compartments. This culture system has been found to be a useful tool in neuroscience studies, such as studies on transport of neurotrophic factors along axons (39, 40), regeneration of axons (34, 41), and interaction of axons with glial cells (34, 42). This culture system is also thought to be useful in studies on neurotropic viruses, although a limited number of virology studies have utilized this system. In fact, it has been successfully used for the study of axonal transport of herpesvirus (43, 44). Using this system, we inoculated axon terminals of primary cultured neurons with the Ni-CE-GFP or CE(NiP)-GFP strain and checked the presence of GFP signals in the cell bodies. The fact that GFP signals were detected in both cell bodies of neurons infected with Ni-CE-GFP and CE(NiP)-GFP strains (Fig. 5C) clearly indicated that both strains have the ability to infect neurons via axon terminals. Notably, at 24 hpi, when GFP signals were first detected in the cell bodies of neurons infected with the Ni-CE-GFP and CE(NiP)-GFP strains, their percentages of GFP-positive cell bodies were comparable (Fig. 5D), suggesting that both strains were transported from axon terminals to cell bodies of neurons with almost identical efficiencies. These data support the theory that Ni-CE P protein does not negatively affect the ability of the virus to be internalized into and/or transported retrogradely in the peripheral nerves.

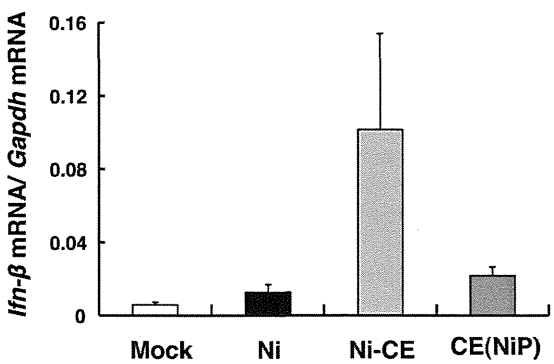

FIG 10 Relative expression levels of the *Ifn*-β gene in thigh muscles of infected mice. Mice were intramuscularly inoculated with 10^6 FFU of the Ni, Ni-CE, or CE(NiP) strain. Thigh muscles were collected at 12 hpi, and total RNAs were extracted from each tissue. The expression level of the *Ifn*-β gene is indicated as the number of copies of specific mRNA per copy of mouse *Gapdh* mRNA. The values in the graph are shown as means ± standard errors of the means.

Previous studies using both street and fixed RABV strains have demonstrated that muscle cells are the infection sites in peripheral tissue (7–10, 45). In this study, we compared *in vivo* replication efficiencies of the Ni-Luc, Ni-CE-Luc, and CE(NiP)-Luc strains in muscle and obtained data strongly suggesting that the Ni P gene, but not the Ni-CE P gene, mediates stable viral replication in muscle (Fig. 6). This finding is supported by the results of *in vitro* experiments showing that the Ni and CE(NiP) strains replicate in cultured muscle cells more efficiently than does the Ni-CE strain (Fig. 7 and 8). Notably, we previously reported that the Ni-CE and CE(NiP) strains grew similarly in mouse and human neuroblastoma cells (4, 30). This indicates that the low replication efficiency of the Ni-CE strain in muscle cells is not due to a defect in the fundamental replication ability.

Previous histopathological studies showed that RABV replicates in muscle cells before infecting peripheral nerves, implying

that viral replication in muscle cells enhances the infection of peripheral nerves (7–10). However, this possibility has not been supported by experimental data showing a correlation between the abilities of the virus to replicate in muscle cells and to infect peripheral nerves. In this study, we demonstrated that the Ni and CE(NiP) strains, which have the ability to infect peripheral nerves *in vivo*, are capable of stably replicating in muscle cells, whereas the Ni-CE strain, with a defect in this ability, is not (Fig. 6, 7, and 8). Hence, we present here the first data indicating that efficiency of RABV replication in muscle cells correlates with the ability of the virus to infect peripheral nerves. This correlation leads to the possibility that replication of RABV in muscle cells is important to present a certain number of infectious viruses to axon terminals of peripheral nerves, consequently enhancing infection of peripheral nerves.

While the results above highlight the contribution of viral replication in muscle cells to the efficient infection of peripheral nerves, several studies using both street and fixed viruses have demonstrated that RABV has the ability to directly infect peripheral nerves without viral replication in muscle cells (11–13, 46, 47). Previous findings that lentiviral vectors pseudotyped with RABV G protein reach the CNS after i.m. inoculation (23, 24) also indicated the lack of necessity of viral replication in muscle cells for infection of peripheral nerves. Notably, our preliminary data demonstrated that when mice were inoculated intramuscularly with a higher dose (i.e., 5×10^7 FFU) of the Ni-CE strain, which has a defect in the ability to replicate in muscle cells, some (~60%) of them developed symptoms such as transient body weight loss (data not shown), strongly suggesting direct infection of peripheral nerves with the Ni-CE strain. We believe that various and complex factors, including the dose and biological characteristics (e.g., viral tropisms to neuron and muscle cells) of the RABV strain used for inoculation affect the efficiency of direct infection of peripheral nerves. In the previous studies described above, this efficiency might have increased under certain conditions, masking the contribution of RABV replication in muscle cells to efficient infection of peripheral nerves.

In response to viral infection, host cells produce type I IFN, including the IFN-α family and IFN-β, which, on binding to IFN receptors on the cell surface, leads cells to an antiviral status by inducing the expression of ISGs encoding antiviral proteins (reviewed in reference 48). It was previously demonstrated that RABV P protein functions to antagonize the host IFN system by inhibiting both cellular signaling pathways for induction of the IFN gene and ISGs (4, 15–21). Based on those findings, we hypothesized that this IFN-antagonistic function of P protein is involved in the different replication efficiencies of CE(NiP) and Ni-CE strains in muscle cells. Consistent with this hypothesis, in muscle G-8 cells, the expression levels of the *Ifn*-β gene and also the *Mx1* and *Oas1* genes, known as ISGs, in CE(NiP)-infected cells were significantly lower than the levels in Ni-CE-infected cells (Fig. 9). In addition, the expression levels of the *Ifn*-β gene in inoculated thigh muscles of CE(NiP)-infected mice tended to be lower than that in the muscles of Ni-CE-infected mice (Fig. 10). Importantly, we found that in Vero cells, which are known to be IFN deficient (49), the Ni-CE-Luc and CE(NiP)-Luc strains expressed almost identical levels of luciferase (data not shown), indicating that the replication efficiencies of the Ni-CE-Luc and CE(NiP)-Luc strains are comparable in the absence of an intact IFN system. These findings strongly suggest that the host IFN system suppresses replication of the Ni-CE strain in muscle cells more efficiently than that of the CE(NiP) strain.

We showed that Ni-CE infection induces the *Ifn*-β gene more efficiently than does CE(NiP) infection in muscle cells (Fig. 9), but the molecular mechanism remains to be elucidated. Brzózka et al. (16) reported that RABV P protein inhibits phosphorylation of interferon regulatory factor 3 (IRF-3), which is an important transcription factor for IFN induction. Since there are five amino acid substitutions in P protein between the Ni and Ni-CE strains (30), these amino acid mutations in Ni-CE P protein might disrupt its function to inhibit IRF-3 phosphorylation in muscle cells. Interestingly, in neuroblastoma SYM-I cells, such a difference between expression levels of the *Ifn*-β gene in Ni-CE- and CE(NiP)-infected cells was not observed (data not shown). Consistent with this, we previously reported that in SYM-I cells, Ni-CE and Ni P proteins expressed from a plasmid equally inhibited activation of the *Ifn*-β promoter induced by infection with Newcastle disease virus (5). These findings suggest that the inhibitory function of Ni-CE P protein in IFN induction is deficient in muscle cells but not in neural cells. Further studies will be required to elucidate the mechanism underlying the cell-type dependency of IFN-antagonistic function of Ni-CE P protein.

Our data strongly suggested that viral evasion of the host IFN system in peripheral tissue is important for neuroinvasiveness of RABV. Importantly, this finding is supported by results of previous studies: it was shown that administration of IFN inducers, such as polyriboinosinic-polyribocytidylic acid containing poly-L-lysine and carboxymethylcellulose [poly(ICLC)] following i.m. challenge with virulent RABV enhances the protective effect of postexposure rabies vaccine in monkeys (50, 51). According to these findings, we believe that the host IFN system in peripheral tissue is a potential target for development of novel prophylactic approaches for rabies.

While we demonstrated the importance of the P gene in neuroinvasiveness of RABV, our data also indicated that the P gene is not the only viral gene determining the levels of neuroinvasiveness of the Ni and Ni-CE strains: the morbidity rate of mice inoculated with the CE(NiP) strain (75 to 80%) was lower than that of mice inoculated with the Ni strain (100%) (Table 2). Notably, the CE(NiN) and CE(NiG) strains caused symptomatic infection in 20 to 40% and 0 to 40% of mice, respectively, suggesting a minor contribution of N and G genes to the neuroinvasiveness. Consistent with the results of previous studies (25–29), the findings of this study indicate that multiple genes are involved in the ability of RABV to cause symptomatic infection after i.m. inoculation, strongly suggesting that the mechanism underlying neuroinvasiveness of RABV is complex.

In conclusion, the present study has demonstrated the importance of the RABV P gene in neuroinvasiveness. Also, we have provided findings strongly suggesting that RABV P protein assists stable viral replication in muscle cells by its IFN-antagonistic function and consequently enhances infection of peripheral nerves. We believe that our findings provide basic information for development of novel prophylactic approaches for rabies and also for establishment of a live rabies vaccine strain with a high level of safety.

ACKNOWLEDGMENTS

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Small Indian mongooses and masked palm civets serve as new reservoirs of *Bartonella henselae* and potential sources of infection for humans

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Abstract

The prevalence and genetic properties of *Bartonella* species were investigated in small Indian mongooses and masked palm civets in Japan. *Bartonella henselae*, the causative agent of cat-scratch disease (CSD) was isolated from 15.9% (10/63) of the mongooses and 2.0% (1/50) of the masked palm civets, respectively. The bacteraemic level ranged from 3.0×10^1 to 8.9×10^3 CFU/mL in mongooses and was 7.0×10^3 CFU/mL in the masked palm civet. Multispacer typing (MST) analysis based on nine intergenic spacers resulted in the detection of five MST genotypes (MSTs 8, 14, 37, 58 and 59) for the isolates, which grouped in lineage 1 with MST genotypes of isolates from all CSD patients and most of the cats in Japan. It was also found that MST14 from the mongoose strains was the predominant genotype of cat and human strains. This is the first report on the isolation of *B. henselae* from small Indian mongooses and masked palm civets. The data obtained in the present study suggest that these animals serve as new reservoirs for *B. henselae*, and may play a role as potential sources of human infection.

Keywords: *Bartonella henselae*, cat-scratch disease, masked palm civet, mongoose, multispacer typing
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Introduction

Bartonella bacteria are small, fastidious, gram-negative, vector-transmitted pathogens. Since the early 1990s, more than 20 species including three subspecies of *Bartonella* have been identified and at least 13 species are known to be zoonotic agents [1,2]. Cat-scratch disease (CSD) is one of the most common zoonoses caused by *Bartonella henselae* and the cat (*Felis catus*) is recognized as the main reservoir for *B. henselae*. The prevalence of the organism in cats varies from 0% in Norway to 68% in the Philippines, and varies according to the housing status of cats (pet or stray) and the geographical location [3]. Except for domestic cats, *B. henselae* was isolated from wild African lions and cheetahs [4]. Antibody to *B. henselae* was also detected in free-ranging and captive wild felids such as bobcats, leopards, jaguars, pumas and tigers [5,6]. These data suggest that wild Felidae are reservoir hosts of *B. henselae* in nature.

Both the small Indian mongoose (*Herpestes auropunctatus*) and the masked palm civet (*Paguma larvata*) belong to the suborder Feliformia of the order Carnivora along with the felids. Since small Indian mongooses were introduced in 1910 from Bangladesh to Okinawa Prefecture, Japan, they have readily adapted to the new environment and have been having serious effects on the unique ecosystem and causing extensive damage to agricultural crops and the poultry industry in the

area [7]. Masked palm civets are widely distributed from Northern India to Southeast Asia and China, and the introduced individuals have also expanded their habitat and caused serious damage to agricultural products and intrusion into human dwellings in Japan [8].

Hence, the increased populations of small Indian mongooses and masked palm civets have resulted in many opportunities for these species to appear in the peridomestic environment and come into contact with either residents or animal control workers. Although these animals present serious risks as sources of zoonoses such as leptospirosis, rabies, severe acute respiratory syndrome, salmonellosis, yersiniosis and campylobacteriosis [9–12], no epidemiological studies on *Bartonella* infection in mongooses and masked palm civets have been conducted.

Several genotyping methods have been developed and applied for the characterization of *Bartonella* isolates. It is reported that multispacer typing (MST) using nine variable intergenic spacers is the most discriminatory genotyping method for *B. henselae* isolates and is used to investigate the relationships between human and cat isolates [13,14].

The aim of the present study was to investigate the prevalence of *Bartonella* species in small Indian mongooses and masked palm civets in Japan. Furthermore, we evaluated the possibility that these animals serve as a source of CSD for humans by MST of the isolates.

Material and Methods

Sample collection

During the period from 2009 to 2012, blood samples were collected from 63 small Indian mongooses in Okinawa Prefecture and 50 masked palm civets in Chiba (n = 26) and Kanagawa (n = 24) Prefectures, Japan. Blood samples from the mongooses and masked palm civets were collected by cardiopuncture after euthanasia following the guidelines for invasive alien species prepared by the Japanese Veterinary Medical Association, and then transferred into EDTA-containing collection tubes. Blood samples from the mongooses were immediately stored at − 70°C, whereas those from the masked palm civets were stored at − 20°C for 2–12 months after collection. The samples were sent to the Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University for examination of *Bartonella*.

Isolation and identification of *Bartonella* bacteria

Frozen blood samples were thawed at room temperature and submitted for the isolation of *Bartonella* species following previously reported procedures [15]. Bacterial colonies were tentatively identified as *Bartonella* species based on colony morphology and the long culture period (>1 week), and subsequently the CFU/mL of blood were calculated by additional quantitative culture. For further characterization, five colonies were picked from each sample and subcultured on fresh blood agar plates using the same conditions as the primary culture.

The genomic DNA of each isolate was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA, USA). Identification of *Bartonella* was performed using *Bartonella*-specific PCR for six housekeeping genes including the 16S ribosomal RNA gene (16S rRNA), the cell division protein gene (*ftsZ*), the citrate synthase gene (*gltA*), the heat-shock protein gene (*groEL*), the riboflavin synthase alpha chain gene (*ribC*) and the RNA polymerase beta subunit-encoding gene (*rpoB*). The primers and PCR conditions used for the PCR amplification of 16S rRNA [16], *ftsZ* [17], *gltA* [18], *groEL* [19], *ribC* [20] and *rpoB* [18] have been previously published.

For DNA sequencing of 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*, the PCR products were purified using a Spin Column PCR product purification kit (Bio Basic Inc., Markham, Ontario, Canada), and then sequenced directly by using dye terminator chemistry and a Genetic Analyzer model 3130 (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer's instructions. The sequence alignments were assembled and edited using the AUTOASSEMBLER program in GENETYX-WIN software, version 9 (Genetyx Corp., Tokyo, Japan), and compared with those of other known *Bartonella* species deposited in the GenBank/EMBL/DDBJ database by using the BLAST program.

Multispacer typing and phylogenetic tree based on nine intergenic spacers

Internal fragments of approximately 300–500 bp of nine intergenic spacers (S1–S9) were amplified by PCR as described previously [13]. Positive and negative controls were prepared using DNA from *B. henselae* Houston-1^T and nuclease-free distilled water, respectively. The PCR products of S1–S9 were purified and sequenced directly. Vector sequencing was applied only when obtaining extra bands for S1. The band with the expected size was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), subcloned using the plasmid pGEM-T Easy vector system (Promega), and sequenced using the same protocol as described for direct sequencing [15]. MST genotypes were determined for ten strains from the mongooses and one strain from the masked palm civet. Ten strains from cats were also subjected to MST analysis. Out of ten cat strains, seven are derived from Okinawa Prefecture where the mongooses were

captured. The other three cat strains are derived from near the areas of Chiba, Kanagawa and Tokyo Prefectures where the masked palm civet was captured. The MST genotype of each strain was defined by the combination of the S1–S9 genotypes. The genotypes of the intergenic spacers and MSTs were assigned numbers according to the previous reports [13,21,22]. When new combinations of intergenic spacers were found for the first time, the genotypes were assigned as novel MST genotypes in the order of detection.

Multiple alignment of the spacer sequences was carried out using the CLUSTALW program. A phylogenetic tree of the concatenated sequences of the nine spacers (S1–S9) was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA4 [23]. Fifty-seven MST genotypes from cat and human strains described in previous reports [13,21,22] were also included in the phylogenetic analysis.

Results

Prevalence and bacteraemic levels of *Bartonella* in small Indian mongooses and masked palm civets

The prevalences of *Bartonella* were 15.9% (10/63) in the small Indian mongooses and 2.0% (1/50) in the masked palm civets. Five isolates from each bacteraemic animal were applied to the genetic characterization of *gltA* because a large number of *Bartonella*-suspected colonies were found in the primary isolation from the animals. Finally, a total of 55 *Bartonella* isolates were obtained from ten mongooses and one masked palm civet. Since all of the isolates were identical in the nucleotide sequence of *gltA*, a representative isolate randomly selected from each bacteraemic animal was used for further genetic characterization. Nucleotide sequence identities of the 11 representative isolates to those of *B. henselae* Houston-1^T were 100% for the 16S rRNA gene, 99.9–100% for *ftsZ*, 100% for *gltA*, 100% for *groEL*, 100% for *ribC*, and 99.6–99.8% for *rpoB*, respectively. Therefore, all of the isolates were identified as *B. henselae*.

Quantitative cultivation indicated that the bacteraemic level varied from 3.0×10^1 to 8.9×10^3 CFU/mL in seven of ten mongooses and was 7.0×10^3 CFU/mL in the masked palm civet (Table 1).

Genotyping and phylogenetic analysis based on MST

The 21 strains (from ten mongooses, one masked palm civet and ten cats) formed eight MST genotypes (MSTs 8, 14, 33, 35, 37, 38, 58 and 59). The strains from the mongooses and the masked palm civet were classified into MSTs 8, 14, 37 and 58 and MST 59, respectively (Table 2). The cat strains from Okinawa, from Chiba, and from Kanagawa and Tokyo Prefectures were classified into MSTs 35, 38 and 58, MST 33, and MST 35, respectively. MST 58, from two mongooses and two cat strains, derived from Okinawa Prefecture and MST 59, from one masked palm civet strain, were novel genotypes. Two distinct S1 bands (nos. 5 and 8) were detected from the four strains of MST 58. All of the MST data obtained in this study were deposited in the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

All of the MST genotypes (MSTs 8, 14, 37, 58 and 59) from the small Indian mongoose and the masked palm civet strains belonged to lineage 1 with the MST genotypes of cat strains from Japan, the Philippines and Thailand, and of CSD patient strains in Japan. Similarly, the MST genotypes (MSTs 33, 35, 38

TABLE 1. Sequence similarities of the genes from the small Indian mongoose and the masked palm civet isolates to those of *Bartonella henselae* Houston-1^T and the bacteraemic levels in the host animals

Animal ID	Strain name	Sequence similarities (%) to *B. henselae* Houston-1^T in: 16S rRNA (1348 bp)[a]	*ftsZ* (788 bp)[a]	*gltA* (312 bp)[a]	*groEL* (1185 bp)[a]	*ribC* (621 bp)[a]	*rpoB* (825 bp)[a]	Bacteraemic level (CFU/mL)[b]
Mongoose								
53	HJ53	100	99.9	100	100	100	99.6	NC[c]
54	HJ54	100	100	100	100	100	99.8	NC[c]
58	HJ58	100	100	100	100	100	99.8	NC[c]
90	HJ90	100	100	100	100	100	99.8	8.0×10^1
91	HJ91	100	99.9	100	100	100	99.6	3.0×10^1
106	HJ106	100	100	100	100	100	99.8	3.0×10^2
107	HJ107	100	99.9	100	100	100	99.6	5.9×10^3
108	HJ108	100	100	100	100	100	99.8	8.9×10^3
109	HJ109	100	99.9	100	100	100	99.6	5.0×10^2
111	HJ111	100	99.9	100	100	100	99.6	1.2×10^3
Civet								
18	PL18	100	100	100	100	100	99.8	7.0×10^3

[a]Length of the sequenced portion of the gene.
[b]Colony forming units/mL of blood.
[c]NC, not countable due to the lack of blood.

TABLE 2. Multispacer typing (MST) genotyping of 21 *Bartonella henselae* isolates from ten small Indian mongooses, one masked palm civet and ten cats

Strain name	Animal source	Prefecture	Genotypes									
			S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
HJ53	Mongoose	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
HJ54	Mongoose	Okinawa	8	2	5	4	1	2	1	1	3	8
HJ58	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ90	Mongoose	Okinawa	4	2	5	4	1	2	1	1	3	14
HJ91	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ106	Mongoose	Okinawa	4	2	5	4	1	2	1	1	3	14
HJ107	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ108	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
HJ109	Mongoose	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
HJ111	Mongoose	Okinawa	5	2	6	5	2	2	1	2	1	37
Oki.cat17	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
Oki.cat26	Cat	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
Oki.cat38	Cat	Okinawa	5	2	6	5	2	2	2	2	1	38
Oki.cat41	Cat	Okinawa	5	2	6	5	2	2	2	2	1	38
Oki.cat48	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
Oki.cat49	Cat	Okinawa	5+8[a]	2	5	4	1	2	1	1	3	58
Oki.cat50	Cat	Okinawa	5	2	6	5	2	2	2	1	1	35
PL18	Civet	Chiba	7	2	5	4	1	2	2	3	3	59
Chi.cat11	Cat	Chiba	4	2	5	4	1	2	2	1	3	33
Kan.cat37	Cat	Kanagawa	5	2	6	5	2	2	2	1	1	35
Tok.cat1	Cat	Tokyo	5	2	6	5	2	2	2	1	1	35

[a]S1 genotype 5+8 indicates that the strain had two different copies of intergenic spacer S1 in its genome.

and 58) of cat strains from four Prefectures were also classified in lineage 1 with the strains from the mongooses and the masked palm civet (Fig. 1).

Discussion

The present study demonstrates for the first time that small Indian mongooses and masked palm civets harbour *B. henselae* in their blood. The prevalence of *B. henselae* was 15.9% (10/63) in the mongooses and 2.0% (1/50) in the masked palm civets. The prevalence of *B. henselae* in masked palm civets was lower than in mongooses. Blood samples from the masked palm civets used in this study were stored at − 20°C, whereas those from the mongooses were stored at − 70°C. It has been suggested that *Bartonella* viability may decrease over time as the result of inadequate conservation of blood samples, and may result in the underestimation of *Bartonella* prevalence based on culturing [4]. Therefore, fresh blood samples or those stored at − 70°C should be used for the cultivation of *Bartonella*.

The levels of bacteraemia in cats experimentally infected with *B. henselae* have ranged from 1×10^1 to 1.7×10^5 [24] or 1.2×10^5 CFU/mL of the blood [25]. In the present study, mongoose and masked palm civet also showed relatively high bacteraemic levels of *B. henselae*: 8.9×10^3 CFU/mL and 7.0×10^3 CFU/mL, respectively. Furthermore, no clinical or pathological abnormalities due to the agent were observed in any of the infected animals, as in bacteraemic cats. These results suggest that the suborder Feliformia composed of mongooses and masked palm civets along with felids serves as a reservoir of *B. henselae*.

Previous studies of the MST genotype of *B. henselae* have shown that all the strains derived from patients with CSD in Japan were categorized in lineage 1 [22]. In the present study, all of the strains from the small Indian mongooses (MSTs 8, 14, 37 and 58) and the masked palm civet (MST 59) also grouped in lineage 1. Yanagihara *et al.* [22] have reported that MSTs 14 and 35 were the predominant genotypes of cat and human strains in Japan. These findings suggest that some of the mongoose strains have similar potential to infect humans as cat strains.

Two mongoose strains (HJ53 and HJ109) and two cat strains (Oki.cat26 and Oki.cat49) from Okinawa Prefecture showed the same genotype and were designated as a novel genotype, MST 58. Furthermore, the prevalence of the bacteria in mongooses was similar to that in cats (18%; 9/50) in Okinawa Prefecture [26]. The main vector of *B. henselae* among cats has been confirmed to be cat fleas (*Ctenocephalides felis*) [27] and a previous study showed that 9.3% (224/2,406) of mongooses in Okinawa Prefecture were infested with cat fleas that have low host specificity [28]. These findings suggest that the *B. henselae* strain with MST 58 may be transmitted between mongooses and cats in the area by direct contact of both animals or by some arthropod vectors such as cat fleas.

Interestingly, MSTs 8 and 37 from mongooses were also reported in cat strains from the USA and the Philippines, respectively [13]; however, strains with those genotypes have not been identified from cats or humans in Japan. It is unclear whether the mongooses were indigenously infected with MSTs

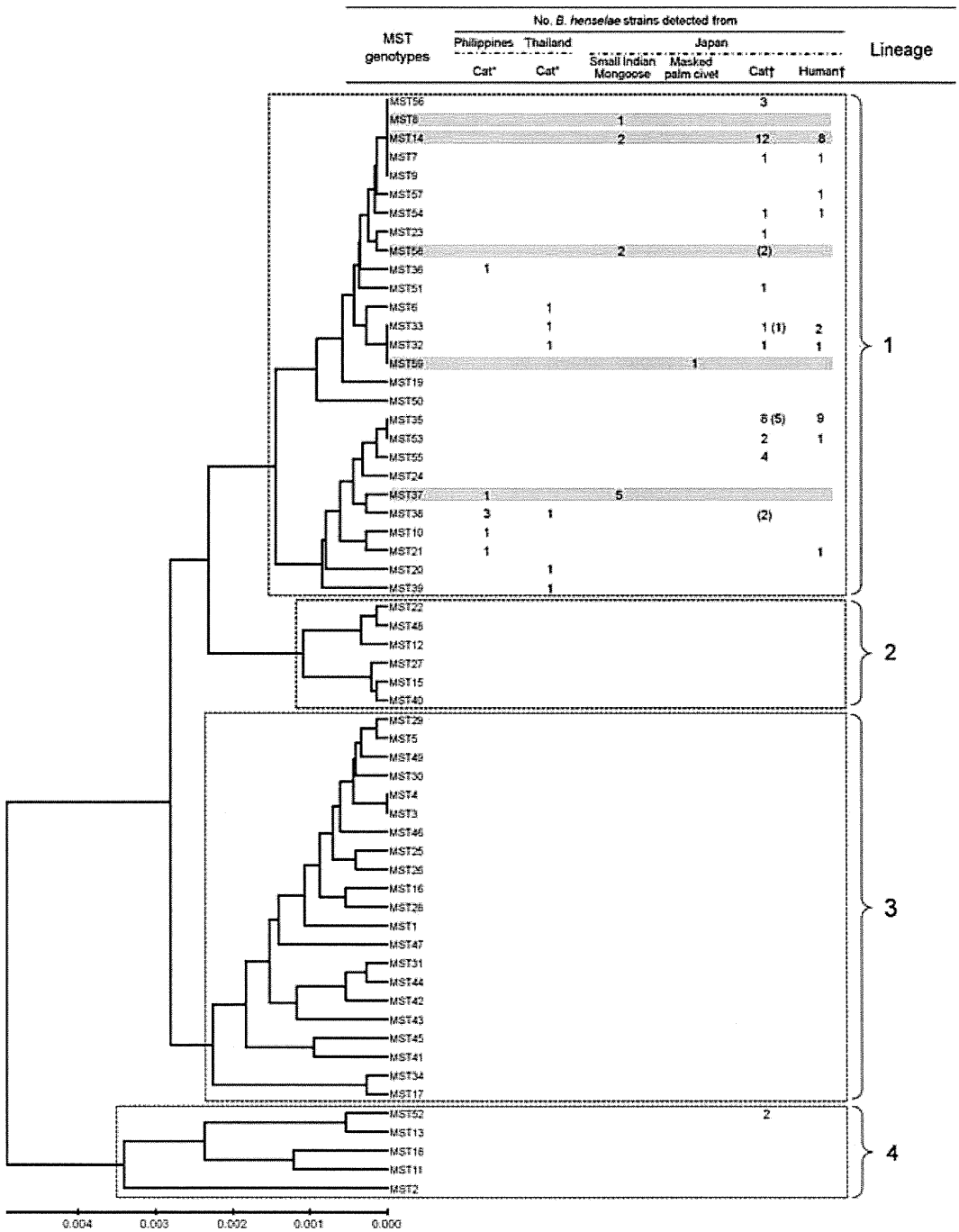

FIG. 1. Phylogenetic tree of *Bartonella henselae* strains from mongooses, masked palm civet, cats and patients with cat-scratch disease (CSD) in Japan based on nine concatenated intergenic spacer sequences. The tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) in MEGA4 software. The *B. henselae* strains isolated from mongooses, a masked palm civet, cats and humans with 57 MST genotypes were included in the analysis. Hatching highlights multispacer typing (MST) genotypes and the numbers of strains from the mongooses, a masked palm civet, cats and humans. The cat strains from the Philippines (*) and Thailand (*) and the cat and human strains from Japan (†) were analysed in the previous reports [13,21,22] and added to this figure. The number of cat strains examined in the present study is shown in parentheses. Dotted rectangles show four lineages of MST genotypes. The scale bar indicates nucleotide substitutions per site.

8 and 37 in the area, so further epidemiological investigations on native mongooses in other Asian countries will allow us to understand the origin of those genotypes of *B. henselae* among Feliformia.

MST 59, detected from the masked palm civet strain, showed a unique genotype. Though the prevalence of cats in Chiba Prefecture where the masked palm civet was captured was 5.0% (1/20, data not shown in the result), the same genotype has not been found in any *B. henselae* strains of the cats from the same area and other prefectures. As only one masked palm civet harbouring the MST 59 genotype was detected in the present study, more samples should be examined to determine whether the genotype is prevalent in animals and humans.

Our investigation showed for the first time that small Indian mongooses and a masked palm civet harboured *B. henselae* and the isolates were grouped into lineage I of MST genotypes with strains derived from cats and from patients with CSD in Japan. Programmes to eradicate introduced mongooses are being carried out in Japan and other countries [29]. Masked palm civets have been sold for human consumption at wild live markets in China [11] and other Asian countries. Interestingly, a CSD case caused by a masked palm civet was reported in 2001 in Japan. In this case, the patient, who was scratched in the left leg by a pet masked palm civet, developed fever and left inguinal lymphadenopathy with high antibody titre (1 : 1024) to *B. hensleae* [30]. Taking into account the similar prevalence to cats in the examined areas, the high bacteraemic levels with no clinical and pathological abnormalities, similar MST genotypes to the cat and human strains of *B. henselae*, and the close contact between humans and these animals, the small Indian mongoose and the masked palm civet in the suborder Feliformia appear to serve as new reservoirs for *B. henselae*, and may play a role as potential sources of human infection.

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Transparency Declaration

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Molecular Epidemiologic Survey of *Bartonella*, *Ehrlichia*, and *Anaplasma* Infections in Japanese Iriomote and Tsushima Leopard Cats

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ABSTRACT: The Iriomote cat (IC; *Prionailurus iriomotensis*) and the Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilura*) are endangered wild felids in Japan. As a part of ongoing conservation activities, we conducted a molecular, epidemiologic survey of *Bartonella*, *Ehrlichia*, and *Anaplasma* infections in wild IC and TLC populations. Blood samples (47 from 33 individual IC; 22 from 13 TLC) were collected between August 2002 and January 2011. Using PCR analysis, we confirmed the presence of *Bartonella henselae* in ICs and *Bartonella clarridgeiae* in TLCs, with prevalences of 6% and 8%, respectively. Using PCR and basic local alignment search tool analyses, we identified *Ehrlichia canis* in both cats and *Anaplasma bovis* in TLCs. The prevalence of *E. canis* was 12% in ICs and 8% in TLCs, and the prevalence of *A. bovis* was 15% in TLCs. This is the first report, to our knowledge, of *B. henselae*, *B. clarridgeiae*, *E. canis*, and *A. bovis* infections in these two endangered species. Continuous monitoring of these pathogens is needed for their conservation.

Key words: *Anaplasma*, *Bartonella*, *Ehrlichia*, Iriomote cat, Tsushima leopard cat.

The Iriomote cat (IC; *Prionailurus iriomotensis*) and the Tsushima leopard cat (TLC; *Prionailurus bengalensis euptilura*) are the only two species or subspecies of wild felids that are indigenous to Iriomote and Tsushima islands in Japan, respectively. Their populations are each estimated to consist of only approximately 100 individuals (Izawa et al., 2009). Although traffic accidents likely pose the biggest threat to Japanese wild cats, another potential threat is infectious diseases. Ectoparasites, such as ticks and lice, have often been observed on TLCs and ICs during our ecologic surveys. Their presence suggests the possibility of arthropod-borne diseases.

Bartonellosis, ehrlichiosis, and anaplasmosis are representative arthropod-borne diseases in felids. *Bartonella* infection was shown in wild felids in North, Central, and South America and in Africa (Molia et al., 2004; Chomel et al., 2006). Domestic cats are susceptible to infection with *Ehrlichia canis* and *Anaplasma phagocytophilum* and display fever, anorexia, and thrombocytopenia. These two pathogens have also been detected in wild felids; however, the virulence of these bacteria, and of *Bartonella*, in these felids is unknown (Filoni et al., 2006). To understand the prevalence of these pathogens, we conducted a molecular epidemiologic survey of *Bartonella*, *Ehrlichia*, and *Anaplasma* in ICs and TLCs.

Capture and sample collection were performed with authorization from the Ministry of the Environment and Agency for Cultural Affairs in Japan. Surveys were performed between August 2002 and January 2011. During that period, 33 ICs on Iriomote island (24°15′–24°25′N, 123°40′–123°55′E) and 13 TLCs on Tsushima island (34°5′–34°42′N, 129°10′–129°30′E) were captured or found dead. Of the 13 ICs found dying or dead, nine were killed in traffic accidents (Table 1). Animals E-60, E-84, and D-43J were found dying or dead and

TABLE 1. Demographics, date of collection, health status at time of acquisition (alive, dead, or dying as a result of automobile accident), and PCR results from Iriomote cats (*Prionailurus iriomotensis*) and Tsushima leopard cats (*Prionailurus bengalensis euptilura*) captured from Iriomote and Tsushima islands from August 2002–January 2011.

Cat identification no.	Gender	Date	Health status	Sample identification no.	PCR results[a]
Iriomote cats					
E-30	Male	23 November 2003	Alive	I-41	—
E-33	Male	26 November 2004	Alive	I-2	—
E-60	Male	27 November 2004	Alive	I-3	—
		4 November 2005	Alive	I-4	—
		4 February 2007	Alive	I-17	BH
		8 February 2010	Dying>dead	I-33	EC
E-61	Female	9 December 2003	Dead	I-1	—
E-67	Male	4 November 2005	Alive	I-5	—
		18 November 2006	Alive	I-13	—
		17 December 2006	Alive	I-39	—
		27 January 2008	Alive	I-22	BH
E-70	Male	6 February 2006	Alive	I-8	—
E-72	Male	7 February 2006	Alive	I-9	—
E-83	Female	17 November 2008	Alive	I-24	—
E-84	Male	03 December 2008	Dead	I-25	—
E-91	Male	14 February 2010	Car-related dying	I-34	—
		17 February 2010	Dead	I-35	—
W-48	Male	7 February 2006	Alive	I-10	—
		20 October2006	Alive	I-38	—
E-71	Male	28 January 2006	Alive	I-7	—
W-87	Female	7 February 2007	Alive	I-21	—
W-99	Male	27 January 2008	Car-related death	I-23	—
W-101	Male	4 February 2007	Alive	I-18	—
W-106	Male	11 December 2005	Alive	I-6	—
		4 February 2007	Alive	I-19	—
W-108	Male	4 February 2007	Alive	I-20	—
W-113	Male	18 November 2006	Alive	I-14	—
W-118	Male	22 October 2006	Alive	I-11	—
W-119J	Female	14 November 2006	Car-related death	I-12	—
W-120	Male	18 November 2006	Alive	I-15	—
W-121	Male	18 November 2006	Alive	I-16	—
W-126	Male	9 January 2010	Alive	I-29	—
W-127	Female	10 January 2010	Alive	I-32	—
		8 January 2011	Alive	I-44	—
W-129	Male	9 January 2010	Alive	I-30	—
		18 December 2010	Alive	I-42	—
		8 January 2011	Alive	I-45	—
W-131J	Male	22 July 2009	Car-related death	I-26	—
W-134	Female	9 January 2010	Alive	I-31	EC
		17 December 2010	Alive	I-43	—
		22 June 2011	Car-related death	I-47	—
W-135	Female	13 April 2010	Car-related death	I-36	—
W-137J	Female	9 September 2010	Car-related death	I-40	EC
W-140	Male	9 January 2011	Alive	I-46	—
D-043J	Male	26 December 2009	Dead	I-27	—
E-89J	Male	3 January 2010	Car-related death	I-28	EC
E-92J	Male	7 May 2010	Car-related death	I-37	—
Tsushima leopard cats					
CFM-20	Female	9 November 2006	Alive	T-5	—
CFS-18	Female	23 August 2002	Alive	T-2	—

TABLE 1. Continued.

Cat identification no.	Gender	Date	Health status	Sample identification no.	PCR results[a]
CFS-26	Female	10 November 2006	Alive	T-7	—
CFT-17	Female	23 August 2002	Alive	T-1	—
CFT-24	Female	12 December 2008	Alive	T-9	—
		24 December 2009	Alive	T-13	AB
		25 December 2010	Alive	T-19	AB
CFT-25	Female	10 November 2006	Alive	T-8	—
		26 December 2009	Alive	T-17	—
CFT-27	Female	12 December 2008	Alive	T-10	—
		24 December 2009	Alive	T-14	AB
		26 December 2010	Alive	T-22	AB
CFT-28	Female	26 December 2009	Alive	T-18	—
CMM-19	Male	23 August 2002	Alive	T-3	EC
		3 November 2005	Alive	T-4	—
		9 November 2006	Alive	T-6	EC
CMS-29	Male	12 December 2008	Alive	T-11	—
		24 December 2009	Alive	T-15	BC
		25 December 2010	Alive	T-20	BC
CMS-32	Male	24 December 2009	Alive	T-16	—
CMT-33	Male	25 December 2010	Alive	T-21	—
MM-22	Male	12 December 2008	Alive	T-12	—

[a] Negative PCR results are indicated with a dash. Positive results are indicated with the initials of pathogens as follows: BH = *Bartonella henselae*; BC = *B. clarridgeiae*; EC = *Ehrlichia canis*; AB = *Anaplasma bovis*.

were reported to the Iriomote Wildlife Conservation Center by local residents. All ICs were severely emaciated; however, direct causes of death could not be identified, even after necropsy. Cat E-84 was found dead at a riverside in a basket used for blue crab fishing and was assumed to have died accidentally. Seven ICs and five TLCs were captured several times; in total, 47 blood samples from ICs and 22 from TLCs were collected.

Total DNA was extracted from 200 μL of each blood sample using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and was used as a template for PCR analyses. To avoid contamination, independent rooms and tools were used for DNA extractions and PCR analysis. As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified to determine the quality of samples. The *GAPDH* gene was successfully amplified from all 69 blood samples, allowing all samples to be used for the PCR-based epidemiologic survey (Fig. 1A).

Nested PCR was applied to amplify the region between the 16S and 23S ribosomal RNA (rRNA) genes from *Bartonella* species (Rampersad et al., 2005), enabling the detection of distinct species based on the size of the amplified DNA fragments. Nested PCR was also used to screen for *Ehrlichia* and *Anaplasma* (Tabar et al., 2008). The sensitivity of PCRs for *Bartonella* and for *Ehrlichia* or *Anaplasma* was determined by using serially diluted plasmid, containing target DNA from *Bartonella henselae* or *Ehrlichia canis*. Both PCR systems had sufficiently high sensitivity to detect as little as one gene copy (Fig. 1B).

Two samples from ICs (E-60 and E-67) and two samples from one TLC (CMS-29) were positive for *Bartonella* DNA (Table 1). Nucleotide sequences of amplified DNA fragments from both ICs showed 100% homology with the *B. henselae* sequence (GenBank accessions AB723703 and AB723704), whereas two clones from TLC (CMS-29) had 100% similarity with a *B. clarridgeiae* sequence, as determined by a basic local alignment search tool (BLAST) analysis (AB723705 and AB723706). Thus, DNA from *B. henselae* and *B. clarridgeiae*

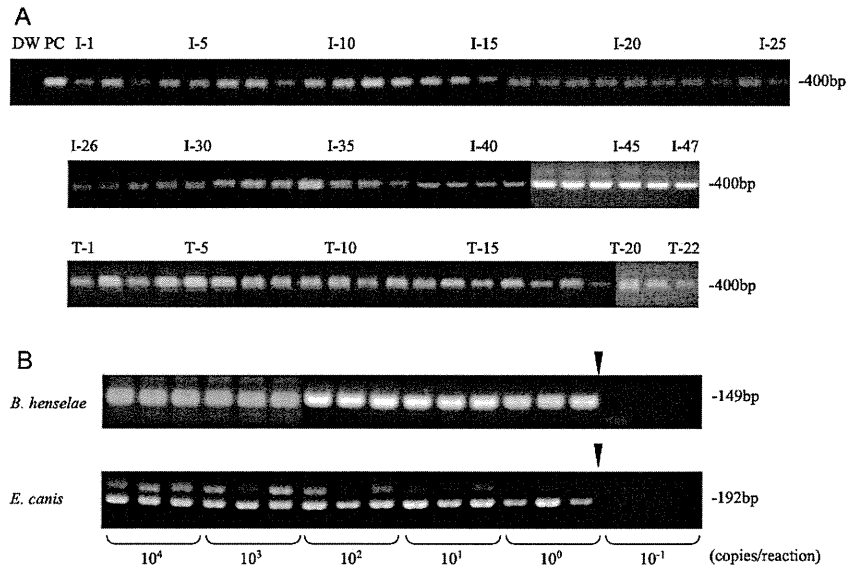

FIGURE 1. Ethidium bromide–stained PCR products after gel electrophoresis of internal control in blood samples obtained from Iriomote cats (*Prionailurus iriomotensis*) and Tsushima leopard cats (*Prionailurus bengalensis euptilura*) during August 2002–January 2011, in Japan, and of detection limit of PCR systems used in this study. (A) The glyceraldehyde-3-phosphate dehydrogenase gene was amplified in all samples, verifying DNA integrity. (B) Determination of the PCR detection limit for *Bartonella*, *Ehrlichia*, and *Anaplasma* species. Arrowheads indicate the detection limit of each PCR. The sample identification is indicated above each lane. DW = distilled water (no template DNA); PC = positive control (genomic DNA from a domestic cat).

was detected in 6% (2 of 33) of the ICs and 8% (1 of 13) of the TLCs, respectively.

Four samples from four ICs (E-60, E-89J, W-134, and W-137J) and six samples from three TLCs (CMM-19, CFT-24, and CFT-27) were positive for *Ehrlichia* and *Anaplasma* by PCR (Table 1). Because of the similarities in the 16S rRNA genes of *Ehrlichia* and *Anaplasma*, further analysis of nearly the complete 16S rRNA gene sequence (ca. 1,370 base pairs) was conducted (Inokuma et al., 2001). All 10 positive samples were successfully amplified. The BLAST analysis showed the nucleotide sequences of six samples (AB723707–AB723712) from four ICs (E-60, E-89J, W-134, and W-137J) and one TLC (CMM-19) were nearly identical (99.6–99.9%) to that of *E. canis*, and the other four samples (AB723713–AB723716) from two TLCs (CFT-24 and CFT-27) were nearly identical (99.6–99.9%) to the corresponding sequence of *A. bovis*. Using phylogenetic analysis, we classified the former six samples as *E. canis*, and the latter four samples grouped with *A. bovis* (Fig. 2). The prevalence of *E. canis* was 12% (4 of 33) in ICs and 8% (1 of 13) in TLCs, respectively. The prevalence of *A. bovis* was 15% (2 of 13) in TLCs.

This is the first study, to our knowledge, to show *Bartonella*, *Ehrlichia*, and *Anaplasma* infections in two Japanese wild cats using molecular techniques. Although *Bartonella* is distributed worldwide, with a high antibody-prevalence in wild felids (Molia et al., 2004; Chomel et al., 2006; Filoni et al., 2006), the prevalence in our PCR-based survey was low in ICs and TLCs. This discrepancy may be due to differences in methodologies that arise when comparing serologic- versus PCR-based surveys (Molia et al., 2004). However, recent PCR-based epidemiologic studies showed that the prevalence may range from 2% to 70% in domestic cats or

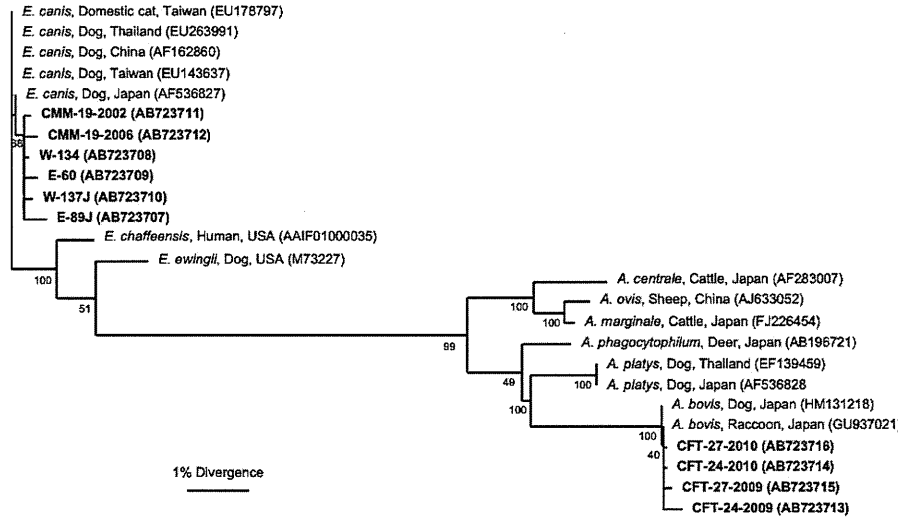

FIGURE 2. Phylogenetic relationship of the partial 16S rRNA gene sequences of *Ehrlichia* and *Anaplasma* isolated from endangered Iriomote cats (ICs; *Prionailurus iriomotensis*) and Tsushima leopard cats (TLCs; *Prionailurus bengalensis euptilura*), in Japan (August 2002–January 2011), and other animals in the database. Six clones of the *Ehrlichia canis* 16S rRNA gene were obtained from four ICs (E-60, E-89J, W-134, and W-137J; Table 1) (AB723707–AB723710) and one TLC (CMM-19) (AB723711 and AB723712). Four clones of the *Anaplasma bovis* 16S rRNA gene were obtained from two TLCs (CFT-24 and CFT-27) (AB723713–AB723716). Accession numbers in DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank or Whole Genome Shotgun (WGS) databases are also indicated in parentheses for other compared sequences: *E. canis* from a cat (*Felis catus*) in Taiwan (EU178797), a dog (*Canis lupus familiaris*) in Taiwan (EU143637), a dog in Thailand (EU263991), a dog in China (AF162860), and a dog in Japan (AF536827); *Ehrlichia chaffeensis* (AAIF01000035); *Ehrlichia ewingii* (M73227); *A. bovis* from a dog in Japan (HM131218) and a raccoon (*Procyon lotor*) in Japan (GU937021); *Anaplasma centrale* (AF283007); *Anaplasma marginale* (FJ226454); *Anaplasma ovis* (AJ633052); *Anaplasma platys* from dogs in Japan (AF536828) and Thailand (EF139459); and *Anaplasma phagocytophilum* (AB196721). Numbers under internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch.

wild felids (Maruyama et al., 2000; Chomel et al., 2006; Pennisi et al., 2010). These findings suggest that the prevalence of *Bartonella* infection may be influenced by regional and ecologic factors. We detected *B. henselae* and *B. clarridgeiae* only in ICs and TLCs, respectively. Additional studies with larger sample sizes are warranted to confirm that observation.

Fleas are a vector of *Bartonella* species between cats (Chomel et al., 1996). Infestation with fleas was rarely detected in ICs and TLCs, and that observation may be related to the low prevalence of *Bartonella* spp. Future prospective, longitudinal studies of ICs and TLCs would be useful to confirm that observation, as some of these animals were found dead or dying. In contrast, recent studies have shown that *Ixodes ricinus* is also a vector of *B. henselae* (Cotté et al., 2008). During the period of this study, >80% of Japanese wild cats were infested with ticks, and at least three tick species in ICs (*Amblyomma testudinarium*, *Haemaphysalis longicornis*, and *Haemaphysalis hystricis*) and four tick species in TLCs (*A. testudinarium*, *Ixodes tanuki*, *Haemaphysalis megaspinosa*, and *Haemaphysalis campanulata*) were identified (Matsuo, pers. comm.). Future surveys of the prevalence of *Bartonella* spp. in ticks from these wild cats would be of value.

Ehrlichia canis–derived DNA in blood is rarely detected by PCR analysis (Filoni et al., 2006). Yet, for unknown reasons, detection rates of *Ehrlichia* in our study were high. Bacteremia should be evaluat-

ed in a larger number of samples and include serologic testing. We are the first, to our knowledge, to detect *A. bovis* DNA in Japanese wild cats. Recently 1.1% of domestic dogs and 0.1% of domestic cats were found to be PCR-positive for *A. bovis* in Japan (Sakamoto et al., 2010; Sasaki et al., 2012). *Anaplasma phagocytophilum* was also reported in ruminants and carnivores (Aktas et al, 2011; Rymaszewska and Adamska, 2011). These findings suggest that *Anaplasma* species have a wide host range, including carnivores. Wild deer in Japan may be infected with *A. bovis* (Kawahara et al., 2006), and heavy tick infestations are observed in wild deer on Tsushima Island. Therefore, we propose that wild deer may serve as a reservoir for *A. bovis* on Tsushima Island. An epidemiologic survey of wild animals on Tsushima Island will be necessary to understand the ecology of the *Anaplasma* species.

It is of great interest to identify which tick species transmit *E. canis* or *A. bovis* to wild cats. *Rhipicephalus sanguineus* is a major vector for *E. canis*; however, we did not observe this tick in either species of wild cat (Groves et al., 1975). Furthermore, *H. longicornis* is suggested to be a major vector for *A. bovis* in Japan, although *H. megaspinosa* and *H. campanulata*, but not *H. longicornis*, were found in TLCs (Kawahara et al., 2006). Future work is planned to identify the tick species responsible for transmitting *E. canis* or *A. bovis* to wild cats.

We detected *B. henselae*, *B. clarridgeiae*, *E. canis*, and *A. bovis* in two endangered species of Japanese wild cats. However, the origin of these pathogens, the prevalence of antibodies against them, and their virulence determinants are yet to be elucidated. Continuous monitoring of *Bartonella*, *Ehrlichia*, and *Anaplasma* infections is required for the conservation of these wild cats.

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