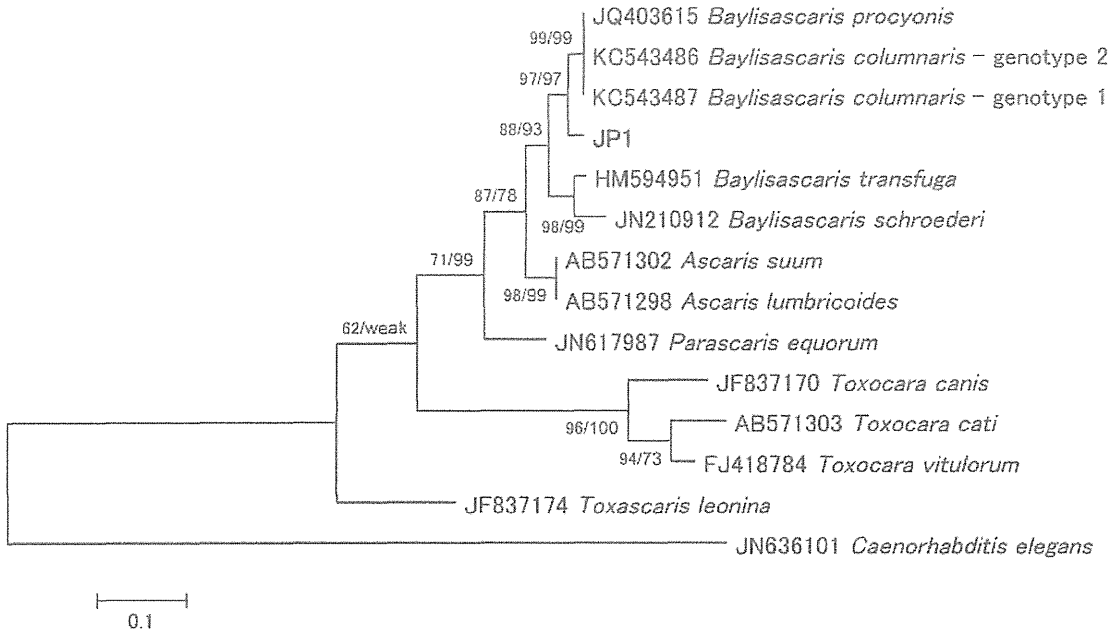

Fig. 3. Dendrogram constructed by neighbor-joining (NJ) and maximum likelihood (ML) methods based on ITS2 nuclear sequences of *Baylisascaris* and related ascarid species; JP1 isolate was examined in the present study; Numbers at the nodes of branches refer to bootstrap values (1,000 pseudoreplications) derived from NJ (first value) and ML analyses. *Weak* bootstrap support was indicated at values < 60%

isolate JP1 from the related species is attributed to 22 – 26 insertions from positions 264 upwards, partially consisting of G-A tandem repeats, the number of which consistently distinguishes *Baylisascaris* species. For nucleotide substitutions, the most polymorphic region for JP1 isolate, comprising 5 of 12 base exchanges, was located between positions 231 – 244 of ITS2 (Fig. 2). Among recorded nucleotide exchanges relative to *B. procyonis*, 9 were transitions and 3 transversions. Resulting transition/transversion (ts/tv) ratio 3:1 for these taxa is generally assumed for closely related organisms.

Phylogenetic trees constructed by NL and ML analyses of ITS2 sequences of the 11 ascarid species revealed the similar topology, with two large clades separating Ascarididae and Toxocaridae isolates. The analyzed JP1 isolate was located in a well-supported subclade (bootstrap 88 % in NJ, 93 % in ML) consisting of *Baylisascaris* representatives, with the two clusters being inferred for this genus. The JP1 isolate has grouped together with *B. procyonis* and *B. columnaris*, with some discrimination from these taxa (Fig. 3). From remaining *Baylisascaris* species, *B. transfuga* and *B. schroederi* located in second cluster, the JP1 isolate differed in 14.3 % and 15.4 % of ITS bases, respectively.

Discussion

In the present study, a peculiar genetic composition of a *Baylisascaris* isolate from a pet kinkajou imported to Japan from South America, consistently differing from the closest *B. procyonis* with public health relevance, was recorded.

The ascarid eggs detected in feces from the kinkajou were morphologically similar to those of *B. procyonis* (raccoon roundworm), with eggs measuring 63-88 μm (mean: 68-76 μm) by 50 – 70 μm (mean: 55-61 μm), and *B. columnaris* (skunk roundworm) with eggs measuring 72.5 ± 4.1 μm by 63.2 ± 7.5 μm (Kazacos *et al.*, 2011; Franssen *et al.*, 2013). Slightly closer values with regard to the JP1 isolate were thus associated with *B. columnaris*, particularly in the length of transverse axis (64.4 ± 1.02 μm in JP1 isolate). Nevertheless, morphometric identification in *Baylisascaris* is often hampered by diversity in size and developmental stages that makes the unequivocal recognition of several species including *B. procyonis* and *B. columnaris* problematic (Berry, 1985; Franssen *et al.*, 2013).

The nuclear rDNA internal transcribed spacer is extensively being used for phylogenetic reconstruction and distinguishing between closely related species, including ascarids (Zhu *et al.*, 2001; Pawar *et al.*, 2012). In the present study, a unique divergence pattern was derived from the sequences of the ITS2 region for the kinkajou isolate suggesting that the nematode could not be entirely allocated to the closest recognized *Baylisascaris* species. Molecular prospecting as a tool for search and evaluation of the existence of separate taxa within the morphospecies has been often addressed to more clarify systematics in ascarid nematodes (Nadler & Huspeth, 2000; Derycke *et al.*, 2010). As Blouin (2002) summarized, the level of fixed sequence difference in ITS between closely related nematodes is relatively frequently ≤ 1 %, unlike mitochondrial DNA, in which helminth species typically differ in at least 10 % of sequences (Vilas *et al.*, 2005). The 7.8 – 8.8 %

range of nucleotide difference between the JP1 isolate and the closest retrieved sequences of *B. procyonis* and *B. columnaris* in ITS2 therefore classified the isolate from the pet kinkajou as referring to *Baylisascaris* sp., with its precise species categorization remaining to be determined.

Another question emerges whether or not the present isolate pertains to any described *Baylisascaris* spp., for which sequencing data are not yet available in GenBank. The genus currently contains eight recognized species (Kazakos, 2013). From these, apart from 4 species with available nuclear sequences, transmissions of *B. melis* linked to badgers absent in South America, and *B. tasmaniensis* with marsupial carnivore hosts endemic for Tasmania, exclude these taxa as plausible for infesting kinkajou in Guyana. From remaining species, *B. laevis*, morphologically similar to *B. columnaris* and *B. procyonis* (Berry, 1985; Anderson, 2000), occurring in large rodents as marmots and ground squirrels, and *B. devosi* occurring in martens and fishers, are transmissible in South American wildlife. A type material from these species is therefore needed to be subjected to further sequence analyses to better clarify the taxonomic issue in the JP1 isolate.

Vilas *et al.* (2005) appointed that because the ITS is a non-coding sequence, frequent deletions and insertions are often present making alignment more complicated. This was also the case of ribosomal data gathered in this study, with the preponderance of insertions in the examined isolate compared to the present *Baylisascaris* sequences. A varying number of G-A tandem repeats can be used as discriminative marker for *Baylisascaris* spp. in the specific zone of the ITS2 region (positions 246 and further). Nine G-A tandem repeats were identified for *B. procyonis*, eight for *B. transfuga*, seven and six for the two *B. columnaris* genotypes, and one for *B. schroederi* that likely represents the ancestral state of the given DNA region (Testini *et al.*, 2011; Lin *et al.*, 2012; Franssen *et al.*, 2013). For the JP1 isolate, 11 tandem contiguous G-A repeats were recorded in the respective locations. Two of them along with the additional interspersed motifs with G, A bases have constituted 22-26 insertions compared to *B. procyonis* and *B. columnaris*, indicating a tendency towards lengthening the ITS2 region. The proposed mechanisms of evolution of repetitive sequences include both intra- or interstrand recombinational effects or mechanisms involving failures in the DNA replication (Platas *et al.*, 2001). Mutational changes can create new motifs that may be propagated by additional slipped-strand mispairing (SSM) events. This was the plausible scenario for amplifying G-A repetitions in *Baylisascaris* spp., which formerly likely arose by chance in ITS2 and could have been further generated by SSM events into longer repeats.

Based on the gathered data, it is likely that the peculiar *Baylisascaris* species circulating in Guyanese zoofauna has been translocated to Japan via the infected kinkajou. Nevertheless, further characterization of *Baylisascaris* isolates from other kinkajou hosts is required, with particular emphasis on adult morphology and the pathogenicity of migrating larvae in paratenic hosts. In addition, to address potential limitations of single locus analyses, both nuclear and mtDNA regions will be targeted in molecular assays of *Baylisascaris* sp. to facilitate species delineation.

Till now, there are over 20 documented cases of ocular larva migrans and severe or even fatal neurological disturbances in humans implied by *B. procyonis* (Galvin *et al.*, 2005; Bauer, 2013). Zoonotic potential of *B. columnaris* is not yet known as serological assays do not discriminate between *Baylisascaris* species (Dangoudoubiyam et al., 2011). Experimental infections showed that *B. procyonis* is more pathogenic to mice than *B. columnaris* owing to faster growth to 1 mm size, which correlates with the first appearance of nervous symptoms (Tiner, 1953; Sheppard & Kazakos, 1997). However, both species are along with *B. melis* (principal host badger) regarded as the most dangerous *Baylisascaris* members because of their serious disease-producing capabilities (Kazakos, 2001).

Given that the *Baylisascaris* sp. from the kinkajou is genetically closely affiliated with *B. procyonis*, the potential risk of human infection with *Baylisascaris* from this host may be considerable. Therefore, transmission-based precautions are needed to prevent human infections of pet kinkajous. It is strongly recommended that owners should take their kinkajous regularly to veterinarian for periodic fecal examination.

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Genetic Characterization of Coronaviruses from Domestic Ferrets, Japan

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We detected ferret coronaviruses in 44 (55.7%) of 79 pet ferrets tested in Japan and classified the viruses into 2 genotypes on the basis of genotype-specific PCR. Our results show that 2 ferret coronaviruses that cause feline infectious peritonitis–like disease and epizootic catarrhal enteritis are enzootic among ferrets in Japan.

An epizootic catarrhal enteritis (ECE) was first recognized in domestic ferrets (*Mustelo putorius furo*) in the United States in 2000 (*1*). The causative agent of ECE was demonstrated to be a novel ferret coronavirus (FRCoV) belonging to the genus *Alphacoronavirus* (*1*,*2*). Ferrets with ECE showed general clinical signs of lethargy, anorexia, and vomiting and had foul-smelling, green mucous–laden diarrhea. A systemic infection of ferrets closely resembling feline infectious peritonitis (FIP) was subsequently reported among ferrets in the United States and Europe. The causative agent was also shown to be an *Alphacoronavirus*, which was named ferret systemic coronavirus (FRSCV) (*3*,*4*); this virus was found to be genetically distinct from those associated with ECE and from 2 viruses assigned to different genotypes (*5*). Other cases of ECE and ferret infectious peritonitis have since been described in the United States and in Europe (*2*–*4*,*6*,*7*). One case of pathology-confirmed FIP-like disease has been described among domestic ferrets in Japan (*8*). The goal of this study was to determine the prevalence of coronavirus among domestic ferrets seen by veterinarians in various parts of Japan.

The Study

Fecal samples were collected during August 2012–July 2013 from 79 ferrets from 10 animal hospitals scattered across 5 prefectures in Japan. Most of the ferrets were brought to veterinarians for clinical signs such as diarrhea, abdominal masses, and hypergammaglobulinemia; some had signs unrelated to coronavirus infection or were asymptomatic (Table 1). The diarrhea tended to be mild, unlike with ECE, and was found in coronavirus-negative and -positive animals.

RNA was extracted from fecal samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription PCR (RT-PCR) was performed by using the QIAGEN OneStep RT-PCR Kit (QIAGEN) using coronavirus consensus primers IN-6 and IN-7, which amplify the open reading frame (ORF) 1b region, encoding RNA-dependent RNA polymerase (RdRp). This primer pair can amplify nucleic acids from many coronaviruses in the subfamily *Coronavirinae* (*9*). Of 79 samples, 33 (41.8%) were positive for coronaviruses by RT-PCR (Table 2). Nucleotide sequences were determined for the amplified fragments and used to construct a phylogenetic tree (Figure 1). The coronaviruses detected in this study belonged to the genus *Alphacoronavirus* but formed a separate species from those of other species. The identities with feline coronavirus, transmissible gastroenteritis virus, porcine respiratory coronavirus, and mink coronavirus were 73.5%–75.9%, 73.5%–76.1%, 73.8%–76.1%, and 80.2%–84.0%, respectively.

On the basis of additional sequence data, a new primer pair was designed: forward FRCoV RdRp-F1 (5′-GTT GGT TGC TGC ACA CAT AG-3′) and reverse FRCoV RdRp-R1 (5′-GGA GAA GTG CTT ACG CAA ATA-3′). Results for RT-PCR using this new primer set showed that 44 (55.7%) of 79 samples were positive for coronavirus, which was a higher number than that obtained by using the published coronavirus consensus primers (55.7% vs. 41.8%) (Table 2). Two samples that had positive results by consensus primers had negative results by the new primers: sample 22 had many mutations in the primer binding site (Figure 1), whereas sample 40 had few mutations.

On the basis of the partial sequences of the spike gene, Wise et al. (*5*) reported that the known ferret coronaviruses could be divided into 2 genotypes: genotype 1, which included the agent of FIP-like disease, and genotype 2, which included the causative agent of ECE. To differentiate between these genotypes in the positive samples from our testing, RT-PCR was carried out by using 2 pairs of genotype-specific primers: forward primer 5′-CTG GTG TTT GTG CAA CAT CTA C-3′ and reverse primer 5′-TCT ATT TGC ACA AAA TCA GAC A-3′ for genotype 1, and forward primer 5′-GGC ATT TGT TTT GAT AAC GTT G-3′ and reverse primer 5′-CTA TTA ATT CGC ACG AAA TCT GC-3′ for genotype 2 (*5*). Among these ferrets, 30 (38.0%) were infected with genotype 1 and 17 (21.5%)

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Table 1. Detection of FRCoV from ferrets with clinical signs, Japan

Sample type	No. (%) samples			
	Diarrhea, n = 34	Hypergammaglobulinemia, n = 6	Abdominal mass, n = 14	Nonrelated signs/ asymptomatic, n = 33
All FRCoV-positive samples†	25 (73.5)	5 (83.3)	7 (50.0)	17 (51.5)
Genoype I samples‡	17 (50.0)	2 (33.3)	4 (28.6)	10 (30.3)
Genoype II samples§	7 (20.6)	1 (16.7)	4 (28.6)	7 (21.2)

*FRCoV, ferret coronavirus; RT-PCR, reverse transcription PCR.
†RT-PCR was carried out by using FRCoV-specific primers.
‡RT-PCR was carried out by using type 1 FRCoV-specific primers (*5*).
§RT-PCR was carried out by using type 2 FRCoV-specific primers (*5*).

with genotype 2; 8 (10.1%) ferrets were infected with both genotypes of coronaviruses (Figure 2). Samples 27 and 28 were from ferrets that lived in the same house and harbored the same ferret coronavirus but that were born on different farms, indicating that horizontal transmission had occurred. The nucleotide sequences of the amplified genes confirmed that these coronaviruses also fell into genotypes 1 and 2 (Figure 2).

Our results indicate that both genotypes of coronavirus have been spreading within the ferret population in Japan for some time, and some ferrets have been coincidentally infected with both genotypes. Of note, most ferrets that were positive for genotype 1 ferret coronavirus in this study did not show FIP-like disease (Table 1), indicating that infection with genotype 1 ferret coronavirus does not always cause FIP-like disease. Genotype 1 ferret coronavirus has also been detected from asymptomatic ferrets in the Netherlands (*11*).

To further investigate virus transmission routes, oral swab specimens were collected from 14 of the 79 ferrets and examined by RT-PCR using primers FRCoV RdRp-F1 and FRCoV RdRp-R1. Five (35.7%) specimens were positive (data not shown), providing a route leading to infection of susceptible animals. Coronaviruses are known to cause both respiratory and intestinal diseases in various animal species; therefore, ferret coronaviruses should be investigated in respiratory disease.

Conclusions

We established a sensitive RT-PCR method using a new primer pair to detect coronavirus sequences and demonstrated that ferret coronaviruses are widespread among ferrets in Japan. We determined the partial nucleotide sequences of the spike gene of 23 strains and found they were clearly divided into 2 genotypes, 1 and 2 (Figure 2). The reported ferret coronaviruses associated with FIP-like disease, designated as genotype 1 by Wise et al. (*5*), all fell within genotype 1 phylogenetically, whereas all published ECE-causing strains fell within genotype 2. This finding leads to a possible conclusion that FIP-like disease–causing strains (i.e., FRSCVs) are variants of what has been designated genotype 1 ferret coronaviruses. Because we found no relationship between the 2 genotypes of ferret coronavirus and the type of disease (Table 1), we cannot determine whether FIP-like and ECE-like ferret coronaviruses circulate independently as distinct entities or evolve, like feline coronaviruses, from more ubiquitous and less pathogenic enzootic strains. Nonetheless, the addition of these 23 new isolates to the phylogenetic tree of ferret coronaviruses tends to support the latter conclusion. Without extensive animal passage studies, virus isolation, and coronavirus-free ferrets, this theory may be difficult to confirm. However, additional evidence tends to link virulent pathotypes of ferret coronaviruses to specific mutational events. Nucleotide sequences of the 3c-like protein genes of FRSCV, MSU-1 (DDBJ/EMBL-Bank/GenBank accession no. GU338456), MSU-S (GU459059), and WADL (GU459058), showed that 2, MSU-1 and WADL, possessed a truncated 3c-like protein gene (*5*), similar to that described for FIP viruses of cats (*12–14*). FIP-causing viruses of cats also contain a second mutation in the spike gene (*15*), which was not investigated in our study. The existence of 2 major genotypes of Japanese ferret coronaviruses is also reminiscent of the serotype I and II feline coronaviruses. Without ferret coronaviruses that can be grown in cell culture, however, such serologic differentiation will be difficult.

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Table 2. Comparison of results for detection of FRCoV in ferret fecal samples by RT-PCR using coronavirus consensus and FRCoV-specific primers, Japan

Coronavirus consensus primers	FRCoV-specific primers		Total no. (%)
	No. positive samples	No. negative samples	
No. positive samples	31	2	33 (41.8)
No. negative samples	13	33	46 (58.2)
Total no. (%)	44 (55.7)	35 (44.3)	79

*FRCov, ferret coronavirus; RT-PCR, reverse transcription PCR.

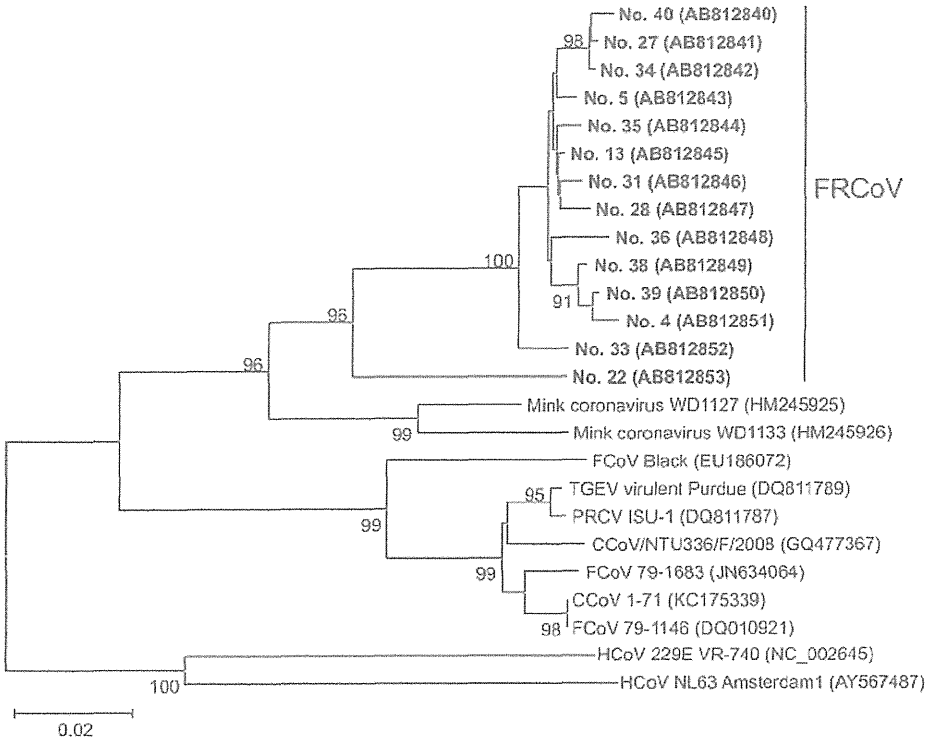

Figure 1. Phylogenetic tree constructed on the basis of the nucleotide sequences of the partial RNA-dependent RNA polymerase–encoding regions of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. Human CoVs (HCoVs) 229E and NL63, which belong to the *Alphacoronavirus* genus, were used as the outgroup. CCoV, canine coronavirus; FCoV, feline coronavirus; TGEV, transmissible gastroenteritis virus; PRCoV, porcine respiratory coronavirus. Scale bar indicates nucleotide substitutions per site.

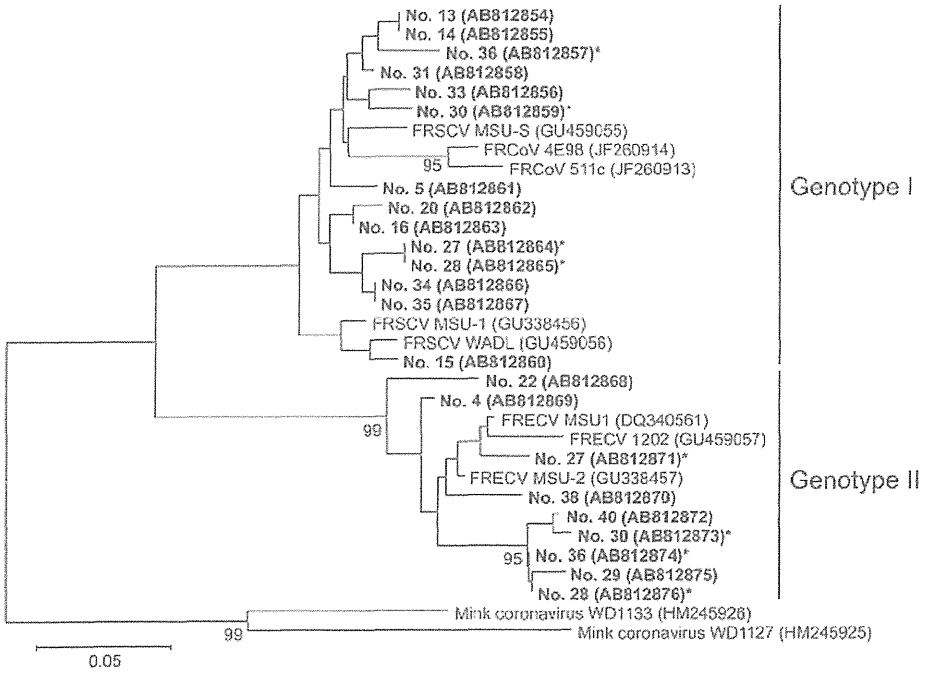

Figure 2. Phylogenetic tree based on the nucleotide sequences of partial S genes of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. Asterisks indicate samples from ferrets infected with FRCoVs of both genotypes1 and 2. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. FRSCV, ferret systemic coronavirus; FRECV, ferret enteric coronavirus. Scale bar indicates nucleotide substitutions per site.

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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 I 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 I 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

			Genotypes									
Strain name	Animal source	Prefecture	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
PLI8	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

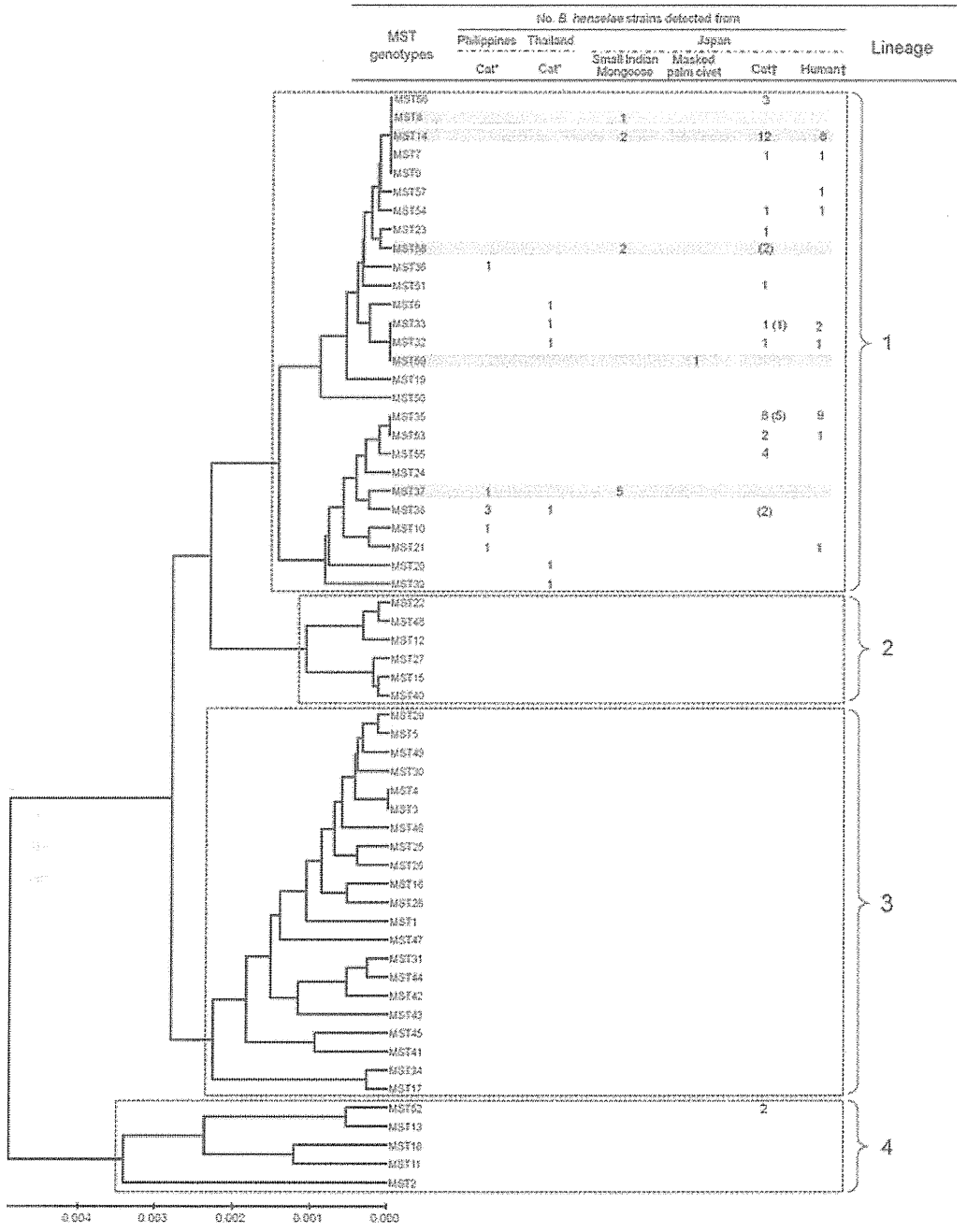

FIG. 1. Phylogenetic tree of *Bartonella henselae* strains from mongooses, masked palm civet, cats and patients with cat-scratch disease (CSD) in Japan based on nine concatenated intergenic spacer sequences. The tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) 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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Bartonella jaculi sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov. and *Bartonella acomydis* sp. nov., isolated from wild Rodentia

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Four novel strains of members of the genus *Bartonella*, OY2-1^T, BR11-1^T, FN15-2^T and KS2-1^T, were isolated from the blood of wild-captured greater Egyptian jerboa (*Jaculus orientalis*), plantain squirrel (*Callosciurus notatus*), fat-tailed gerbil (*Pachyuromys duprasi*) and golden spiny mouse (*Acomys russatus*). All the animals were imported to Japan as pets from Egypt, Thailand and the Netherlands. The phenotypic characterization (growth conditions, incubation periods, biochemical properties and cell morphologies), DNA G+C contents (37.4 mol% for strain OY2-1^T, 35.5 mol% for strain BR11-1^T, 35.7 mol% for strain FN15-2^T and 37.2 mol% for strain KS2-1^T), and sequence analyses of the 16S rRNA genes indicated that those strains belong to the genus *Bartonella*. Sequence comparisons of *gltA* and *rpoB* genes suggested that all of the strains should be classified as novel species of the genus *Bartonella*. In phylogenetic trees based on the concatenated sequences of five loci, including the 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and the ITS region, and on the concatenated deduced amino acid sequences of three housekeeping genes (*ftsZ*, *gltA* and *rpoB*), all strains formed distinct clades and had unique mammalian hosts that could be discriminated from other known species of the genus *Bartonella*. These data strongly support the hypothesis that strains OY2-1^T, BR11-1^T, FN15-2^T and KS2-1^T should be classified as representing novel species of the genus *Bartonella*. The names *Bartonella jaculi* sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov. and *Bartonella acomydis* sp. nov. are proposed for these novel species. Type strains of *Bartonella jaculi* sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov. and *Bartonella acomydis* sp. nov. are OY2-1^T (=JCM 17712^T=KCTC 23655^T), BR11-1^T (=JCM 17709^T=KCTC 23909^T), FN15-2^T (=JCM 17714^T=KCTC 23657^T) and KS2-1^T (=JCM 17706^T=KCTC 23907^T), respectively.

The genus *Bartonella* is classified in the class *Alphaproteobacteria*, order *Rhizobiales* and family *Bartonellaceae*, and at the time of writing consists of 24 species and three subspecies. The bacteria in the genus *Bartonella* show fastidious, Gram-negative, aerobic and slow-growing properties. *Bartonella* strains have been documented to infect erythrocytes of various mammals, especially rodents in North America (Kosoy *et al.*, 1997), Asia (Ying *et al.*, 2002; Inoue *et al.*, 2008; Inoue *et al.*, 2010), Australia (Gundi *et al.*, 2009) and Europe (Birtles *et al.*, 1995; Tea *et al.*, 2004). We have already reported that rodents and squirrels imported to Japan as pets from Asia, North America, Europe and the Middle and Near East carried organisms of the genus *Bartonella* at a high prevalence (26.0 %). In the

The GenBank/EMBL/DDBJ accession numbers for the partial sequences of the 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and ITS regions of the four type strains described in this paper are as follows: *Bartonella jaculi* OY2-1^T, AB602527, AB602539, AB444975, AB529934 and AB602557, respectively; *Bartonella callosciuri* BR11-1^T, AB602530, AB602542, AB602551, AB529931 and AB602560, respectively; *Bartonella pachyuromydis* FN15-2^T, AB602531, AB602543, AB444978, AB602555 and AB602561, respectively; *Bartonella acomydis* KS2-1^T, AB602533, AB602545, AB444979, AB529942 and AB602563, respectively. Accession numbers for partial sequences of the above five loci in the other four isolates described in this paper (OY5-1, BR1-1, FN18-1 and KS7-1) are summarized in Table 1.

A supplementary figure and supplementary table are available with the online version of this paper.

previous study, a total of 407 isolates were obtained from the 142 bacteraemic animals and the isolates were classified into 10 genogroups consisting of four zoonotic known species of the genus *Bartonella* and six novel species of the genus *Bartonella* according to the phylogenetic analysis based on the sequence of *gltA* gene (Inoue *et al.*, 2009). The purpose of the present study was to further characterize four of the six novel species of the genus *Bartonella* by biochemical, morphological and genetic approaches, including multilocus sequencing analysis of four housekeeping genes and the 16S–23S rRNA intergenic spacer region. Furthermore, we compared the specific hosts of the isolates with species of the genus *Bartonella* with validly published names because Bartonellae have evolved towards adaptation to specific mammalian hosts (Chomel *et al.*, 2009).

Four novel species of the genus *Bartonella* were isolated from the rodents and squirrels from Egypt, Thailand and the Netherlands, and two strains from each animal species were selected from the each clade in the phylogenetic tree of the *gltA* gene for further analysis. The strains and their animal sources are summarized in Table 1. Each strain was grown on heart infusion agar plates (Difco) containing 5 % defibrinated rabbit blood (HIA) at 35 °C in a moist atmosphere under 5 % CO_2 for 14 days. After Gram staining, each strain was observed by light microscopy (Olympus) at × 1000 magnification. The surface structures of the cells were analysed by transmission electron microscopy (model JEM1200EX; JEOL) at 100 kV with negative staining. Cytochrome oxidase test strips (Nissui) were used for evaluating the oxidase activity of the bacteria. Catalase activity was examined by mixing fresh colonies that had been cultured on rabbit blood–chocolate HIA plates for 14 days at 35 °C in a moist atmosphere containing 5 % CO_2 with 3 % H_2O_2 on a glass slide. A total of 23 biochemical characteristics were assessed by using a MicroScan Rapid Anaerobe Panel (Dade Behring) according to the manufacturer's instructions as previously described (Welch *et al.*, 1993).

Genomic DNA was extracted from each strain by using the Instagene Matrix (Bio-Rad) according to the manufacturer's instructions. Partial sequences of the 16S rRNA-encoding gene (16S rRNA), the cell division protein gene (*ftsZ*), the citrate synthase gene (*gltA*), the RNA polymerase beta-subunit-encoding gene (*rpoB*) and 16S–23S rRNA intergenic spacer region (ITS region) were amplified by PCR as described previously (Birtles & Raoult, 1996; Heller *et al.*, 1997; Renesto *et al.*, 2001; Houpikian & Raoult, 2001; Zeaiter *et al.*, 2002). The sequence data were submitted to GenBank and the accession numbers were obtained (Table 1). In addition, the full-length sequence of another cell division protein gene (*ftsY*) was determined and the DNA G+C content (mol%) of each strain was estimated by the sequence as previously described (Fournier *et al.*, 2006; Fournier *et al.*, 2007; Mediannikov *et al.*, 2008). The primers for amplification of the full-length *ftsY* gene were originally designed in this study (forward primer, 5′-CHGGAACAATTGTTMAAGC-3′; reverse primer, 5′-CYAATGGHCCCATTTCYAAG-3′; sequencing primer 1, 5′-ACTAGGDGCAGATGCYGC-3′; sequencing primer 2, 5′-TYGGCAAAGTCAGARGC-3′). PCR amplification for *ftsY* gene was performed as follows: a 5 min denaturation at 95 °C was followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 47 °C and extension for 60 s at 72 °C.

PCR products were purified by using a Spin Column PCR product purification kit (Bio Basic) then sequenced directly by using dye terminator chemistry and a Genetic Analyzer model 3130 (Applied Biosystems).

For the phylogenetic analysis, the sequence data for the 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and the ITS region of the strains were aligned and subsequently compared with those of type strains of known species of the genus *Bartonella* from GenBank by using the CLUSTAL W program in MEGA 4.0.2 software (Tamura *et al.*, 2007). The phylogenetic tree was constructed by the neighbour-joining method from the concatenated sequences of the 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and the ITS region with the Jukes–Cantor parameters distance model. The phylogenetic tree of the concatenated deduced amino acid sequences of the *ftsZ*, *gltA* and *rpoB* gene products was also constructed by using the neighbour-joining method and the evolutionary distances were computed using the Poisson correction method. Support for the nodes in the trees was assessed by bootstrapping with 1000 replicates. *Brucella melitensis* 16M^T was used as an outgroup.

All of the strains grown on HIA formed smooth, transparent to grey–whitish colonies of 1−2 mm in diameter. Colonies of most strains were round, but colonies of strains KS2-1^T and KS7-1 were irregular. Gram-negative, coccobacilli to short rod-shaped bacteria were observed for all of the strains by light microscopy after 14 days culture. Electron microscopic examination showed that strains OY2-1^T, BR11-1^T, FN15-2^T and KS2-1^T were similar in cell size: the length ranging from 1.15 to 1.45 μm and width ranging from 0.52 to 0.59 μm, respectively (Fig. 1). The strains had no flagella, but pili-like structures around the cell body were observed in the four strains (Fig. 2).

All of the strains were negative for oxidase, catalase and urease activities. Nitrate reduction, indole production and indoxyl phosphate degradation were not observed in any of the strains. None of the strains hydrolysed trehalose and most of *p*-nitrophenyl substrates: *N*-acetyl-β-D-glucosaminide, α-D-galactopyranoside, β-D-galactopyranoside, α-D-glucopyranoside, β-D-glucopyranoside, α-L-fucopyranoside and α-D-mannopyranoside. Strains FN15-2^T, FN18-1, KS2-1^T and KS7-1 hydrolysed bis-*p*-nitrophenyl phosphate, but strains OY2-1^T, OY5-1, BR1-1 and BR11-1^T did not. Amino acid arylamidase activities towards leucine, methionine, lysine (alkaline as well as acidic), glycine, glycylglycine, arginine and tryptophan were observed in all the strains, but there was no activity for pyrrolidonyl. All of the strains except BR1-1 and BR11-1^T showed the ability to degrade L-proline-β-naphthylamide. The results of these biochemical tests are similar to those for other members of

Table 1. Host animals, GenBank accession numbers and sequence similarities (%) of five loci in four novel species of the genus *Bartonella*

Abbreviations: B. a, *B. alsatica*; B. b, *B. birtlesii*; B. c, *Bartonella clarridgeiae*; B. d, *B. doshiae*; B. e, *B. elizabethae*; B. g, *B. grahamii*; B. h, *B. henselae*; B. k, *Bartonella koehlerae*; B. que, *Bartonella queenslandensis*; B. qt, *Bartonella quintana*; B. r, *B. rattaustraliani*; B. s, *B. silvatica*; B. tay, *Bartonella taylorii*; B. tri, *B. tribocorum*; B. v. a, *Bartonella vinsonii* subsp. *arupensis*; B. v. v, *Bartonella vinsonii* subsp. *vinsonii*.

Species	Host (scientific name)	Strain	GenBank accession number [most closely related species of the genus (sequence similarity, %)]				
			16S rRNA (1363 bp)	*ftsZ* (788 bp)	*gltA* (312 bp)	*rpoB* (825 bp)	ITS region (1251 bp)
Bartonella jaculi	Greater Egyptian jerboa (*Jaculus orientalis*)	OY2-1^T	AB602527 [B. k (99.2)]	AB602539 [B. v. v (92.5)]	AB444975 [B. v. a (93.6)]	AB529934 [B. qt (90.9)]	AB602557 [B. tay (75.3)]
		OY5-1	AB602528 [B. k (99.0)]	AB602540 [B. v. v (92.5)]	AB444976 [B. v. a (93.3)]	AB602554 [B. qt (90.9)]	AB602558 [B. tay (75.3)]
Bartonella callosciuri	Plantain squirrel (*Callosciurus notatus*)	BR1-1	AB602529 [B. que, B. s (99.3)]	AB602541 [B. a (93.3)]	AB444977 [B. v. v (94.6)]	AB529929 [B. a (91.5)]	AB602559 [B. v. v (71.8)]
		BR11-1^T	AB602530 [B. que, B. s (99.3)]	AB602542 [B. a (93.3)]	AB602551 [B. v. v (94.6)]	AB529931 [B. a (91.5)]	AB602560 [B. v. v (71.8)]
Bartonella pachyuromydis	Fat-tail gerbil (*Pachyuromys duprasi*)	FN15-2^T	AB602531 [B. tri (99.4)]	AB602543 [B. a (93.1)]	AB444978 [B. b, B. v. v (91.3)]	AB602555 [B. v. v (91.7)]	AB602561 [B. d (70.9)]
		FN18-1	AB602532 [B. que (99.3)]	AB602544 [B. a (92.3)]	AB602552 [B. b, B. v. v (91.3)]	AB529949 [B. v. v (91.7)]	AB602562 [B. d (71.6)]
Bartonella acomydis	Golden spiny mouse (*Acomys russatus*)	KS2-1^T	AB602533 [B. r (99.3)]	AB602545 [B. a (92.8)]	AB444979 [B. b (92.6)]	AB529942 [B. b (93.4)]	AB602563 [B. e (62.7)]
		KS7-1	AB602534 [B. r (99.3)]	AB602546 [B. a (92.9)]	AB602553 [B. b (92.6)]	AB602556 [B. b (93.4)]	AB602564 [B. e (62.9)]

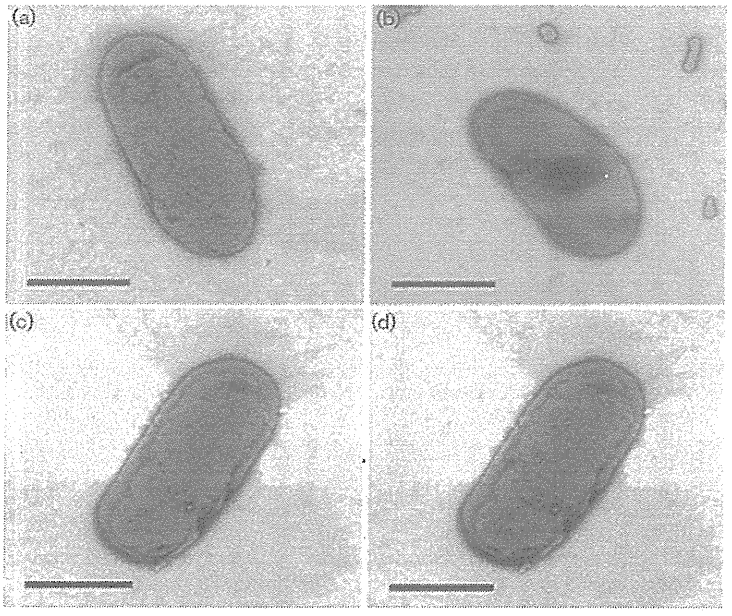

Fig. 1. Transmission electron micrographs of negatively stained cells of (a) *Bartonella jaculi* sp. nov. strain OY2-1^T, (b) *Bartonella callosciuri* sp. nov. strain BR1-1^T, (c) *Bartonella pachyuromydis* sp. nov. strain FN15-2^T and (d) *Bartonella acomydis* sp. nov. strain KS2-1^T. Bars, 500 nm.

the genus *Bartonella* (Table S1, available in IJSEM online); however, the profiles cannot be used for the differentiation of species of the genus *Bartonella* because of the relatively inert nature of Bartonellae (Dehio *et al.*, 2001; Bermond *et al.*, 2002). All of the morphological and biochemical characteristics of the four novel *Bartonella* species are summarized in Table S1.

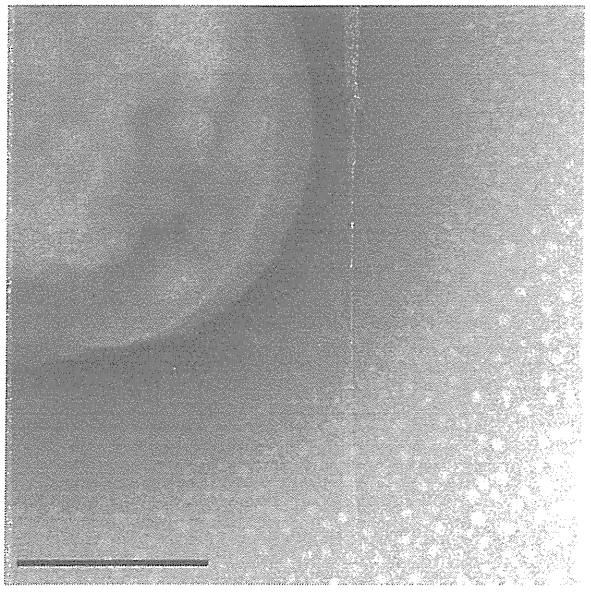

Fig. 2. Transmission electron micrograph showing the pili-like structures around the cell body of strain FN15-2^T. The structures were confirmed in all strains tested. Bar, 200 nm.

The G+C contents of the *ftsY* sequences ranged from 35.5 mol% for BR11-1^T to 37.4 mol% for OY2-1^T. These values were found to be similar to those for other known species of the genus *Bartonella*: 37 mol% for *Bartonella alsatica* (Heller *et al.*, 1999), 38 mol% for *Bartonella birtlesii* (Bermond *et al.*, 2000), *Bartonella bovis*, *Bartonella capreoli* (Bermond *et al.*, 2002) and *Bartonella tribocorum* (Heller *et al.*, 1998), 40 mol% for *Bartonella japonica*, *Bartonella silvatica* (Inoue *et al.*, 2010) and *Bartonella grahamii* (Birtles *et al.*, 1995) and 41 mol% for *Bartonella doshiae* (Birtles *et al.*, 1995), *Bartonella elizabethae* and *Bartonella henselae* (Daly *et al.*, 1993).

The DNA fragments of the five loci examined were sequenced and the sequence data were compared with those from the type strains of other known species of the genus *Bartonella*. Sequence similarities of the five loci in the strains to the corresponding type strains of species of the genus *Bartonella* are shown in Table 1. The highest similarity values were 94.6 % for *gltA* and 93.4 % for *rpoB*. These values were lower than the cut-off value for discriminating species of the genus *Bartonella* determined by La Scola *et al.* (2003), indicating that the strains studied represent distinct and novel species of the genus *Bartonella*.

The strains OY2-1^T and OY5-1, BR1-1 and BR11-1^T, FN15-2^T and FN18-1 and KS2-1^T and KS7-1 formed distinct clusters from other known species of the genus *Bartonella* by phylogenetic analysis of concatenated sequences of the five loci. In the relationship between species of the genus *Bartonella* and the major mammalian hosts, each of four novel species of the genus *Bartonella* had a unique host, suggesting that these strains have each evolved towards adaptation to a specific environmental and ecological niche and have specialized to optimize with a given host (Fig. 3).

The data support the hypothesis that all of the strains should be classified as distinct species of the genus *Bartonella*.

In addition, the phylogenetic analysis based on deduced amino acid sequences of proteins encoded by the *ftsZ*, *gltA* and *rpoB* genes (Fig. S1) showed that these four novel species of the genus *Bartonella* also formed distinct clades from other known species of the genus *Bartonella* as did the phylogenetic tree derived from the concatenated sequences of the five loci (Fig. 3).

The results in this study strongly indicate that four novel species of the genus *Bartonella* are clearly distinct from other known species of the genus *Bartonella*. We propose the names *Bartonella jaculi* sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov. and *Bartonella acomydis* sp. nov. for the strains OY2-1^T and OY5-1, BR1-1 and BR11-1^T, FN15-2^T and FN18-1 and KS2-1^T and KS7-1, respectively.

Description of *Bartonella jaculi* sp. nov.

Bartonella jaculi (ja'cu.li. N.L. gen. n. *jaculi* of *Jaculus*, isolated from *Jaculus orientalis*).

After 14 days of incubation on HIA at 35 °C in a moist atmosphere under 5 % CO_2, colonies appear small (1–2 mm in diameter), round, grey–whitish, smooth and umbonate. Electron microscopic examination reveals small bacilli with pili-like structures but without flagella. The cell size is 1.45 μm in length and 0.59 μm in width. The strain does not have oxidase, catalase and urease activities. The strain does not show the ability to reduce nitrate, the ability to produce indole and ability to degrade indoxyl phosphate. The strain does not hydrolyse *p*-nitrophenyl substrates and trehalose. The strain exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, glycylglycine, arginine, tryptophan and proline. Can be distinguished from other known

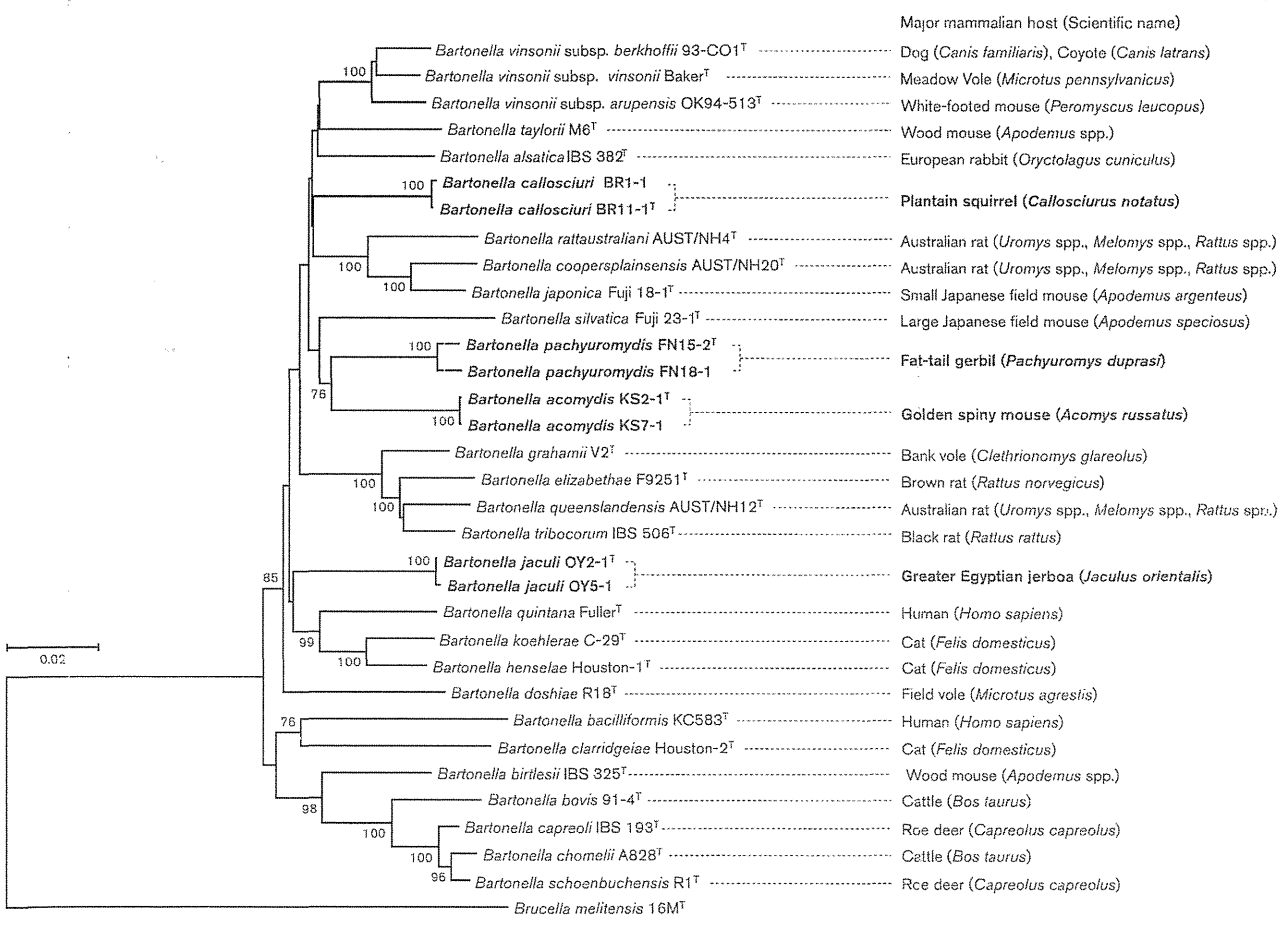

Fig. 3. Phylogenetic relationships based on the concatenated sequences of the 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and the ITS region of the four novel species and other known species of the genus *Bartonella*. The phylogenetic tree was constructed by using the neighbour-joining method with the Jukes–Cantor parameters distance model. The tree was rooted by using *Brucella melitensis* 16M^T as an outgroup. Bootstrap values (percentages of 1000 replications) with greater than 70 % confidence are indicated at the tree nodes. Bar, 0.02 substitutions per nucleotide position.

species of the genus *Bartonella* by the concatenated sequence of its 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and ITS region and has a unique mammalian host, *Jaculus orientalis*.

The type strain is OY2-1^T (=JCM 17712^T=KCTC 23655^T), isolated from the blood of *Jaculus orientalis* mice. The DNA G+C content of the type strain is 37.4 mol% based on the *ftsY* gene sequence. Strain OY5-1 (=JCM 17713=KCTC 23656), isolated from the same source, is a second strain of the species.

Description of *Bartonella callosciuri* sp. nov.

Bartonella callosciuri (cal.lo.sci'u.ri. N.L. gen. n. *callosciuri* of *Callosciurus*, isolated from *Callosciurus notatus*).

After 14 days of incubation on HIA at 35 °C in a moist atmosphere under 5% CO_2, colonies appear small (1–2 mm in diameter), round, grey–whitish, smooth and umbonate. Electron microscopic examination reveals small bacilli with pili-like structures, but without flagella. The cell size is 1.15 μm in length and 0.56 μm in width. The strain does not have oxidase, catalase and urease activities. The strain does not show the ability to reduce nitrate, the ability to produce indole and the ability to degrade indoxyl phosphate. The strain does not hydrolyse *p*-nitrophenyl substrates and trehalose. The strain exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, glycylglycine, arginine and tryptophan, but not proline. Can be distinguished from other known species of the genus *Bartonella* by the concatenated sequences of its 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and ITS region and has a unique mammalian host, *Callosciurus notatus*.

The type strain is BR11-1^T (=JCM 17709^T=KCTC 23909^T), isolated from the blood of *Callosciurus notatus* squirrels. The DNA G+C content of the type strain is 35.5 mol% based on the *ftsY* gene sequence. Strain BR1-1 (=JCM 17708=KCTC 23910), isolated from the same source, is a second strain of the species.

Description of *Bartonella pachyuromydis* sp. nov.

Bartonella pachyuromydis (pa.chy.u.ro'my.dis. N.L. gen. n. *pachyuromydis* of *Pachyuromys*, isolated from *Pachyuromys duprasi*).

After 14 days of incubation on HIA at 35 °C in a moist atmosphere under 5% CO_2, colonies appear small (1–2 mm in diameter), irregular shaped, grey–whitish, smooth and umbonate. Electron microscopic examination reveals small bacilli with pili-like structures but without flagella. The cell size is 1.35 μm in length and 0.59 μm in width. The strain does not have oxidase, catalase and urease activities. The strain does not show the ability to reduce nitrate, the ability to produce indole and the ability to degrade indoxyl phosphate. The strain hydrolyses bis-*p*-nitrophenyl phosphate, but not other *p*-nitrophenyl substrates and trehalose. The strain exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, glycylglycine, arginine, tryptophan and proline. Can be distinguished from other known species of the genus *Bartonella* by the concatenated sequences of its 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and ITS region and has a unique mammalian host, *Pachyuromys duprasi*.

The type strain is FN15-2^T (=JCM 17714^T=KCTC 23657^T), isolated from the blood of *Pachyuromys duprasi* mice. The DNA G+C content of the type strain is 35.7 mol% based on the *ftsY* gene sequence. Strain FN18-1 (=JCM 17715=KCTC 23911), isolated from the same source, is a second strain of the species.

Description of *Bartonella acomydis* sp. nov.

Bartonella acomydis (a.co'my.dis. N.L. gen. n. *acomydis* of *Acomys*, isolated from *Acomys russatus*).

After 14 days of incubation on HIA at 35 °C under 5% CO_2, colonies appear small (1–2 mm in diameter), irregular shaped, grey–whitish, smooth and umbonate. Electron microscopic examination reveals small bacilli with pili-like structures but without flagella. The cell size is 1.39 μm in length and 0.52 μm in width. The strain does not have oxidase, catalase and urease activities. The strain does not show the ability to reduce nitrate, the ability to produce indole and the ability to degrade indoxyl phosphate. The strain hydrolyses bis-*p*-nitrophenyl phosphate but not other *p*-nitrophenyl substrates and trehalose. The strain exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, glycylglycine, arginine, tryptophan and proline. Can be distinguished from other known species of the genus *Bartonella* by the concatenated sequences of its 16S rRNA, *ftsZ*, *gltA* and *rpoB* genes and ITS region and has a unique mammalian host, *Acomys russatus*.

The type strain is KS2-1^T (=JCM 17706^T=KCTC 23907^T), isolated from the blood of *Acomys russatus* mice. The DNA G+C content of the type strain is 37.2 mol% based on the *ftsY* gene sequence. Strain KS7-1 (=JCM 17707=KCTC 23908), isolated from the same source, is a second strain of the species.

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