Bartonella japonica sp. nov. and *Bartonella silvatica* sp. nov., isolated from *Apodemus* mice

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Two bacterial strains, Fuji 18-1^T and Fuji 23-1^T, were isolated from the blood of the small Japanese field mouse (*Apodemus argenteus*) and the large Japanese field mouse (*Apodemus speciosus*), respectively, specimens of which were captured in the forest of Mount Fuji, Japan. Phenotypic characterization (growth conditions, incubation periods, biochemical properties and cell morphologies), DNA G + C contents (40.1 mol% for strain Fuji 18-1^T and 40.4 mol% for strain Fuji 23-1^T) and sequence analyses of the 16S rRNA genes indicated that both strains were members of the genus *Bartonella*. Using *rpoB* and *gltA* sequencing analysis, the highest sequence similarities between strains Fuji 18-1^T, Fuji 23-1^T and other recognized species of the genus *Bartonella* showed values considerably lower than 91.4 % and 89.9 % in the *rpoB* gene and 89.1 % and 90.4 % in the *gltA* gene, respectively. It is known that similarities of 95.4 % for the *rpoB* gene and 96.0 % for the *gltA* gene can be applied as cut-off values for the designation of novel species of the genus *Bartonella*. In a phylogenetic tree based on the merged set of concatenated sequences of seven loci [16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the intergenic spacer region (ITS)], strains Fuji 18-1^T and Fuji 23-1^T formed a distinct clade from other recognized species of the genus *Bartonella*. These data support the classification of strains Fuji 18-1^T and Fuji 23-1^T as novel species of the genus *Bartonella*. The names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov. are proposed for these novel species. The type strains of *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov. are Fuji 18-1^T (=JCM 15567^T=CIP 109861^T) and Fuji 23-1^T (=JCM 15566^T=CIP 109862^T), respectively.

Abbreviations: ITS, intergenic spacer region; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank accession numbers for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the 16S–23S rRNA intergenic spacer region (ITS) from *Bartonella japonica* sp. nov. Fuji 18-1^T are AB440632, AB440633, AB242289, AB440634, AB440635, AB242288, and AB498007, respectively. GenBank accession numbers for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and ITS sequences from *Bartonella silvatica* sp. nov. Fuji 23-1^T are AB440636, AB440637, AB242287, AB440638, AB440639, AB242292, and AB498008, respectively.

Sequence similarities of the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and ITS region sequences between strains Fuji 18-1^T, Fuji 23-1^T and other species of the genus *Bartonella*, a maximum-parsimony phylogenetic tree based on the concatenated sequence of the seven loci and neighbour-joining trees of the seven loci are available as supplementary material with the online version of this paper.

The genus *Bartonella* is classified in the class *Alphaproteobacteria*, order *Rhizobiales* and family *Bartonellaceae* and, at the time of writing, comprised 19 recognized species and three subspecies. The bacteria in the genus *Bartonella* are small, fastidious, slow-growing Gram-negative rods (Brenner *et al.*, 1993; Birtles *et al.*, 1995). Species of the genus *Bartonella* are known to infect erythrocytes and endothelial cells of various mammals, such as humans, cats, dogs, ruminants, wild rabbits and wild rodents (Dehio, 2005). We previously described the prevalence of species of the genus *Bartonella* among 685 wild rodents in Japan (Inoue *et al.*, 2008). Based on phylogenetic trees constructed with the *rpoB* and *gltA* gene sequences and the criteria for the definition of a species of the genus *Bartonella* (La Scola *et al.*, 2003), two strains were identified as possible novel species of this genus. Strains Fuji 18-1^T and Fuji 23-1^T were isolated from *Apodemus argenteus* and *Apodemus speciosus* mice captured in the Mount Fuji forest in Japan. In the present study, these strains were characterized by biochemical, morphological and genetic approaches, including multilocus sequencing analysis of six housekeeping genes and the 16S–23S rRNA intergenic spacer region (ITS).

Strains Fuji 18-1^T and Fuji 23-1^T were grown on heart infusion agar plates (HIA; Difco) containing 5% (w/v) defibrinated rabbit blood at 35 °C with 5% CO₂ for 14 days. Gram staining was assessed by light microscopy (Olympus) at ×1000 magnification. Cell morphology was observed by transmission electron microscopy (model JEM1200EX; JEOL) at 100 kV with negative staining.

Biochemical characteristics were assessed by using a MicroScan Rapid Anaerobe Panel (Dade Behring Inc.) according to the manufacturer's instructions as described previously (Welch *et al.*, 1993). Cytochrome oxidase test strips (Nissui) were used for evaluating the oxidase activity of the strains. Catalase activity was examined by mixing fresh colonies which had been cultured for 14 days at 35 °C on 5% rabbit blood chocolate HIA plates with 3% H₂O₂ on a glass slide.

DNA G+C content was determined by HPLC (Mesbah & Whitman, 1989). Mean values of the G+C content (±SD) were calculated based on assays conducted in triplicate.

Genomic DNA was extracted using the Instagene Matrix (Bio-Rad) according to the manufacturer's instructions. Six housekeeping genes, 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB*, and ITS fragments were amplified by PCR as previously described (Birtles & Raoult, 1996; Heller *et al.*, 1997; Renesto *et al.*, 2001; Houpiikian & Raoult, 2001; Zeaiter *et al.*, 2002a, b; Inoue *et al.*, 2009). The PCR products were purified using a Spin Column PCR Product Purification kit (Bio Basic). Direct DNA sequencing of the purified PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) on a Genetic Analyzer (model 3130; Applied Biosystems). For the phylogenetic

analysis, sequence data were aligned with those of type strains of other species of the genus *Bartonella* (Table 1) that were available in GenBank by using CLUSTAL W software (Thompson *et al.*, 1994) in the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees based on six housekeeping genes and the ITS region were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987). The nucleotide substitution rates were calculated by Kimura's two-parameter distance model (Kimura, 1980). Bootstrap analysis was carried out on 1000 replications of the dataset (Felsenstein, 1985). *Brucella melitensis* 16M^T was chosen as the outgroup. The phylogenetic trees of the concatenated sequence data for the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region were constructed using the NJ and maximum-parsimony (MP) methods (Fitch, 1971) with the Jukes–Cantor parameter model (Jukes & Cantor, 1969) by using the MEGA4 program.

Strains Fuji 18-1^T and Fuji 23-1^T grown on HIA formed smooth, transparent to grey-whitish colonies of 1–2 mm in diameter. Gram-negative coccobacilli to short rod-shaped cells were observed by light microscopy after 14 days culture. The cell morphologies of both strains were similar and no flagella or pili were observed by electron microscopy (Fig. 1). Cell sizes were 0.74 µm in length and 0.36 µm in width for strain Fuji 18-1^T and 1.16 µm in length and 0.43 µm in width for strain Fuji 23-1^T.

Both strains were oxidase- and catalase-negative and neither exhibited urease activity nor hydrolysed trehalose. They both hydrolysed bis-*p*-nitrophenyl phosphate, but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Both strains had amino acid arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan. Strain Fuji 23-1^T exhibited L-proline-β-naphthylamide activity but strain Fuji 18-1^T did not. Both strains had glycylglycylarylamidase activity, but not pyrrolidonyl arylamidase activity. These biochemical results are typical for members of the genus *Bartonella* (Bermond *et al.*, 2000, 2002; Maillard *et al.*, 2004); however, the profiles cannot be applied routinely and reliably 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).

The DNA G+C contents of strains Fuji 18-1^T and Fuji 23-1^T were 40.1±0.6 mol% (mean±SD) and 40.4±0.5 mol% (mean±SD), respectively. These values were similar to those of other species of the genus *Bartonella* (Bermond *et al.*, 2000, 2002).

DNA fragments of all seven loci examined were sequenced and the sequence data were compared with the type strains of other species of the genus *Bartonella*. The 16S rRNA gene sequence similarities of strains Fuji 18-1^T and Fuji 23-1^T to other *Bartonella* species ranged from 96.5% (*Bartonella bacilliformis*) to 98.8% (*Bartonella grahamii*) and 96.7% (*Bartonella bacilliformis*) to 98.8% (*Bartonella koehlerae*), respectively (see Supplementary Table S1 in IJSEM Online).

Table 1. GenBank accession numbers of the seven loci for the *Bartonella* species used in this study

Species	GenBank accession numbers						
	16S rRNA	ftsZ	gltA	groEL	ribC	rpoB	ITS
<i>Bartonella japonica</i> sp. nov. Fuji 18-1 ^T (=JCM 15567 ^T =CIP 109861 ^T)	AB440632	AB440633	AB242289	AB440634	AB242288	AB242288	AB498007
<i>Bartonella silvatica</i> sp. nov. Fuji 23-1 ^T (=JCM 15566 ^T =CIP 109862 ^T)	AB440636	AB440637	AB242287	AB440638	AB440639	AB242292	AB498008
<i>B. alsatica</i> IBS 382 ^T (=CIP 105477 ^T)	AJ002139	AF467763	AF204273	AF299357	AY116630	AF165987	AF312506
<i>B. bacilliformis</i> KC583 ^T (=ATCC 35685 ^T)	Z11683	AB292602	AB292601	Z11683	AJ236918	AF165988	CP000524
<i>B. birtlesii</i> IBS 325 ^T (=CIP 106294 ^T)	AF204274	AF467762	AF204272	AF355773	AY116632	AB196425	AY116640
<i>B. bovis</i> 91-4 ^T (=CIP 106692 ^T)	AF293391	AF467761	AF293394	AF071194	AY116637	AY166581	AY116638
<i>B. capreoli</i> IBS 193 ^T (=CIP 106691 ^T)	AF293389	AB290192	AF293392	AB290190	AB290194	AB290188	AB498009
<i>B. chomelii</i> A828 ^T (=CIP 107869 ^T)	AY254309	AB290193	AY254308	AB290191	AB290195	AB290189	AB498010
<i>B. clarridgeiae</i> Houston-2 cat ^T (=ATCC 51734 ^T)	AB292603	AF141018	U84386	AF014831	AB292604	AF165990	AF312497
<i>B. doshiae</i> R18 ^T (=NCTC 12862 ^T)	Z31351	AF467754	Z70017	AF014832	AY116627	AF165991	AJ269786
<i>B. elizabethae</i> F9251 ^T (=ATCC 49927 ^T)	L01260	AF467760	Z70009	AF014834	AY116633	AF165992	L35103
<i>B. grahamii</i> V2 ^T (=NCTC 12860 ^T)	Z31349	AF467753	Z70016	AF014833	AY166583	AF165993	AJ269785
<i>B. henselae</i> Houston-1 ^T (=ATCC 49882 ^T)	BX897699	AF061746	BX897699	AF014829	AJ132928	AF171070	L35101
<i>B. koehlerae</i> C-29 ^T (=ATCC 700693 ^T)	AF076237	AF467755	AF176091	AY116641	AY116634	AY166580	AF312490
<i>B. quintana</i> Fuller ^T (=ATCC VR-358 ^T)	M11927	AB292605	Z70014	AB290196	AJ236917	AF165994	L35100
<i>B. schoenbuchensis</i> R1 ^T (=NCTC 13165 ^T)	AJ278187	AF467765	AJ278183	AY116642	AY116628	AY167409	AY116639
<i>B. taylorii</i> M6 ^T (=CIP 107028 ^T)	Z31350	AF467756	Z70013	AF304017	AY116635	AF165995	AJ269788
<i>B. tribocorum</i> IBS 506 ^T (=CIP 105476 ^T)	AJ003070	AF467759	AJ005494	AF304018	AB292600	AF165996	AF312505
<i>B. vinsonii</i> subsp. <i>arupensis</i> OK 94-513 ^T (=ATCC 700727 ^T)	AF214558	AF467758	AF214557	AF304016	AY116631	AY166582	AF312504
<i>B. vinsonii</i> subsp. <i>berkhoffii</i> 93-CO1 ^T (=ATCC 51672 ^T)	L35052	AF467764	U28075	AF014836	AY116629	AF165989	AF167988
<i>B. vinsonii</i> subsp. <i>vinsonii</i> Baker ^T (=ATCC VR-152 ^T)	M73230	AF467757	Z70015	AF014835	AY116636	AF165997	L35102

La Scola *et al.* (2003) reported that *rpoB* and *gltA* were the most appropriate genes for discriminating species of the genus *Bartonella* and proposed that gene sequence similarities <95.4 % in *rpoB* and <96.0 % in *gltA* between recognized species of the genus *Bartonella* could be used as cut-off values for the designation of novel *Bartonella* species. The highest sequence similarities between strains Fuji 18-1^T, Fuji 23-1^T and other species of the genus *Bartonella* showed values considerably lower than 91.4 % (strain Fuji 18-1^T compared with *Bartonella alsatica*) and 89.9 % (strain Fuji 23-1^T/*B. alsatica*) for the *rpoB* gene and 89.1 % (strain Fuji 18-1^T/*Bartonella vinsonii* subsp. *arupensis*) and 90.4 % (strain Fuji 23-1^T/*Bartonella taylorii*) for the *gltA* gene. Thus, strains Fuji 18-1^T and Fuji 23-1^T fulfil the requirements for classification as novel species of the genus *Bartonella*.

Strains Fuji 18-1^T and Fuji 23-1^T also showed considerably lower gene sequence similarities for the remaining four loci, *ftsZ*, *groEL*, *ribC* and ITS, when compared with other recognized species of the genus *Bartonella* (Supplementary Table S1). In the phylogenetic tree based on the merged set of concatenated sequences of seven loci, strains Fuji 18-1^T and Fuji 23-1^T formed a distinct clade with other species of the genus *Bartonella* (Fig. 2). The MP tree based on the concatenated sequence of the seven loci and the NJ trees

based on sequence analyses of the seven loci also revealed that strains Fuji 18-1^T and Fuji 23-1^T were clearly separated from all other species of the genus *Bartonella* (see Supplementary Figs S1 and S2 in IJSEM Online).

In conclusion, sequence similarities of the *rpoB* and *gltA* genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1^T and Fuji 23-1^T as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

Description of *Bartonella japonica* sp. nov.

Bartonella japonica [ja.po'ni.ca. N.L. fem. adj. *japonica* of Japan, where the host rodent, the small Japanese field mouse (*Apodemus argenteus*), from which the strain was isolated, is widely distributed].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO₂, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 0.74 × 0.36 µm. Oxidase- and catalase-negative, does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Exhibits arylamidase activity

Fig. 1. Transmission electron micrograph of cells of (a) *Bartonella japonica* sp. nov. strain Fuji 18-1^T and (b) *Bartonella silvatica* sp. nov. strain Fuji 23-1^T. Bars, 200 nm.

towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan, but not to proline. Glycylglycylarylamidase activity is present, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 18-1^T (=JCM 15567^T=CIP 109861^T), was isolated from the blood of *Apodemus argenteus* mice. The DNA G+C content of the type strain is 40.1 mol%.

Description of *Bartonella silvatica* sp. nov.

Bartonella silvatica [sil.va'ti.ca. L. fem. adj. *silvatica* of the forest where the host rodent, the large Japanese field mouse (*Apodemus speciosus*), from which the strain was isolated, was captured].

After 14 days incubation on HIA at 35 °C in a moist atmosphere under 5 % CO₂, colonies appear small (1–2 mm in diameter), round, grey-whitish and smooth. Cells are small bacilli without flagella or pili and are 1.16 × 0.43 µm. Oxidase- and catalase-negative. Does not exhibit urease activity or hydrolyse trehalose. Hydrolyses bis-*p*-nitrophenyl phosphate but not *p*-nitrophenyl *N*-acetyl β-D-glucosaminide. Exhibits arylamidase activity towards leucine, methionine, lysine (alkaline as well as acidic), glycine, proline, arginine and tryptophan. Glycylglycylarylamidase activity, but no pyrrolidonyl arylamidase activity. Can be distinguished from other species of the genus *Bartonella* by the 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* gene and ITS region sequences.

The type strain, Fuji 23-1^T (=JCM 15566^T=CIP 109862^T), was isolated from the blood of *Apodemus speciosus* mice. The DNA G+C content of the type strain is 40.4 mol%.

Acknowledgements

This work was supported in part by grants for the Academic Frontier Project 'Surveillance and Control for Zoonoses' and Grant-in-aid for Scientific Research (20580343) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Fig. 2. Phylogenetic relationship of species of the genus *Bartonella* inferred from concatenated sequences of 16S rRNA, *ftsZ*, *gltA*, *groEL*, *ribC* and *rpoB* genes and the ITS region. The phylogenetic tree was constructed by using the NJ method with the Jukes–Cantor parameter model. The tree was rooted by the use of *Brucella melitensis* 16M^T as an outgroup. Bootstrap values (percentages of 1000 replications) above 70 % are indicated at the nodes. Bar, 0.01 estimated nucleotide substitutions per site.

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