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

Kai Inoue,1 Hidenori Kabeya,1 Hatsumi Shiratori,2 Kenji Ueda,2 Michael Y. Kosoy,3 Bruno B. Chomel,4 Henri-Jean Boulouis5 and Soichi Maruyama1

Correspondence
Soichi Maruyama
maruyama.soichi@nihon-u.ac.jp

1Laboratory of Veterinary Public Health, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-8510, Japan
2Life Science Research Center, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan
3Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, USA
4Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA
5UMR BIPAR/AFSSA, Institut National de la Recherche Agronomique, Ecole Nationale Vétérinaire d’Alfort/UVPM, 7 avenue du Général de Gaulle, 94704 Maisons-Alfort Cedex, France

Two bacterial strains, Fuji 18-1T and Fuji 23-1T, 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 18-1T and 40.4 mol% for strain Fuji 23-1T) 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 18-1T, Fuji 23-1T 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 18-1T and Fuji 23-1T formed a distinct clade from other recognized species of the genus Bartonella. These data support the classification of strains Fuji 18-1T and Fuji 23-1T 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 18-1T (=JCM 15567T=CIP 109861T) and Fuji 23-1T (=JCM 15566T=CIP 109862T), 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 18-1T 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 23-1T are AB440636, AB440637, AB242287, AB440638, AB440639, and AB498008, respectively.

Sequence similarities of the 16S rRNA, ftsZ, gltA, groEL, ribC and rpoB genes and ITS region sequences between strains Fuji 18-1T, Fuji 23-1T 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 trees 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 18-1T and Fuji 23-1T 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 18-1T and Fuji 23-1T 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; Houpiikan & Raoult, 2001; Zeaier 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* 16Mᵀ 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 18-1T and Fuji 23-1T grown on HIA formed smooth, transparent to grey-whitish colonies of 1–2 mm in diameter. Gram-negative cocccobacilli 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 18-1T and 1.16 μm in length and 0.43 μm in width for strain Fuji 23-1T.

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 23-1T exhibited l-proline-β-naphthylamide activity but strain Fuji 18-1T did not. Both strains had glycylglycylarlamidase 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 18-1T and Fuji 23-1T 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 18-1T and Fuji 23-1T 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).
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 18-1T, Fuji 23-1T and other species of the genus *Bartonella* showed considerably lower than 91.4% (strain Fuji 18-1T/strain Fuji 23-1T 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 ITS genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1T and Fuji 23-1T 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 exhibit urease activity or hydrolyse trehalose. Hydrolyses *p*-nitrophenyl *p*-nitrophenyl phosphate but not *p*-nitrophenyl N-acetyl β-D-glucosaminide. Exhibits arylamidase activity based on sequence analyses of the seven loci also revealed that strains Fuji 18-1T and Fuji 23-1T 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 18-1T and Fuji 23-1T as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

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 18-1T, Fuji 23-1T and other species of the genus *Bartonella* showed considerably lower than 91.4% (strain Fuji 18-1T/strain Fuji 23-1T 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 ITS genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1T and Fuji 23-1T as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

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 18-1T, Fuji 23-1T and other species of the genus *Bartonella* showed considerably lower than 91.4% (strain Fuji 18-1T/strain Fuji 23-1T 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 ITS genes and the phylogenetic analyses of seven different loci support the classification of strains Fuji 18-1T and Fuji 23-1T as novel species of the genus *Bartonella*, for which we propose the names *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., respectively.

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

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bartonella japonica</em> sp. nov. Fuji 18-1T (=ICM 15567 =JCM 15566)</td>
<td>AB440632 AB440633 AB242289 AB440634 AB242288 AB242288 AB498007</td>
</tr>
<tr>
<td><em>Bartonella japonica</em> sp. nov. Fuji 23-1T (=ICM 15566 =JCM 15567)</td>
<td>AB440636 AB440637 AB242287 AB440638 AB440639 AB242292 AB498008</td>
</tr>
<tr>
<td><em>B. alatica</em> IBS 382T (=ATCC 105477)</td>
<td>AJ002139 AF467763 AF204273 AF299357 AY116630 AF165987 AF312506</td>
</tr>
<tr>
<td><em>B. bacilliformis</em> KC583T (=ATCC 35685)</td>
<td>Z11683 AB292602 AB292601 Z11683 AJ269198 AF165988 CP000524</td>
</tr>
<tr>
<td><em>B. birtlesii</em> IBS 325T (=ATCC 106294)</td>
<td>AF204274 AF467762 AF204272 AF355773 AY116632 B196425 AY116640</td>
</tr>
<tr>
<td><em>B. bovis</em> 91-4T (=ATCC 106692)</td>
<td>AF293391 AF467761 AF293394 AY071194 AY116637 AY166581 AY116638</td>
</tr>
<tr>
<td><em>B. capreoli</em> IBS 193T (=ATCC 106691)</td>
<td>AF293389 AB290192 AF293392 AB290190 AB290194 AB290188 AB498009</td>
</tr>
<tr>
<td><em>B. chomelii</em> A828T (=ATCC 107869)</td>
<td>AY254309 AB290193 AY254308 AB290191 AB290189 AB498010</td>
</tr>
<tr>
<td><em>B. clarridgeiae</em> Houston-2 catT (=ATCC 51734)</td>
<td>AB292603 AF141018 U84386 AF014831 AB292604 AF165990 AF312497</td>
</tr>
<tr>
<td><em>B. dohiae</em> R1T (=NCTC 12862)</td>
<td>Z13351 AF467754 Z70017 AF014832 AY116627 AF165991 AY267986</td>
</tr>
<tr>
<td><em>B. elizabethae</em> F9251T (=ATCC 49927)</td>
<td>L01260 AF467760 Z70009 AF014834 AY116633 AF165992 L35103</td>
</tr>
<tr>
<td><em>B. grahamii</em> V2T (=NCTC 12860)</td>
<td>Z13349 AF467753 Z70016 AF014833 AY166583 AF165993 AY267985</td>
</tr>
<tr>
<td><em>B. henselae</em> Houston-1T (=ATCC 49882)</td>
<td>BY897699 AF061746 BX897699 AF014829 AJ132928 AY171070 L35101</td>
</tr>
<tr>
<td><em>B. koehnei</em> C-29T (=ATCC 700693)</td>
<td>AF076237 AB290193 AF076236 AF165991 AY116641 AY166580 AF312490</td>
</tr>
<tr>
<td><em>B. quintana</em> FullerT (=ATCC VR-358)</td>
<td>M11927 AB292605 Z70014 AB290196 AJ269176 AF165994 L35100</td>
</tr>
<tr>
<td><em>B. schoenbuchensis</em> RI T (=NCTC 13165)</td>
<td>AJ278187 AF467765 AJ278183 AY116642 AY166428 AY167409 AY116639</td>
</tr>
<tr>
<td><em>B. taylorii</em> M6T (=CIP 107028)</td>
<td>Z31350 AF467756 Z70013 AF304017 AY116635 AF165995 AY267988</td>
</tr>
<tr>
<td><em>B. tribocorum</em> IBS 506T (=CIP 105476)</td>
<td>AJ003070 AF467759 AJ003072 AF304018 AB292600 AF165996 AF312505</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. <em>aranensis</em> OK 94-513T (=ATCC 700727)</td>
<td>AF214558 AF467758 AF214557 AF304016 AY166581 AF165993 AF312504</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. <em>berkhoffii</em> 93-CO1T (=ATCC 51672)</td>
<td>L35052 AF467764 U28075 AF014836 AY116629 AF165989 AF167988</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. <em>vinsonii</em> Baker T (=ATCC VR-152T)</td>
<td>M73230 AF467757 Z70015 AF014835 AY116629 AF165997 L35102</td>
</tr>
</tbody>
</table>
towards leucine, methionine, lysine (alkaline as well as acidic), glycine, arginine and tryptophan, but not to proline. Glycylglycylarylamidase activity is present, but no pyrrolidonyl ary lamidase 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<sup>T</sup> (=JCM 15567<sup>T</sup>=CIP 109861<sup>T</sup>), 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 % CO<sub>2</sub>, 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<sup>T</sup> (=JCM 15566<sup>T</sup>=CIP 109862<sup>T</sup>), 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<sup>T</sup> as an out-group. Bootstrap values (percentages of 1000 replications) above 70 % are indicated at the nodes. Bar, 0.01 estimated nucleotide substitutions per site.

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


