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Bartonella apis sp. nov., a honey bee gut symbiont of the class *Alphaproteobacteria*

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Running title: Description of a bee gut symbiont

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Sequence deposition: The 16S rRNA gene sequences and protein-coding gene sequences of the bacterial strains PEB0122^{T}, PEB0149, PEB0150, BBC0104, and BBC0108 from *Apis mellifera*, and the uncultured Rhizobiales bacterium from *Herpagnathos saltator* are deposited in GenBank with accession numbers KP987849 – KP987886 and KT315729 – KT315734.

Abstract

Here, we report culturing and characterization of an alphaproteobacterium of the order *Rhizobiales,* isolated from the gut of the honey bee, *Apis mellifera*. Strain PEB0122^T shares >95 % 16S rRNA sequence similarity with species of the genus *Bartonella,* a group of mammalian pathogens transmitted by bloodsucking arthropods. Phylogenetic analyses showed that PEB0122^T and related strains from the honey bee gut form a sister clade of the genus *Bartonella*. Optimal growth of strain PEB0122^T was obtained on solid media supplemented with defibrinated sheep blood under microaerophilic conditions at 35–37 °C, which is consistent with culturing characteristics of other *Bartonella* species. Reduced growth of strain PEB0122^T also occurred under aerobic conditions. The rod-shaped cells of strain PEB0122^T had a mean length of 1.2–1.8 μm and revealed hairy surface structures. Strain PEB0122^T was positive for catalase, cytochrome *c* oxidase, urease, and nitrate reductase. The fatty acid composition was comparable to those of other *Bartonella* species, with palmitic acid ($C_{16:0}$) and isomers of 18- and 19-carbon chains being the most abundant. The genomic G+C content of PEB0122^T was determined to be about 45.5%. The high sequence similarity with *Bartonella* species and its close phylogenetic position suggest that strain PEB0122^T is a novel species within the genus *Bartonella*. We propose the name *Bartonella apis* sp. nov. The type strain is PEB0122^T (=NCIMB 14961^T, =DSM 29779^T).

Main text

Non-culture-based analyses of 16S rRNA gene sequences have shown that the hindgut of adult honey bees (genus *Apis*) is inhabited by a relatively small number of bacterial species inlcuding two *Firmicutes*, two *Gammaproteobacteria,* two *Alphaproteobacteria,* one *Bifidobacterium,* and one *Betaproteobacterium* (Cox-Foster *et al.*, 2007; Martinson *et al.*, 2011; Moran *et al.*, 2012; Sabree *et al.*, 2012). These bacteria are consistently present in honey bees worldwide (Ahn *et al.*, 2012; Babendreier *et al.*, 2007; Jeyaprakash *et al.*, 2003), and a subset has also been found in the gut of various bumble bee species (genus *Bombus*) (Cariveau *et al.*, 2014; Martinson *et al.*, 2011). In other environments, these bacteria have so far not been detected which suggests specific adaptation to the gut of social bees. Genome sequencing projects have provided first insights into the functional capabilities of bee gut bacteria (Ellegaard *et al.*, 2015; Engel *et al.*, 2012; Kwong *et al.*, 2014a, b). Furthermore, cultures of most community members have been established and species names proposed, such as *Snodgrassella alvi* (*Betaproteobacteria*), *Gilliamella apicola* and *Frischella perrara* (*Gammaprotoebacteria*), *Bifidobacterium asteroides* (*Actinobacteria*), or *Lactobacillus apis* and *Lactobacillus mellis* (*Firmicutes*) (Engel *et al.*, 2013; Killer *et al.*, 2014; Kwong & Moran, 2013; Olofsson *et al.*, 2014). However, species descriptions of the two *Alphaproteobacteria* are still lacking. Based on 16S rRNA analyses, they seem to belong to distinct lineages of *Alphaproteobacteria* and were therefore referred to as ‘Alpha-1’ and ‘Alpha-2’ (Cox-Foster et al., 2007). ‘Alpha-2’ was further sub-divided into ‘Alpha-2.1’ and ‘Alpha-2.2’ representing two distinct clades within the family of *Acetobacteriaceae* (Corby-Harris *et al.*, 2014; Martinson *et al.*, 2011). Phylotype ‘Alpha-1’ belongs to the order *Rhizobiales* and appears to be closely related to the genus *Bartonella* (Martinson *et*

al., 2011). The latter constitutes a group of facultative intracellular pathogens that persist in the bloodstream of a wide range of mammals. Some *Bartonella* species can colonize human hosts and cause severe diseases such as Carrion's disease or bacillary angiomatosis (Harms & Dehio, 2012). Transmission of these mammalian pathogens is facilitated by bloodsucking arthropods. To our knowledge, all currently described *Bartonella* species have either been isolated from the bloodstream of mammals or from bloodsucking arthropods. In contrast, 'Alpha-1' has so far only been detected in the gut of honey bees.

Another group of *Rhizobiales* that seems to be closely related to 'Alpha-1' are bacteria detected in the gut of herbivorous tropical ants from diverse species. The function of these ant-associated *Rhizobiales* has so far remained elusive, but several lines of evidence suggest that they might play a role in the nitrogen uptake of their host (Russell *et al.*, 2009).

Here we report the cultivation and characterization of 'Alpha-1', strain PEB0122^T, from *A. mellifera* and propose the name *Bartonella apis* sp. nov.

Strain PEB0122^T was isolated together with strains PEB0149 and PEB0150 from homogenized guts of the Western honey bee, *A. mellifera*. Adult worker bees were captured in West Haven, CT, USA, and immobilized by chilling at 4 °C. Thereafter, the guts were dissected with sterile forceps and homogenized in PBS by bead-beating. Diluted homogenates were plated on tryptic soy agar supplemented with 5 % defibrinated sheep blood (Blood agar, Hardy Diagnostics) and incubated at 37 °C in an atmosphere enriched with 5 % CO_2. Bacterial colonies were visible after 2 - 3 days and were identified by amplification and sequencing of the partial 16S rRNA gene. To this end, PCR was performed on the bacterial colony with the universal 16S rRNA gene primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and

1492R (5'-GGTTACCTTGTTACGACTT-3'). DNA from colonies was denatured in a thermocycler by boiling at 95 °C for 10 min, followed by 35 cycles of amplification (95 °C for 20 s, 54 °C for 30 s and 72 °C for 40 s) and 5 min of final elongation at 72 °C. Sequencing of amplicons was performed using the dideoxy chain-termination method and capillary gel electrophoresis on an Applied Biosystems 3730xl DNA Genetic Analyzer. Resulting sequences were trimmed based on the ABI sequencer traces. CLUSTALW alignments (Thompson *et al.*, 1994) revealed that the 16S rRNA sequences of strains PEB0122^{T}, PEB0149 and PEB0150 are identical. We next searched the sequences with MegaBLAST against the non-redundant nucleotide collection database of NCBI. All three strains shared >97 % similarity with 16S rRNA sequences deposited as 'Uncultured *Rhizobiales* bacteria' from *A. mellifera* and related bee species. The closest described taxa in the MegaBLAST analysis were species of the genus *Bartonella*, including several human pathogens. To conduct a more detailed comparative analysis, we retrieved the full-length sequences of the 16S rRNA, *gltA*, and *rpoB* genes of strains PEB0122^{T}, PEB0149 and PEB0150 from ongoing genome sequencing projects (data not show). CLUSTALW alignments confirmed that strains PEB0122^{T}, PEB0149 and PEB0150 (1,528 sites) have identical 16S rRNA genotypes and share high sequence similarities with species of the genus *Bartonella,* ranging from 95.9 % (*Bartonella chomelii* A828^{T}) to 97.6 % ('*Bartonella tamiae*' Th239^{T}) (Table S1). In contrast, sequence similarities for conserved fragments of the *gltA* gene (327-bp fragment) and the *rpoB* gene (825-bp fragment) (La Scola *et al.*, 2003) were much lower (Table S1). Strain PEB0122^{T} exhibited sequence similarities with other *Bartonella* species ranging from 77.4 % to 82.0 % for the *gltA* fragment and from 75.6 % to 79.1 % for the *rpoB* fragment. This is clearly below the sequence similarity cutoffs of 96.0 % (*gltA* fragment) and 95.4 % (*rpoB*

123 fragment) which were proposed by La Scola *et al.* (2003) to discriminate *Bartonella*
124 species from each other. In contrast to the comparison with previously described
125 *Bartonella* species, sequence similarities between strain PEB0122^T and the two other
126 strains isolated from the bee gut, PEB0149 and PEB0150, were much higher, ranging
127 from 98.2 % to 98.8 % (*gltA* fragment) and 98.7% to 99.5 % (*rpoB* fragment), and
128 thus suggesting that they belong to the same species (Table S1).
129 A phylogenetic tree inferred from aligned 16S rRNA sequences corroborated
130 the findings from the sequence similarity analyses. Strains PEB0122^T, PEB0149 and
131 PEB0150 clustered together with 16S rRNA sequences previously detected in *Apis*
132 spp. and with two strains, BBC0104 and BBC0108, which we had isolated from the
133 gut of honey bees in Switzerland (Fig. 1). The honey bee-specific strains form a clade
134 that is basal to species of the genus *Bartonella*. The phylogenetic clustering of strains
135 from honey bees and the basal position of this cluster within the genus *Bartonella* are
136 both supported by bootstrap values ≥80 %. The most closely related *Bartonella*
137 species is '*B. tamiae*', forming a sister clade to the sequences from honey bees (Fig.
138 1). However, this relationship is not supported by the bootstrap analysis (i.e. values
139 <80 %), probably because the 16S rRNA sequence similarities between honey bee
140 strains and different *Bartonella* species are all in the same range (Table S1). The 16S
141 rRNA phylogeny also showed that sequences of *Rhizobiales* from various ant species
142 are more distant from the honey bee-specific strains than *Bartonella* spp. They form a
143 separate monophyletic clade basal to the honey bee-specific clade and the genus
144 *Bartonella* (Fig. 1). Accordingly, pairwise 16S rRNA sequence similarities were
145 lower for strain PEB0122^T with the ant strains (93.3 % to 93.9 %) than with
146 *Bartonella* spp. (95.9 % to 97.6 %) (Table S1).

147 Because of the high similarity of 16S rRNA sequences and the resulting low
148 bootstrap values for most branches in the tree, we inferred another phylogeny based
149 on concatenated nucleotide sequences of eight phylogenetic marker genes (*alaS*,
150 COG0013; *uvrC*, COG0322; *recN*, COG 0497; *pyrG*, COG0504; *ffh/srp*, COG0541;
151 *uvrB*, COG0556; *radA*, COG1066; *typA*, COG1217). These genes occur in a single
152 copy in the majority of bacteria and have proven to be suitable for taxonomic
153 classification of bacteria in previous publications (Ciccarelli *et al.*, 2006; Engel *et al.*,
154 2012, 2013, 2014; Mende *et al.*, 2013; Sorek *et al.*, 2007). Sequences of PEB0122^{T},
155 PEB0149, PEB0150 and an uncultured *Rhizobiales* spp. from the ant, *Herpagnathos*
156 *saltator*, were obtained from ongoing genome sequencing projects. The phylogenetic
157 marker gene tree confirmed the overall topology of the 16S rRNA tree (Fig. 2): (i)
158 The honey bee strains formed a monophyletic clade basal to species of the genus
159 *Bartonella*, (ii) '*B. tamiae*' was the next closely related species, and (iii) the
160 *Rhizobiales* spp. from the ant, *H. saltator,* formed a more distant sister lineage basal
161 to the honey bee-specific strains and the genus *Bartonella*. Due to the limited number
162 of available sequences less taxa were included in this analysis than in the 16S rRNA
163 analysis. In summary, our phylogenies and comparative sequence analyses suggest
164 that strains of 'Alpha-1' belong to the genus *Bartonella* (i.e. 16S rRNA identity
165 >95%), but form a distinct clade that is basal to other *Bartonella* spp. Sequence
166 similarities of *gltA* and *rpoB* gene fragments are clearly below the defined species
167 cutoff (La Scola *et al.*, 2003), indicating that the strains isolated from the honey bee
168 gut present a novel *Bartonella* species. Accession numbers of sequences used for
169 these analyses are listed in Table S2. With 45.5 mol%, strains PEB0122^{T}, PEB0149,
170 and PEB0150 exhibit a markedly elevated DNA G+C content compared to other
171 *Bartonella* spp., which typically exhibit G+C content in the range of 37.8 – 41.8

172 mol%. G+C content information of strains PEB0122^T, PEB0149 and PEB0150 was
173 obtained from the unpublished genome sequencing projects.
174 To further describe the honey bee-specific strains, we studied growth
175 characteristics and conducted a number of phenotypic and morphological analyses.
176 Due to the identical 16S rRNA genotype of the three isolated strains, we restricted our
177 analyses to strain PEB0122^T. Growth of strain PEB0122^T was tested under different
178 conditions and compared to '*B. tamiae*' Th239^T (Kosoy *et al.*, 2008) and *Bartonella*
179 *henselae* Houston-1^T (Sölder *et al.*, 1995) For the inoculation of agar plates, equal
180 volumes of bacteria were resuspended in PBS and adjusted to the same optical density
181 at 600 nm. The following growth media were tested: Colombia agar base
182 (bioMérieux), tryptic soy agar (TSA; BD), brain hearth infusion agar (BHIA; BD),
183 heart infusion agar (HIA; BD) and mannitol medium (1.25 g yeast extract, 6.25 g
184 mannitol, 3.75 g agar, 0.75 g bacto peptone) complemented or not complemented
185 with 5 % defibrinated sheep blood. For all strains, optimal growth was observed on
186 Colombia agar base with 5 % defibrinated sheep blood (CBA) at 37 °C in an 5 % CO_2
187 incubator (i.e. microaerophilic condition). On media without supplemented blood,
188 growth of neither strain PEB0122^T nor the other two *Bartonella* species could be
189 observed. The dependence on blood to grow on agar plates is a typical characteristic
190 of *Bartonella* spp.. For strain PEB0122^T, formation of opaque white colonies with a
191 size of 0.2 – 0.4 mm was observed after 2 – 3 days of incubation. In contrast, colonies
192 of '*B. tamiae*' and *B. henselae* were only observed after 4 and 5 days, respectively, and
193 were generally smaller in size compared to strain PEB0122^T (Fig. S1). Noteworthy,
194 strain PEB0122^T also exhibited slow growth under aerobic conditions, i.e. in air,
195 while neither '*B. tamiae*' nor *B. henselae* showed any reasonable growth under these
196 conditions. None of the three strains grew under anaerobic conditions. Temperature

197 sensitivity was tested on CBA in CO_2Gen Compact sachets (OXOID), creating a
198 microaerophilic environment with about 15 % O_2 and 6 % CO_2. In the tested
199 temperature range of 25 – 40 °C, all strains grew optimally between 35 – 37 °C. In
200 none of the tested growth conditions, haemolytic activity was observed during
201 incubation on CBA. All subsequent assays and analyses were performed with bacteria
202 grown under optimal conditions, i.e. on CBA in a 5% CO_2 incubator at 37 °C. Cell
203 morphology of strain PEB0122^T was analyzed with differential interference contrast
204 (DIC) microscopy and transmission electron microscopy (TEM). For the former, cells
205 were harvested from plates after they had grown for 2 days, resuspended in PBS
206 (optical density at 600 nm ≈ 1), placed on a microscopy slide covered with a thin
207 layer of agar, and incubated for 1 hour with DAPI (5 mg / ml) in order to stain the
208 DNA. Rod-shaped cells were the predominant morphology of strain PEB0122^T, '*B.*
209 *tamiae*' Th239^T and *B. henselae* Houston-1^T (Fig. S1). However, with cell lengths
210 ranging between 1.2 – 1.8 µm, the rods of PEB0122^T were shorter than those of
211 '*B. tamiae*' (1.2 - 2 µm) but longer than those of *B. henselae* (0.7 – 1.4 µm). We did
212 not observe any filamentous or coccoid-like morphologies for strain PEB0122^T. For
213 the TEM studies, cells were grown for 2 days and then fixed in 2.5 % glutaraldehyde
214 and 4 % formaldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed in 1 % osmium
215 oxide in H_2O and *en bloc* stained with 1 % uranyl acetate. Then, the samples were
216 dehydrated in a graded series of ethanol and embedded in Epon. Ultrathin sections
217 were post-stained with uranyl acetate and lead citrate and observed in a TEM at 80
218 keV (CM10, FEI Company, Eindhoven, The Netherlands). Pictures were taken using
219 the Morada camera (Olympus, SIS, Munster, Germany). Cells of PEB0122^T appeared
220 rod-shaped in TEM and revealed a two-layered cell envelope typical for gram-
221 negative bacteria (Fig. 3). The length was between 1.2 – 1.8 µm confirming results

222 from DIC microscopy. The width was about 0.5 µm. Hairy structures could be
223 detected on the surface of some cells covering parts of the cell envelope. The presence
224 of filamentous structures in the extracellular matrix of bacterial cells could suggest
225 the presence of flagella. Several *Bartonella* species, including the closely related '*B.*
226 *tamiae*', are known to harbor flagella (Dehio *et al.*, 2001; Dehio & Engel, 2009)
227 making their presence also likely in strain PEB0122^T. Electron-dense spots were
228 observed in the cytoplasm of cells of strain PEB0122^T. These could originate from
229 metal deposits.

230 For biochemical and metabolic characterization, we performed several
231 standard assays on strains PEB0122^T, PEB0149, '*B. tamiae*' Th239^T, and *B. henselae*
232 Houston-1^T (Table 1). Catalase activity was tested by the direct addition of bacteria
233 onto a drop of 3 % H_2O_2. The observation of foaming as a result of O_2 gas production
234 was used as an indication for a catalase-positive strain. The presence of the
235 cytochrome *c* oxidase was determined by spreading bacteria on Whatman paper with
236 a drop of 1% N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and observing the
237 development of a characteristic blue-violet color which indicates oxidation of TMPD
238 by cytochrome *c* oxidase. PEB0122^T, PEB0149 and '*B. tamiae*' Th239^T were positive
239 for catalase activity, while *B. henselae* Houston-1^T was negative. All four strains were
240 positive for cytochrome *c* oxidase. Using the Microgen^{TM} GnA+B-ID System kit
241 (Microgen Bioproducts), we further tested fermentation of different substrates
242 (glucose, mannitol, xylose, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose,
243 adonitol, raffinose and salicin), reduction of nitrate, H_2S production, the activity of
244 urease, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase,
245 tryptophan deaminase (TDA), and proteolytic enzymes, indole production from
246 tryptophan, acetoin production from glucose, utilization of citrate (as the only carbon

247 source) and β-galactosidase hydrolysis of ONPG. Both 'Alpha-1' strains, PEB0122^T
248 and PEB0149, revealed the same metabolic characteristics in these test. In contrast to
249 '*B. tamiae*' and *B. henselae*, they were positive for xylose and arabinose fermentation,
250 nitrate reduction, and urease activity. All tests that gave differential results between
251 the four tested strains are summarized in Table 1.

252 We also tested antibiotic susceptibility of strains PEB0122^T and PEB0149, *B.*
253 *tamiae*' Th239^T and *B. henselae* Houston-1^T. Filter discs (BD Sensi-Discs) were
254 spotted with defined amounts of antibiotics and put directly onto CBA plates
255 inoculated with approximately the same number of bacteria. The following antibiotics
256 were tested: kanamycin, tetracycline, oxytetracycline, chloramphenicol and
257 rifampicin at concentrations of 10, 20 and 30 µg per disc, and ampicillin,
258 erythromycin, gentamicin and streptomycin at concentrations of 10 and 20 µg per
259 disc. Plates were cultivated for 6 days before they were inspected for zones without
260 bacterial growth around the discs. The antibiotic-susceptible *Escherichia coli* strain
261 K-12 MG1655 was cultivated on TSA in aerobic condition at 37 °C for one day and
262 used as a positive control for the activity of the antibiotics. Strains were considered to
263 be resistant when no clearance zone appeared around the filter disc. Strain PEB0122^T
264 was found to be resistant to oxytetracycline (20 µg) and, as indicated by slower
265 growth, weakly resistant to tetracycline (20 µg), ampicillin (20 µg), and
266 chloramphenicol (20 µg) (Table 1). Strain PEB0122^T was susceptible to all other
267 tested types of antibiotics and concentrations. A similar pattern was found for strain
268 PEB0149, while '*B. tamiae*' Th239^T and *B. henselae* Houston-1^T showed marked
269 differences in resistance (Table 1).

270 Fatty acid analyses of strains PEB0122^T and '*B. tamiae*' Th239^T were carried
271 out by the Identification Service of the DSMZ, Braunschweig, Germany, and

compared to previously published profiles of other *Bartonella* spp., including *B. henselae* Houston-1^T, *B. quintana* WA-1 and *B. bacilliformis* ATCC 35685^T (Clarridge *et al*. 1995). The most abundant cellular fatty acids of strain PEB0122^T were palmitic acid ($C_{16:0}$), $C_{18:1}\omega 7c$ and $C_{19:1}$CYCLO$\omega 8c$. This was also the case for '*B. tamiae*' Th239^T, except that the proportions were shifted towards markedly higher amounts of $C_{19:1}$CYCLO$\omega 8c$ and fewer amounts of palmitic acid. Previously published fatty acid compositions of *Bartonella* spp. (Clarridge *et al*. 1995) showed similar cellular fatty acid compositions with palmitic acid ($C_{16:0}$) and isomers of 18- or 19-carbon fatty acids being the most abundant (Table 2). Overall, strain PEB0122^T could be distinguished from other *Bartonella* species by a higher content of palmitic acid ($C_{16:0}$) and margaric acid ($C_{17:0}$).

In conclusion, sequence similarity and phylogenetic position suggest that the closely related strains PEB0122^T, PEB0149 and PEB0150 belong to a novel species of the genus *Bartonella.* Distinct growth characteristics and a number of biochemical properties discriminate strains PEB0122^T and PEB0149 from other *Bartonella* species. Thus, we conclude that these strains can be classified within a novel species of the genus *Bartonella* for which we propose the name *Bartonella apis* sp. nov..

Description of *Bartonella apis* sp. nov.

Bartonella apis (aˈpis. L. gen. fem. n. apis from honey bee, the genus name of the honey bee *Apis mellifera*, referring to the insect host of this *Bartonella* species).

Cells of this Gram-staining-negative bacterium are rod-shaped with a length of 1.2 – 1.8 µm and width of about 0.5 µm. Optimal growth is achieved on CBA under

295 microaerophilic atmosphere enriched with 5 % CO_2 at 35 – 37 °C. Suboptimal growth
296 is also observed under aerobic conditions. No growth is observed under anaerobic
297 conditions. After 2 – 3 days of cultivation, smooth, round, opaque-white colonies with
298 a diameter of 0.2 – 0.4 mm are formed. Positive for catalase and cytochrome *c*
299 oxidase. Positive for urease, nitrate reductase and fermentation of glucose, xylose, and
300 arabinose, but negative for H_2S production, lysine decarboxylase, ornithine
301 decarboxylase, arginine dihydrolase, tryptophan deaminase (TDA), proteolysis, indole
302 production from tryptophan, acetoin production from glucose, utilization of citrate, β-
303 galactosidase hydrolysis of ONPG, and fermentation of mannitol, inositol, sorbitol,
304 rhamnose, sucrose, lactose, adonitol, raffinose and salicin. The most abundant cellular
305 fatty acids are palmitic acid ($C_{16:0}$) and isomers of 18- and 19-carbon chains.

306 The type strain is PEB0122^T (=NCIMB 14961^T, =DSM 29779^T), isolated from the
307 gut of Western honey bee, *A. mellifera*, from West Haven, CT, USA. The genomic
308 DNA G+C content of the strain is 45.5 mol%.

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310

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Tables

Table 1. Differential characteristics of strain PEB0122^T, strain PEB0149, '*B. tamiae*' Th239^T and *B. henselae* Houston-1^T.

Characteristic	1	2	3	4
Oxygen requirement	MA, (A)	MA, (A)	MA	MA
Catalase	+	+	+	−
Urease	+	+	−	−
Reduction of nitrate	+	+	−	−
Positive fermentation substrates	glucose xylose arabinose	glucose xylose arabinose	glucose sucrose	−
Antibiotic susceptibility				
Kanamycin	S	S	S	10 (W)
Tetracycline	20 (W)	30 (W)	30	S
Oxytetracycline	20	30 (W)	30	S
Ampicillin	20 (W)	20 (W)	30	S
Chloramphenicol	20 (W)	10	30	S
Erythromycin	S	S	30	S
Gentamicin	S	S	S	10
Rifampicin	S	S	10 (W)	S
Streptomycin	S	S	S	10

Strains: 1, strain PEB0122^T; 2, strain PEB0149; 3, '*B. tamiae*' Th239^T; 4, *B. henselae* Houston-1^T. All data are from this study.

A, aerobe; MA, microaerophilic; +, positive reaction; −, negative reaction.

Antibiotic susceptibility assay: S, susceptible; numbers correspond to the antibiotic concentrations to which tested strains were resistant or weakly resistant (W), amounts lower than 10 μg and more than 30 μg were not tested.

458 **Table 2.** Fatty acid compositions (in percentage) of strain PEB0122^T in comparison

459 with related *Bartonella* species.

Fatty acids (%)	1	2	3*	4*	5*
$C_{12:0}$	5.5	3.6	-	-	-
$C_{16:0}$	37.8	25.5	21.5	16.1	28.3
$C_{16:1}\omega7c/C_{15:0}$ iso2OH	1.6	0.4	0.5	0.4	21.2
$C_{17:0}$	5.1	1.2	0.2	2.1	0
$C_{18:0}$	6.0	8.7	27.8	27.8	1.5
$C_{18:1}\omega7c$	16.7	9.6	48.3†	51.4†	39.9†
$C_{19:1}$CYCLO$\omega8c$	24.6	48.4			
others	2.6	2.5			

460

461 Strains: 1, strain PEB0122^T; 2, '*B. tamiae*' Th239^T; 3, *B. henselae* Houston-1^T; 4,

462 *B. quintana* WA-1; 5, *B. bacilliformis*-1 ATCC 35685^T. Bacterial cultures of strain

463 PEB0122^T and '*B. tamiae*' were grown on CBA with 5 % sheep blood at 37 °C in 5 %

464 CO_2 for two days. Values <1 % have been omitted. Data are from this study, unless

465 indicated otherwise.

466 * Data obtained from Clarridge *et al*. (1995).

467 † Value was described as "Summed feature 7" in the original study, which likely

468 comprises isomers of 18- and 19-carbon chains.

469

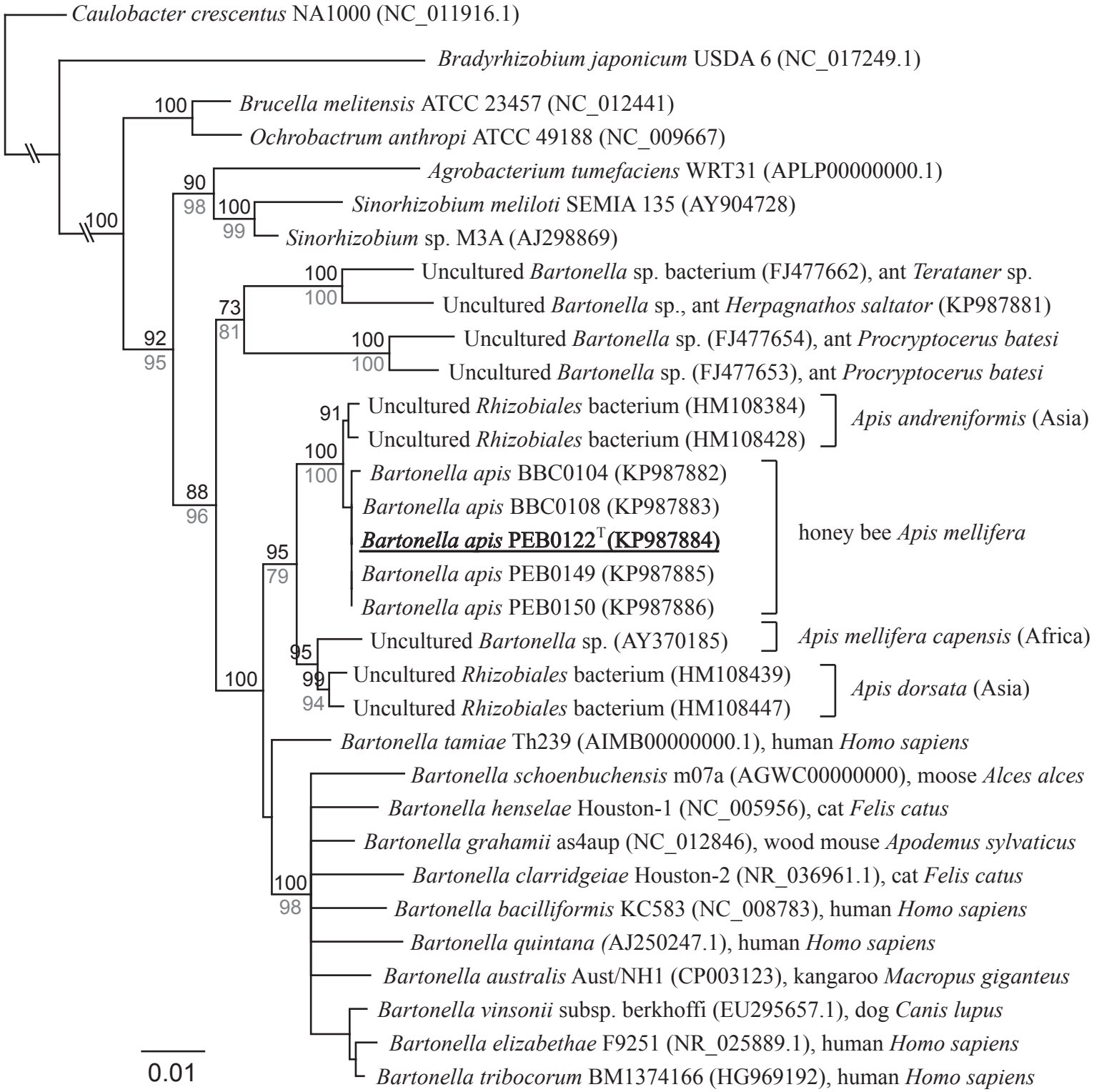
Figure legends

Figure 1. Neighbor-joining tree based on a CLUSTALW alignment of cropped 16S rRNA gene sequences (1,364 aligned sites). The tree was inferred with MEGA 6.0 (Tamura *et al.*, 2013) using the Jukes-Cantor model. 1000 bootstrap trees were inferred and values ≥80 % are shown above branches. Numbers below branches indicate bootstrap values ≥80 % (100 replicates) resulting from a maximum-likelihood (ML) analysis using the general time reversible model (GTR; +I = 0.717, +G = 0.492) as the best available model according to jModelTest2 (Darriba *et al.*, 2012; Guindon *et al.*, 2010). Information about host species of *Bartonella* spp. was taken from Sato *et al.* (2013). Accession numbers of sequences are listed in Table S2. Scale bar, 0.005 substitutions per site.

Figure 2. Maximum-likelihood (ML) tree based on eight concatenated phylogenetic marker genes: *alaS, uvrC, recN, pyrG, ffh*/*srp, uvrB, radA, and typA*. Nucleotide sequences of the eight genes were aligned on protein level with CLUSTALW, back-translated, cropped and concatenated in Geneious® 6.1.8 (Kearse *et al.*, 2012). The ML tree was inferred using the GTR model (+I = 0.319, +G = 0.884). 100 bootstrap trees were calculated and values ≥80 % are shown above branches. Accession numbers of sequences are listed in Table S2. Scale bar, 0.05 substitutions per site.

Figure 3. Transmission electron micrograph of strain PEB0122^{T}. Thin sections were observed by TEM. The cells show a membrane structure characteristic for gram-negative bacteria. The surface of some bacteria is partially covered with fine hairy

494 structures (black arrow head) of approximately 30 nm in length. Next to the cells,
495 flagella-like structures are visible (black arrow). The electron-dense spots in the
496 bacteria could indicate metal deposits (white arrow). Scale bar, 1 μm.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Bradyrhizobium japonicum USDA 6 (NC_017249.1)
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Brucella melitensis ATCC 23457 (NC_012441)
Ochrobactrum anthropi ATCC 49188 (NC_009667)
Agrobacterium tumefaciens WRT31 (APLP00000000.1)
90
98
100
Sinorhizobium meliloti SEMIA 135 (AY904728)
100
99
Sinorhizobium sp. M3A (AJ298869)
100
Uncultured *Bartonella* sp. bacterium (FJ477662), ant *Terataner* sp.
100
73
Uncultured *Bartonella* sp., ant *Herpagnathos saltator* (KP987881)
81
92
100
Uncultured *Bartonella* sp. (FJ477654), ant *Procryptocerus batesi*
95
100
Uncultured *Bartonella* sp. (FJ477653), ant *Procryptocerus batesi*
91
Uncultured *Rhizobiales* bacterium (HM108384)
Apis andreniformis (Asia)
Uncultured *Rhizobiales* bacterium (HM108428)
100
100
Bartonella apis BBC0104 (KP987882)
Bartonella apis BBC0108 (KP987883)
88
96
***Bartonella apis* PEB0122^{T} (KP987884)**
honey bee *Apis mellifera*
95
79
Bartonella apis PEB0149 (KP987885)
Bartonella apis PEB0150 (KP987886)
Uncultured *Bartonella* sp. (AY370185)
Apis mellifera capensis (Africa)
95
Uncultured *Rhizobiales* bacterium (HM108439)
100
99
Apis dorsata (Asia)
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Uncultured *Rhizobiales* bacterium (HM108447)
Bartonella tamiae Th239 (AIMB00000000.1), human *Homo sapiens*
Bartonella schoenbuchensis m07a (AGWC00000000), moose *Alces alces*
Bartonella henselae Houston-1 (NC_005956), cat *Felis catus*
Bartonella grahamii as4aup (NC_012846), wood mouse *Apodemus sylvaticus*
Bartonella clarridgeiae Houston-2 (NR_036961.1), cat *Felis catus*
100
98
Bartonella bacilliformis KC583 (NC_008783), human *Homo sapiens*
*Bartonella quintana (*AJ250247.1), human *Homo sapiens*
Bartonella australis Aust/NH1 (CP003123), kangaroo *Macropus giganteus*
Bartonella vinsonii subsp. berkhoffi (EU295657.1), dog *Canis lupus*
Bartonella elizabethae F9251 (NR_025889.1), human *Homo sapiens*
Bartonella tribocorum BM1374166 (HG969192), human *Homo sapiens*
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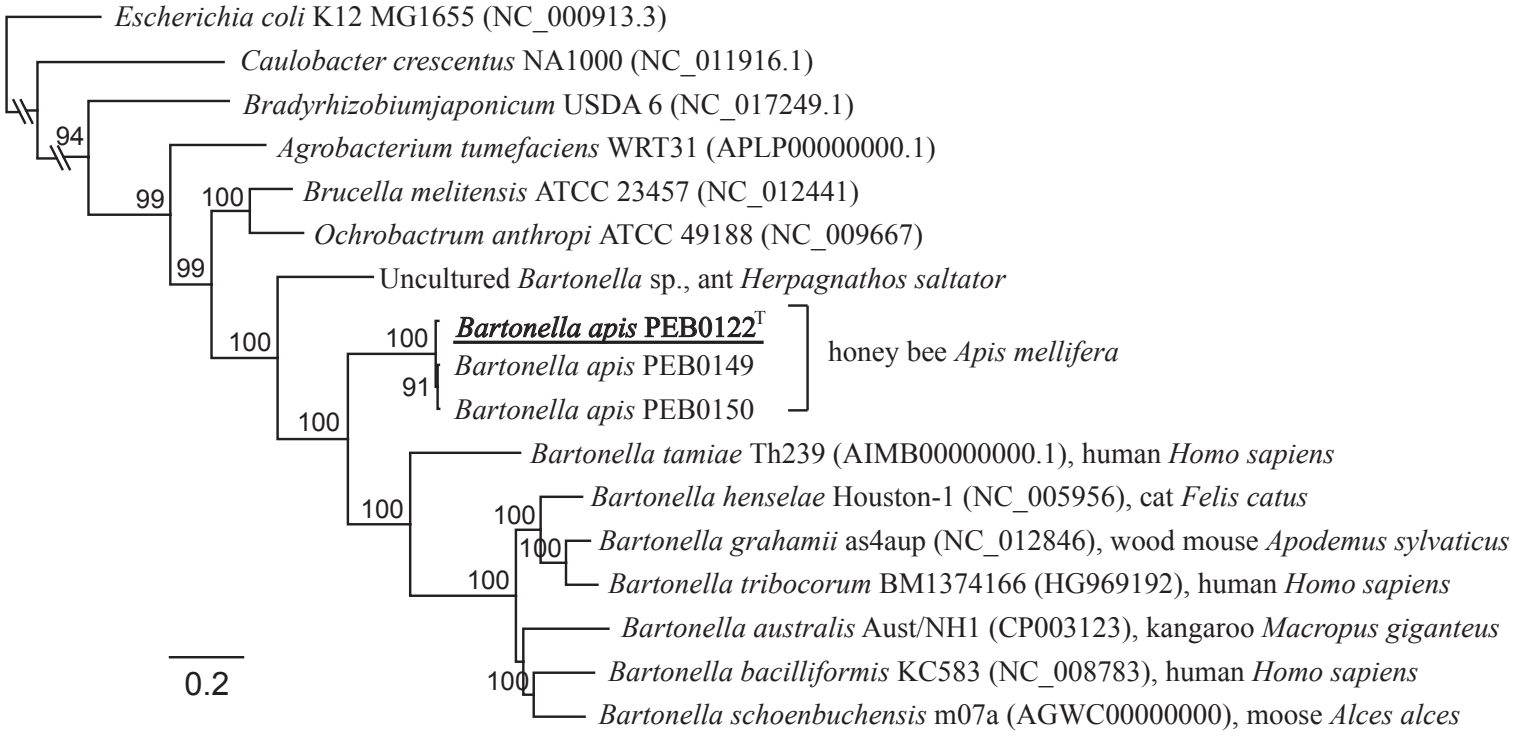
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Bradyrhizobiumjaponicum USDA 6 (NC_017249.1)
Agrobacterium tumefaciens WRT31 (APLP00000000.1)
Brucella melitensis ATCC 23457 (NC_012441)
Ochrobactrum anthropi ATCC 49188 (NC_009667)
Uncultured *Bartonella* sp., ant *Herpagnathos saltator*
***Bartonella apis* PEB0122**^{T}
Bartonella apis PEB0149
Bartonella apis PEB0150
honey bee *Apis mellifera*
Bartonella tamiae Th239 (AIMB00000000.1), human *Homo sapiens*
Bartonella henselae Houston-1 (NC_005956), cat *Felis catus*
Bartonella grahamii as4aup (NC_012846), wood mouse *Apodemus sylvaticus*
Bartonella tribocorum BM1374166 (HG969192), human *Homo sapiens*
Bartonella australis Aust/NH1 (CP003123), kangaroo *Macropus giganteus*
Bartonella bacilliformis KC583 (NC_008783), human *Homo sapiens*
Bartonella schoenbuchensis m07a (AGWC00000000), moose *Alces alces*
94
99
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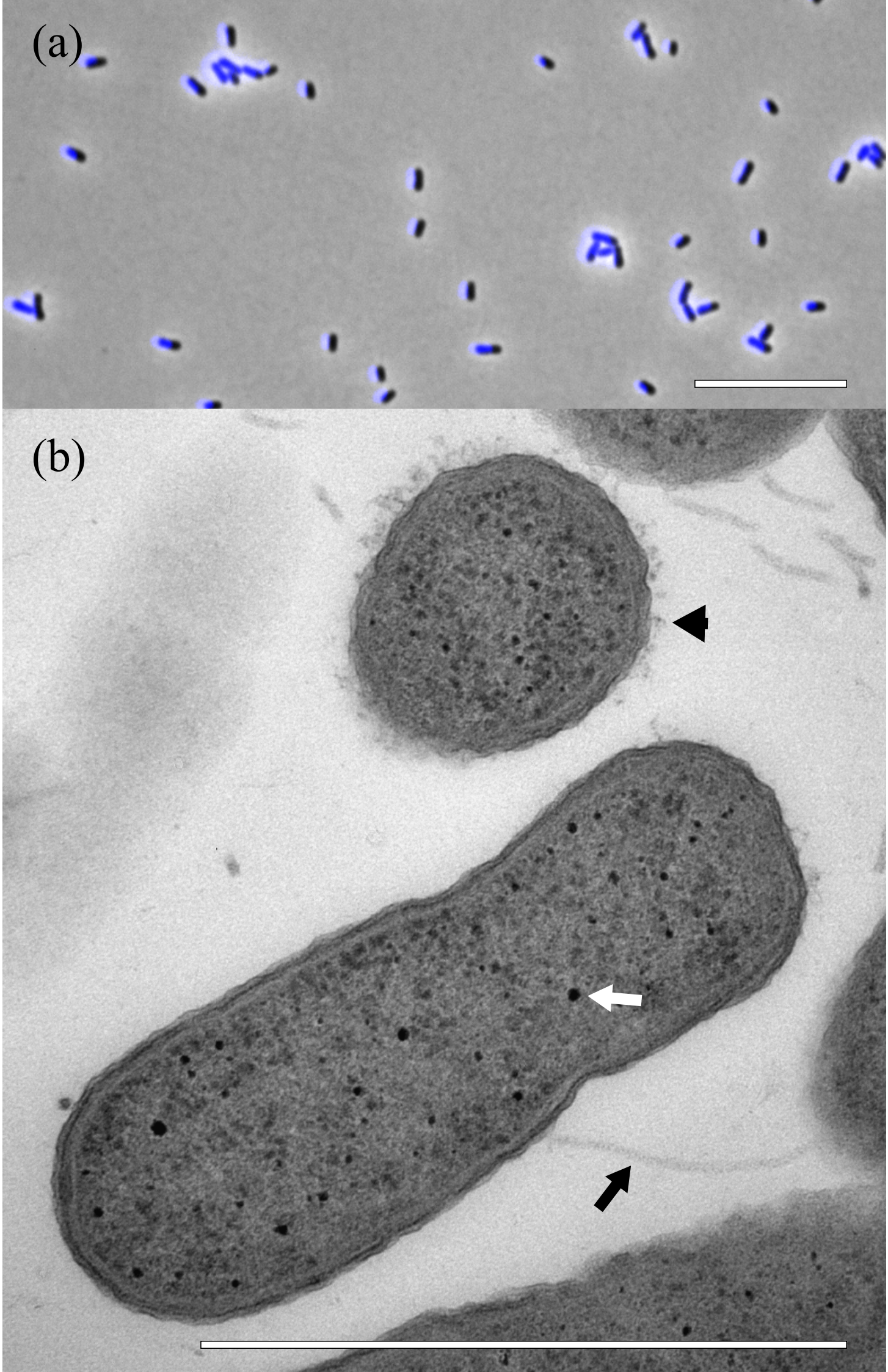
(a)
(b)

Supplementary data for:

Bartonella apis sp. nov., a honey bee gut symbiont from the class *Alphaproteobacteria*

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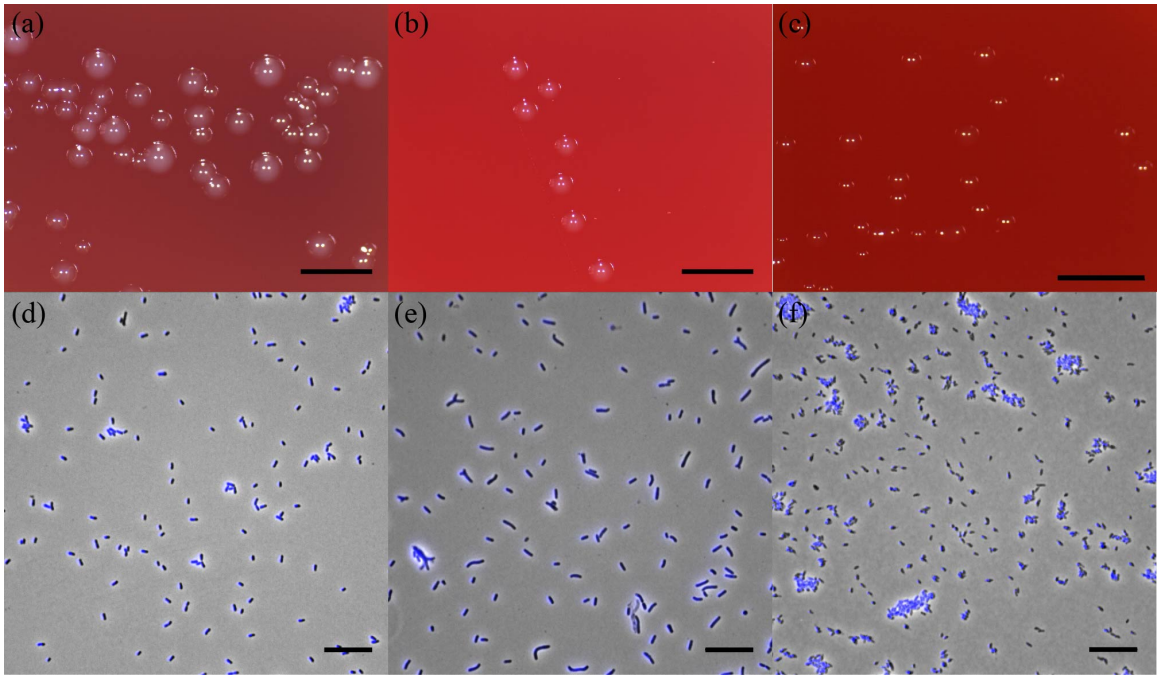

Figure S1. Colony morphology and single cell images of strain PEB0122^{T} (a, d), '*B. tamiae'* Th239^{T} (b, e) and *B. henselae* Houston-1^{T} (c, f) after 4 days of growth on CBA supplemented with 5 % blood, 37 °C, 5 % CO_2. Images (a), (b) and (c) were taken with a stereomicroscope (Leica, EZ4HD). Scale bars, 1 mm. Microscopy analysis of single cells (d), (e) and (f) was performed with a Nikon microscope (D5100) under DIC and UV mode and merged to display both the cell shape and DNA. Scale bars,10 µm.

		Sequence similarities (%) for *B. apis* PEB0122 (T)		
Species	Strain	16S rRNA	*gltA* (327 bp)	*rpoB* (825 bp)
B. apis	**PEB0122 (T)**	100	100	100
B. apis	PEB0149	100	98.8	99.5
B. apis	PEB0150	100	98.2	98.7
B. apis	BBC0108	100	-	-
B. apis	BBC0104	99.8	-	-
Uncultured *Rhizobiales* bacterium (from *Apis andreniformis)* (3)*	-	99.5	-	-
Uncultured *Rhizobiales* bacterium (from *Apis andreniformis)* (4)*	-	99.1	-	-
Uncultured *Rhizobiales* bacterium (from *Apis dorsata)* (1)*	-	98.2	-	-
Uncultured *Bartonella* sp. (from *Apis mellifera capensis)**	-	98	-	-
Uncultured *Rhizobiales* bacterium (from *Apis dorsata)* (2)*	-	97.9	-	-
'B. tamiae'	Th239 (T)	97.6	80.4	79.1
B. grahamii	V2 (T)	97.3	81	76.8
B. vinsonii subsp. *vinsonii**	Baker (T)	97.2	81	77.3
B. vinsonii subsp. *arupensis*	OK94-513 (T)	97.1	81.7	77.3
B. birtlesii	IBS 325 (T)	97.1	81.7	77.3
B. doshiae	R18 (T)	97.1	80.4	77.1
B. koehlerae	C-29 (T)	97.1	78.3	76.5
*B. callosciuri**	BR11-1 (T)	97	80.1	77.4
*B. queenslandensis**	AUST/NH12 (T)	97	78.9	77.2
B. rattaustraliani	AUST/NH4 (T)	97	81.3	76.9
B. florencae	R4 (T)	96.9	82	78.3
B. henselae	Houston-1 (T)	96.9	78.9	77.3
B. elizabethae	F9251 (T)	96.9	81.3	77.5
B. tribocorum	IBS 506 (T)	96.9	80.1	77.2
B. bacilliformis	KC583 (T)	96.9	78.9	76
*B. capreoli**	IBS 193 (T)	96.8	80	76.9
*B. japonica**	Fuji 18-1 (T)	96.8	78.6	76
*B. silvatica**	Fuji 23-1 (T)	96.8	79.2	76.8
*B. taylorii**	M6 (T)	96.8	80.4	77.5
B. alsatica	IBS 382 (T)	96.8	82	76.9
*B. acomydis**	KS2-1 (T)	96.7	81	77.4
*B. pachyuromydis**	FN15-2 (T)	96.7	81.7	76.6
*B. jaculi**	OY2-1 (T)	96.7	78.6	76.2
B. clarridgeiae	Houston-2 (T)	96.7	78.3	76.1
B. rochalimae	ATCC BAA-1498 (T)	96.7	77.4	76.6
B. senegalensis	OS02 (T)	96.6	80.1	75.6
'B. australis'	Aust/NH1 (T)	96.5	81	76.7
*B. quintana**	Fuller (T)	96.5	79.2	76.5
*B. coopersplainsensis**	AUST/NH20 (T)	96.4	78	76.3
*B. schoenbuchensis**	R1 (T)	96.4	78	76.9
B. bovis	91-4 (T)	96.4	80.1	76.3
B. vinsonii subsp. *berkhoffii*	93-C01 (T)	96	81	78.3
*B. chomelii**	A828 (T)	95.9	78.3	77.5
Brucella melitensis	bv. 1 str. 16M (T)	95.1	77.7	81.2
Uncultured *Bartonella* sp. (from *Herpagnathos saltator)*	-	93.9	79.5	80.5
Uncultured *Bartonella* sp. (from *Terataner* sp.)*	-	93.8	-	-
Uncultured *Bartonella* sp. (from *Procryptocerus batesi)* (5)*	-	93.6	-	-
Uncultured *Bartonella* sp. (from *Procryptocerus batesi)* (6)*	-	93.3	-	-

Table S1. Sequence similarities (%) of 16S rRNA, *gltA* and *rpoB* genes for strain PEB0122^{T} with closely related strains, including previously described *Bartonella* species, strains from the honey bee gut, and sequences obtained from different ant species. Pairwise sequence similarities are based on ClustalW alignments. For the analysis of 16S rRNA genes, all sequences were full-length or nearly full-length (marked with an asterisk). For the analysis of *gltA* and *rpoB* genes, 327-bp and 825-bp long fragments were used, respectively. These partial sequences were proposed by La Scola *et al.* (2003) to discriminate *Bartonella* species.

Species	Strain	Genome	16S rRNA	*gltA*	*rpoB*	*alaS*	*ffh/srp*	*pyrG*	*radA*	*recN*	*typA*	*uvrB*	*uvrC*
B. acomydis	KS2-1 (T)	-	AB602533	AB529942	AB444979	-	-	-	-	-	-	-	-
B. alsatica	IBS 382 (T)	JH725020	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. apis	**PEB0122 (T)**	**-**	**KP987884**	**KT315729**	**KT315732**	**KP987850**	**KP987854**	**KP987859**	**KP987865**	**KP987862**	**KP987870**	**KP987874**	**KP987878**
B. apis	PEB0149	-	KP987885	KT315730	KT315733	KP987851	KP987855	KP987857	KP987867	KP987863	KP987871	KP987875	KP987879
B. apis	PEB0150	-	KP987886	KT315731	KT315734	KP987852	KP987856	KP987860	KP987868	KP987864	KP987872	KP987876	KP987880
B. apis	BBC0104	-	KP987882	-	-	-	-	-	-	-	-	-	-
B. apis	BBC0108	-	KP987883	-	-	-	-	-	-	-	-	-	-
'B. australis'	Aust/NH1 (T)	NC_020300	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. bacilliformis	KC583 (T)	NC_008783	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. birtlesii	IBS 325 (T)	CM001557	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. bovis	91-4 (T)	NZ_CM001844	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. callosciuri	BR11-1 (T)	-	AB602530	AB529931	AB602551	-	-	-	-	-	-	-	-
B. capreoli	IBS 193 (T)	-	AF293389	AB290188	AF293392	-	-	-	-	-	-	-	-
B. chomelii	A828 (T)	-	AY254309	KM215705	KM215690	-	-	-	-	-	-	-	-
B. clarridgeiae	Houston-2 (T)	JADC01000001	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. coopersplainsensis	AUST/NH20 (T)	-	EU111759	EU111792	EU111803	-	-	-	-	-	-	-	-
B. doshiae	R18 (T)	JH725094	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. elizabethae	F9251 (T)	JH725933	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. florencae	R4 (T)	HE997451	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. grahamii	V2 (T)	JACX01000001	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. henselae	Houston-1 (T)	NC_005956	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. jaculi	OY2-1 (T)	-	AB602527	AB529934	AB444975	-	-	-	-	-	-	-	-
B. japonica	Fuji 18-1 (T)	-	AB440632	AB242288	AB242289	-	-	-	-	-	-	-	-
B. koehlerae	C-29 (T)	KL407334	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. pachyuromydis	FN15-2 (T)	-	AB602531	AB602555	AB444978	-	-	-	-	-	-	-	-
B. queenslandensis	AUST/NH12 (T)	-	EU111755	EU111787	EU111798	-	-	-	-	-	-	-	-
B. quintana	Fuller (T)	-	M73228	AF165994	Z70014	-	-	-	-	-	-	-	-
B. rattaustraliani	AUST/NH4 (T)	NZ-CALW02000001	genome	EU111793	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. rochalimae	ATCC BAA-1498 (T)	KL407337	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. schoenbuchensis	R1 (T)	PRJEA41447	AJ278187	AJ167409	AJ278183	genome	genome	genome	genome	genome	genome	genome	genome
B. senegalensis	OS02 (T)	HE997540	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. silvatica	Fuji 23-1 (T)	-	AB440636	AB242292	AB242287	-	-	-	-	-	-	-	-
'B. tamiae'	Th239 (T)	JH725147	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. taylorii	M6 (T)	-	Z31350	AF165995	Z70013	-	-	-	-	-	-	-	-
B. tribocorum	IBS 506 (T)	NC_010161	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. vinsonii subsp. *arupensis*	OK94-513 (T)	JH725037	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. vinsonii subsp. *berkhoffii*	93-C01 (T)	JACY01000001	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
B. vinsonii subsp. *vinsonii*	Baker (T)	-	NR_037056	AF165997	Z70015	-	-	-	-	-	-	-	-
Brucella melitensis	bv. 1 str. 16M (T)	NC_0331817	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome	genome
Uncultured *Bartonella* sp. (from *Apis mellifera capensis)*	-	-	AY370185	-	-	-	-	-	-	-	-	-	-
Uncultured *Bartonella* sp. (from *Herpagnathos saltator)*	-	-	KP987881	KT852960	KT852961	KP987849	KP987853	KP987858	KP987866	KP987861	KP987869	KP987873	KP987877
Uncultured *Bartonella* sp. (from *Procryptocerus batesi)* (5)	-	-	FJ477653	-	-	-	-	-	-	-	-	-	-
Uncultured *Bartonella* sp. (from *Procryptocerus batesi)* (6)	-	-	FJ477654	-	-	-	-	-	-	-	-	-	-
Uncultured *Bartonella* sp. (from *Terataner* sp.*)*	-	-	FJ477662	-	-	-	-	-	-	-	-	-	-
Uncultured *Rhizobiales* bacterium (from *Apis andreniformis)* (3)	-	-	HM108384	-	-	-	-	-	-	-	-	-	-
Uncultured *Rhizobiales* bacterium (from *Apis andreniformis)* (4)	-	-	HM108428	-	-	-	-	-	-	-	-	-	-
Uncultured *Rhizobiales* bacterium (from *Apis dorsata)* (1)	-	-	HM108439	-	-	-	-	-	-	-	-	-	-
Uncultured *Rhizobiales* bacterium (from *Apis dorsata)* (2)	-	-	HM108447	-	-	-	-	-	-	-	-	-	-

Table S2. Accession numbers of gene sequences used in this study.