Bats as Reservoir Hosts of Human Bacterial Pathogen, *Bartonella mayotimonensis*

Technical Appendix

**Bat Sampling for Peripheral Blood, Fecal Droppings, and Ectoparasites**

Bats were caught with a combination of mist nets and harp trap (Animal Ethics Committee license no. ESLH-YM-2007-01055). Two mist nets were positioned on each side of the harp trap. A Sussex Autobat siren (I), which produces species-specific ultrasound social calls, was placed in the center of the harp trap to attract the bats. This multitrap combination was placed across the flying corridor of bats commuting between roosts and foraging areas. Caught bats were visually identified to species, banded, and measured for mass and forearm length. The tail skin membrane was wiped with cotton sticks soaked in 75% (v/v) ethanol. The blood sample was collected into a 75-μL heparinized capillary tube from the interfemoral vein after lancing with a 25-gauge needle. Blood samples were stored on ice until culturing. Fur ectoparasites collected from bats were surface sterilized for 15 min in 75% (v/v) ethanol followed by a wash with phosphate-buffered saline (PBS). The ectoparasites were stored dry at –80°C until isolation of DNA. Fecal droppings were collected from holding bags where the bats were kept during the capture period or straight from the bats during handling. All bats were released after sampling.

**Metagenomic Analysis of Fecal DNA**

Fecal samples were processed in the Herbarium laboratory at the University of Turku (Turku, Finland), where so far only plant specimens have been handled. Fecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA, catalog no.51504). Negative control extraction containing all the chemicals but no fecal pellet was performed alongside to monitor for contamination of the extraction chemicals. The DNA fragmentation and library preparation was performed in the TegLab facilities (Laboratory of Genetics,
University of Turku) following Ion Torrent user guide (publication part no. 4471989 rev. B). Negative control reaction was performed to monitor for contamination of the chemicals used. Adapter ligation success was visually inspected under UV light using a 2% (w/v) agarose gel stained with 0.5 μg/mL (w/v) of ethidium bromide. The DNA library was amplified with the following setup: 5 μL library was added to a master mix consisting of 5 U of Herculase II polymerase (Agilent Technologies, Santa Clara, CA, USA, catalog no. 600677), 1× Herculase II reaction buffer, 25 mM each dNTP, 10 μM each primer, and added PCR-grade water up to 50 μL. Amplification step generated millions of DNA copies, which include the binding sites necessary for subsequent Ion Torrent sequencing. The thermocycling profile included a 30 s denaturation at 98°C followed by 15 cycles consisting of a 20 s denaturation at 98°C, a 30 s annealing at 64°C, and a 30 s elongation at 72°C. Final elongation was conducted at 72°C for 5 min. To clean the amplified library of leftover adapters and primer-dimers, size-selection was done by separating the entire library using 2% (w/v) Size-Select Agarose E-Gel and E-Gel Electrophoresis System (Life Technologies, Carlsbad, CA, USA, catalog nos. G6610-02 and G6500) following the manufacturer’s instructions. The library pool stock was then diluted to a final concentration of 26 pM. For template preparation, an 18-μL aliquot of the library dilution (≈2.8 × 10^8 molecules) was transferred into the sequencing reaction setup. Emulsion PCR and Ion Torrent Sequencing was carried out on a 314 chip according to the manufacturer’s protocol (publication part no. 4471974 rev. C). Performance of the Ion Torrent Personal Genome Machine is shown in Technical Appendix Figure 1. The resulting reads were trimmed of sequencing adapters and poor-quality parts by using 0.05 error probability limit and then the reads <50 bp were excluded by using the software Geneious Pro (Geneious version 6.1, Biomatters) available at www.geneious.com/. Subsequent analyses were carried out by using super computer clusters at the IT Center for Science (Espoo, Finland, www.csc.fi) and at Finnish Grid Infrastructure (www.csc.fi/english/collaboration/projects/fgi). Sequences were assigned to GenBank reference database sequences using the BLASTN 2.2.25+ algorithm. MetaGenome Analyzer software (MEGAN v4.70.4) available at http://ab.inf.uni-tuebingen.de/software/megan/ was used to visualize the results.

**Isolation of Bartonella from Peripheral Blood**

Blood samples were cultured within 3–6 hours after blood sampling. Blood-filled heparinized capillary tubes were emptied into 500 μL of PBS on ice. Broad-spectrum antifungal
compound amphotericin B (Fungizone; Sigma, catalog no. A2942) was added at a concentration of 10 μg/mL (w/v). 400 μL aliquots of the blood samples were cultured on Columbia Blood Agar Base (CBA) (Difco, catalog no. 279240) supplemented with 5% (v/v) of defibrinated sheep blood. The remaining samples were stored at –80°C for DNA isolation. The plates were incubated in a humified 5% CO2 atmosphere at 37°C up to 1 month. Individual colonies from the primary plates (passage 0) were subcultured on fresh CBA blood plates. After 1 week of incubation as described above, the clonal isolates were suspended in 1 mL of Todd Hewitt Broth (Beckton Dickinson, Franklin Lakes, NJ, USA, catalog no. 249210) supplemented with 0.5% (w/v) yeast extract (Biokar Diagnostics, Beauvais, France, catalog no. A1202HA) [THY] and 25% (v/v) of glycerol. These solutions were stored at –80°C as passage 1 stocks.

Extraction of DNA from Bat Ectoparasites, Blood, and *Bartonella* Isolates

Ectoparasites were mechanically disrupted with Kimble Kontes pellet pestle (Sigma) in 200 μL PBS. One hundred microliters of bat blood–PBS solution (see above) was diluted with 100 μL PBS. First, the samples were incubated for 10 min at room temperature in 2% (w/v) sodium dodecyl sulphate, and then, after 3 U Proteinase K (Finnzymes) was added, in a shaker at 60°C for 2 h. After incubation, 150 μL of saturated NaCl (6 M) was added, the samples were vortexed for 30 sec and centrifugated at 16100 rcf for 30 min. From the supernatant, the DNA was precipitated with 200 μL of isopropanol overnight at –20°C. The next day, the precipitated DNA was pelleted with centrifugation at 16100 rcf and washed with 200 μL of ice cold 70% (v/v) ethanol. The DNA pellets were air-dried and dissolved in sterile water. Passage 2 clonal isolates were harvested from 5-day-old CBA blood plates into sterile PBS. Bacteria were pelleted by centrifugation (16100 rcf, 2 min). Bacterial pellets were resuspended in 1 mL of 25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA (pH 8.0) containing 500,000 U of lysozyme and 100 U of RNAse A. The suspensions were incubated at 37°C for 2 h. Sodium dodecyl sulphate was added to 1.0% (w/v), and the proteins were removed by 2 phenol and subsequent 2 chloroform precipitations. 0.11 volume of 3 M NaOAc (pH 5.2) was added. The DNA was precipitated, washed and dissolved as above, except 2.2 volumes of ice-cold 99% (v/v) ethanol was added to precipitate the DNA.
**Bartonella and Ectoparasite PCR Analyses**

The PCR reactions were carried out in a total volume of 50 mL, containing 2 mM primers (Technical Appendix Table 2), 50 mM of each dNTP, 1 U of DyNAzyme II DNA Polymerase (Thermo Scientific), and 100–250 ng of template DNA or water (negative control). DNA from *Bartonella henselae* Houston-1 was used as a positive *Bartonella* control. All of the PCRs were run under the same conditions with an initial denaturation at 95°C for 1 min, followed by denaturation at 95°C for 30 s, annealing at 55°C for 15 s, and extension at 72°C for 1 min. Amplification was completed by 39 additional cycles at 72°C for 1 min and final extension at 72°C for 10 min.

**Transmission Electron Microscopy**

Bacteria were harvested from 5-day-old CBA blood plates into sterile PBS. Bacteria were pelleted by centrifugation (16100 ref, 2 min) and fixed with 5% (v/v) glutaraldehyde in 0.16 M s-collidine buffer pH 7.4. Bacterial pellets were embedded in epoxy resin, and the blocks were cut by using an ultra microtome (Leica Ultracut UCT). 70-nm ultrathin sections were mounted on formvar-coated copper grids. The ultrathin sections were stained with 1% (w/v) uranyl acetate for 30 min at 20°C and 0.3% (w/v) lead citrate for 3 min at 20°C. The grids were examined using electron microscopes JEM-1200EX and JEM-1400 Plus, JEOL, Tokio, Japan.

**Nucleotide Sequence and Phylogenetic Analyses**

To incorporate all *Bartonella* species and Candidatus *B. mayotimonensis* into the type strain phylogeny (Figure 2) and the pairwise genetic distance value calculations (Technical Appendix Table 4), *rpoB* sequences were trimmed to 406-bp fragments (corresponds to nucleotide positions 246–651 of *B. alsatica rpoB*, AF165987), *gltA* sequences down to 311–312-bp fragments (corresponds to nucleotide positions 4–315 of *B. alsatica gltA*, AF204273), 16S rRNA sequences down to 483–85-bp fragments (corresponds to nucleotide positions 881–1365 of *B. alsatica rpoB*, AJ002139), and *ftsZ* sequences down to 280-bp fragments (corresponds to nucleotide positions 61–340 of *B. alsatica ftsZ*, AF467763). GenBank accession numbers of the type strain sequences are shown in Technical Appendix Table 5. Phylogenetic analysis of the worldwide bat-colonizing *Bartonella* strains (Figure 3) was performed by using the *gltA* sequences trimmed down to 253-bp fragments (corresponds to
nucleotide positions 4–256 of *B. alsatica gltA*, AF204273). Phylogenetic analyses were performed by using Molecular Evolutionary Genetics Analysis (MEGA) 5.2.1 (www.megasoftware.net/). To this end, the sequences were first aligned with ClustalW. The neighbor-joining trees were constructed by using the maximum composite likelihood method with 1,000 replicas. The maximum-likelihood trees were constructed using the Tamura-Nei method with 1,000 replicas and nearest-neighbor-interchange as the maximum-likelihood heuristic method with the default option to construct the initial tree.

Reference

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Technical Appendix Table 1. Bat sampling and PCR-detection and isolation of *Bartonella* spp.*

| Sample type, bat species | Capture date | Capture location | Band no., sex, age, mass, average of left and right forearm | Body condition index† | No. clonal blood isolates | PCR of blood samples on rpoB | Fur ectoparasites‡ | PCR of ectoparasite samples on rpoB |
|-------------------------|--------------|------------------|----------------------------------------------------------|------------------------|-----------------------------|-----------------------------|------------------|-----------------------------------|
| **Fecal**               |              |                  |                                                          |                        |                             |                             |                  |                                   |
| *Myotis daubentonii*    | 2010 Jun 3   | 60° 26' 41" N, 22° 03' 15" E | 2018, M, adult, 8.8 g, 38.5 mm§ | 0.229                   | ND                          | ND                          | ND                | ND                                |
| *Myotis daubentonii*    | 2011 Jul 20  | 60° 21' 31" N, 22° 13' 26" E | 2140, M, adult, 8.0 g, 38.1 mm | 0.210                   | ND                          | ND                          | ND                | ND                                |
| *Myotis daubentonii*    | 2011 Jul 18  | 60° 26' 54" N, 21° 59' 27" E | 2758, M, juvenile, ND, 37.5 mm | ND                      | ND                          | ND                          | ND                | ND                                |
| *Myotis daubentonii*    | 2011 Jul 19  | 60° 26' 54" N, 21° 59' 27" E | 2768, M, juvenile, ND, 36.4 mm | ND                      | ND                          | ND                          | ND                | ND                                |
| *Myotis daubentonii*    | 2011 Jul 24  | 60° 12' 45" N, 21° 51' 18" E | 2771, F, adult, 7.8 g, 34.7 mm | 0.225                   | ND                          | ND                          | ND                | ND                                |
| *Myotis daubentonii*    | 2011 Jul 24  | 60° 12' 45" N, 21° 51' 18" E | 2772, F, adult, 14.3 g, 40 mm | 0.358                   | ND                          | ND                          | ND                | ND                                |
| *Myotis brandti*        | 2011 Jul 27  | 60° 26' 54" N, 22° 06' 29" E | 2786, F, adult, 7.9 g, 33.9 mm | 0.233                   | ND                          | ND                          | ND                | ND                                |
| *Eptesicus nilssonii*   | 2011 Jul 31  | 60° 26' 54" N, 22° 06' 29" E | 2788, M, adult, 9.7 g, 39.0 mm | 0.249                   | ND                          | ND                          | ND                | ND                                |
| *Myotis brandti*        | 2011 Jul 31  | 60° 26' 54" N, 22° 06' 29" E | 2781, F, juvenile, 7.9 g, 35.7 mm | 0.221                   | ND                          | ND                          | ND                | ND                                |
| **Blood**               |              |                  |                                                          |                        |                             |                             |                  |                                   |
| *Eptesicus nilssonii*   | 2012 Aug 6   | 60° 27' 14" N, 22° 17' 05" E | 2369, F, adult, 10.8 g, 41.5 mm | 0.260                   | –                           | –                           | –                 | –                                |
| *Myotis mystacinus*     | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1156, F, juvenile, 6.2 g, 34.9 mm | 0.178                   | –                           | rpoB-4                      | –                 | –                                |
| *Myotis brandti*        | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | no band, M, juvenile ND, ND | ND                      | –                           | –                           | Siphonaptera (n = 1) | rpoB-4               |
| *Eptesicus nilssonii*   | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1157, F, adult, 10.1 g, 38.1 mm | 0.265                   | 6 clones, all rpoB-1#      | ND                          | Siphonaptera (n = 1) | rpoB-1               |
| *Eptesicus nilssonii*   | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1168, F, adult, 9.6 g, 39.5 mm | 0.243                   | –                           | –                           | –                 | –                                |
| *Myotis brandti*        | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1159, M, adult, 6.3 g, 35.2 mm | 0.179                   | –                           | –                           | –                 | –                                |
| *Myotis daubentonii*    | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1160, M, juvenile, 8.1 g, 37.1 mm | 0.218                   | 1 clone, rpoB-3            | ND                          | Penicillidia monoceros (n = 1) | rpoB-5           |
| *Myotis mystacinus*     | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1161, F, juvenile, 8.2 g, 37.7 mm | 0.218                   | –                           | –                           | Penicillidia monoceros (n = 1) | –                  |
| *Myotis mystacinus*     | 2012 Aug 25  | 59° 55' 34" N, 22° 24' 51" E | 1162, F, adult, 6.8 g, 34.4 mm | 0.198                   | –                           | –                           | –                 | –                  |
| *Myotis daubentonii*    | 2012 Aug 27  | 60° 26' 54" N, 21° 59' 27" E | 2569, M, juvenile, 7.7 g, 37.3 mm | 0.206                   | –                           | Penicillidia monoceros (n = 1) | rpoB-2           |
| *Myotis daubentonii*    | 2012 Aug 27  | 60° 26' 54" N, 21° 59' 27" E | 2570, M, juvenile, 7.8 g, 37.4 mm | 0.209                   | –                           | –                           | –                 | –                  |
| *Myotis daubentonii*    | 2012 Aug 27  | 60° 26' 54" N, 21° 59' 27" E | 2571, M, juvenile, 7.7 g, 37.9 mm | 0.203                   | –                           | –                           | –                 | –                  |
| *Myotis daubentonii*    | 2012 Sep 3   | 60° 26' 54" N, 21° 59' 27" E | 2572, F, adult, 9.0 g, 36.7 mm | 0.245                   | –                           | Nyctenibia kolenati (n = 2) | –                 | –                  |
| *Myotis daubentonii*    | 2012 Sep 3   | 60° 26' 54" N, 21° 59' 27" E | 2573, M, juvenile, 7.9 g, 36.9 mm | 0.214                   | 2 clones, both rpoB-3      | ND                          | –                 | –                  |
| *Myotis daubentonii*    | 2012 Sep 3   | 60° 26' 54" N, 21° 59' 27" E | 2574, M, juvenile, 7.5 g, 36 mm | 0.208                   | 4 clones, all rpoB-2       | ND                          | –                 | –                  |
| *Myotis daubentonii*    | 2012 Sep 3   | 60° 26' 54" N, 21° 59' 27" E | 2575, M, juvenile, 7.4 g, 37.8 g | 0.196                   | 3 clones, all rpoB-3       | ND                          | Nyctenibia kolenati (n = 1) | rpoB-2           |
| *Myotis daubentonii*    | 2012 Sep 3   | 60° 26' 54" N, 21° 59' 27" E | 2576, M, juvenile, 7.4 g, 36.8 mm | 0.201                   | 12 clones, all rpoB-3      | ND                          | Nyctenibia kolenati (n = 1) | –                  |

* M, male; F, female; ND, not determined; –, negative results.
†Mass divided with the average of the left and right forearm.
‡Visual identification to the order Siphonaptera during sampling. Species identification of the flies additionally based on mitochondrial cytochrome c oxidase subunit I barcode analysis at http://v3.boldsystems.org/.
§Individual of the metagenomic fecal sample.
#The detected *Bartonella* spp. rpoB allele 1 - 5.
| Oligo | Target genetic marker, oligo orientation | Sequence 5′→3′ | Reference |
|-------|-----------------------------------------|----------------|-----------|
| Bartonella spp. | | | |
| fD1 16S rRNA gene, forward | AGAGTTTGATCCTGGCTCAG | (1) |
| rP2 16S rRNA gene, reverse | ACGGCTACCTTGTTAGCCTT | (1) |
| Bart/16–23F 16S-23S rRNA intergenic spacer region (ISR), forward | TTGATAAGCGTGAGGTCGGAGG | (2) |
| Bart/16–23R 16S-23S rRNA intergenic spacer region (ISR), reverse | CAAAGCAGGTGCTCTCCCAG | (2) |
| prAPT0243 GltA gene, forward | GCCATGTCTGCTTTTTATCA | This study |
| BhCS.781p GltA gene, forward | AATGCAAAAAGAACAGTAAACA | (3) |
| BhCS.1137n GltA gene, reverse | GATGTGCATCCTACGCATTATGG | (4) |
| prAPT0245 RpoB gene, forward | AATGGTGCCTCAGCACGTATAAG | (4) |
| prAPT0247 FtsZ gene, forward | GCTTCAAGGAGTTGATTTGTTGTTGCCAAT | This study |
| prAPT0258 FtsZ gene, reverse | ACGACCATTTACGCTAAACAGACAC | This study |
| ssrA-F SsrA gene, forward | GCTGATGTAATAAATGGCAGATTTAAA | (5) |
| ssrA-R SsrA gene, reverse | GCTTCTGTTGCCAGGTG | (5) |
| prPE23 VirB4 gene, forward | GAGGTTGGCCGCCCACCATG | (6) |
| prPE24 VirB4 gene, reverse | GAGGTTGGCCGCCCACCATG | (6) |

Ectoparasites

| Oligo | Target genetic marker, oligo orientation | Sequence 5′→3′ | Reference |
|-------|-----------------------------------------|----------------|-----------|
| ZBJ-ArtF1c Mitochondrial cytochrome c oxidase subunit I (COI) | AGATATTGGAACWTTATATTTTATTTTTTGG | (7) |
| ZBJ-ArtR2c Mitochondrial cytochrome c oxidase subunit I (COI) | WACTAATCAATTWCCAAATTCCTCC | (7) |

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| Bat strain | Marker (sequenced length)* | GenBank accession no. | Closely related Bartonella spp. or Candidatus-status Bartonella spp., percentage of similarity (bat strain/reference strain), GenBank accession no. of the reference strain |
|------------|---------------------------|----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1157/3     | 16S rRNA (485 bp)         | KF003116, KF003117   | Nondisc, 100% (485/485) Candidatus B. mayotimonensis, 85.7% (239/279) FJ376735, 95.8% (387/404) FJ376736, 92.9% (553/659) AF214557, 96% (267/278) FJ376734 |
|            | rpoB (406 bp)             | KF003118             | Candidatus B. mayotimonensis, 95.8% (387/404) FJ376736, B. alsatica, 95.3% (385/404) AF165987, B. vinsonii subsp. arupensis, 95.1% (385/405) AY166582 |
|            | gltA (595 bp)             | KF003115             | B. vinsonii subsp. arupensis, 92.9% (553/659) AF214557, Candidatus B. mayotimonensis, 92.8% (552/595) FJ376732, B. taylorii, 92.6% (551/595) Z70013 |
|            | ftsZ (511 bp)             | KF003121             | Candidatus B. mayotimonensis, 96% (267/278) FJ376734, B. washoensis, 93.2% (476/511) AB292598, Nondisc, 92.6% (472/511) |
|            | ssrA (254 bp)             | KF003119             | B. washoensis, 97.2% (247/254) JN029786, B. grahamii, 95.7% (243/254) JN029795, Nondisc, 95.3% (242/254) |
| 1160/1     | 16S rRNA (485 bp)         | KF003123, KF003124   | B. japonica, 100% (485/485) AB440632, Candidatus B. mayotimonensis, 83.3% (235/282) FJ376735, Nondisc, 99.8% (484/485) |
|            | rpoB (406 bp)             | KF003125             | Candidatus B. mayotimonensis, 97.0% (393/405) FJ376736, B. vinsonii subsp. arupensis, 93.6% (380/406) AY166582, B. alsatica, 93.6% (379/405) AF165987 |
|            | gltA (595 bp)             | KF003122             | Candidatus B. mayotimonensis, 91.4% (544/595) FJ376732, B. vinsonii subsp. arupensis, 90.8% (540/595) AF214557, Nondisc, 90.6% (539/595) |
|            | ftsZ (511 bp)             | KF003128             | Candidatus B. mayotimonensis, 95.3% (265/278) FJ376734, Nondisc, 91.2% (466/511), B. phoceensis, 91.0% (465/511) AY515135 |
|            | ssrA (253 bp)             | KF003126             | B. washoensis, 96.4% (244/253) JN029786, Nondisc, 96.0% (243/253), B. grahamii, 95.3% (241/253) JN029795 |
| 2574/1     | 16S rRNA (485 bp)         | KF003130, KF003131   | Nondisc, 100% (485/485), B. quintana, 91.9% (372/405) AF165994, Nondisc, 99.8% (484/485) |
|            | rpoB (406 bp)             | KF003132             | B. quintana, 91.9% (372/405) AF165994, Nondisc, 91.1% (370/406), B. koehlerae, 91.8% (546/595) AF176091 |
|            | gltA (595 bp)             | KF003129             | B. koehlerae, 91.8% (546/595) AF176091, B. henselae, 91.3% (543/595) CAF27442, B. quintana, 90.4% (538/595) Z70014 |
|            | ftsZ (511 bp)             | KF003135             | Candidatus B. mayotimonensis, 88.5% (451/511), Nondisc, 90.6% (452/511) AF467757, B. grahamii, 87.7% (448/511) AF467753 |
|            | ssrA (253 bp)             | KF003133             | B. vinsonii subsp. arupensis, 94.9% (240/253) JN029783, B. vinsonii subsp. vinsonii, 94.5% (240/253) JN029795 |

*Type strain ssrA sequences are not available for all species and Candidatus B. mayotimonensis.
†Nondisc, a nondiscriminatory marker (>2 Bartonella species or Candidatus-status Bartonella species have the same sequence similarity with the bat strain.
Table 4. Pairwise genetic distance values of the concatenated rpoB, gltA, 16S rRNA and 16S rRNA sequences. Lowest genetic distance values of the bat strains compared with the Brucella abortus outbreak strain 9-941, the Bartonella type strains (Technical Appendix Table 5), and the Candidatus B. mayotimonensis patient strain are underlined.

| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| **rpoB** | **gltA** | **16S rRNA** | **16S rRNA** |
| 1 | 0.236 | 0.088 | 0.087 |
| 2 | 0.236 | 0.088 | 0.087 |
| 3 | 0.236 | 0.088 | 0.087 |
| 4 | 0.236 | 0.088 | 0.087 |
| 5 | 0.236 | 0.088 | 0.087 |
| 6 | 0.236 | 0.088 | 0.087 |
| 7 | 0.236 | 0.088 | 0.087 |
| 8 | 0.236 | 0.088 | 0.087 |
| 9 | 0.236 | 0.088 | 0.087 |
| 10 | 0.236 | 0.088 | 0.087 |
| 11 | 0.236 | 0.088 | 0.087 |
| 12 | 0.236 | 0.088 | 0.087 |
| 13 | 0.236 | 0.088 | 0.087 |
| 14 | 0.236 | 0.088 | 0.087 |
| 15 | 0.236 | 0.088 | 0.087 |
| 16 | 0.236 | 0.088 | 0.087 |
| 17 | 0.236 | 0.088 | 0.087 |
| 18 | 0.236 | 0.088 | 0.087 |
| 19 | 0.236 | 0.088 | 0.087 |
| 20 | 0.236 | 0.088 | 0.087 |
| 21 | 0.236 | 0.088 | 0.087 |
| 22 | 0.236 | 0.088 | 0.087 |
| 23 | 0.236 | 0.088 | 0.087 |
| 24 | 0.236 | 0.088 | 0.087 |
| 25 | 0.236 | 0.088 | 0.087 |
| 26 | 0.236 | 0.088 | 0.087 |
| 27 | 0.236 | 0.088 | 0.087 |
| 28 | 0.236 | 0.088 | 0.087 |
| 29 | 0.236 | 0.088 | 0.087 |
| 30 | 0.236 | 0.088 | 0.087 |
| 31 | 0.236 | 0.088 | 0.087 |
| 32 | 0.236 | 0.088 | 0.087 |
| 33 | 0.236 | 0.088 | 0.087 |
| 34 | 0.236 | 0.088 | 0.087 |
| 35 | 0.236 | 0.088 | 0.087 |
| 36 | 0.236 | 0.088 | 0.087 |

1. B. abortus
2. B. australis
3. B. bacilliformis
4. B. birtlesii
5. B. bovis
6. B. caipori
7. B. chomelii
8. B. clarridgeiae
9. B. cooperensis
10. B. dshlie
11. B. elizabethae
12. B. grahamei
13. B. henselae
14. B. japonica
15. B. koehlerae
16. B. melitensis
17. B. phoenicis
18. B. queenslandensis
19. B. quintana
20. B. rattiu
21. B. rattius
22. B. rogelii
23. B. suis
24. B. silvatica
25. B. tatai
26. B. taylorii
27. B. trubonar
28. B. virchowii
29. B. virchowii
30. B. wiesenhorn
31. B. wiesenhorn
32. Candidatus B. mayotimonensis
33. Bat strain 11573
34. Bat strain 11601
35. Bat strain 2574/1
36. Brucella abortus 9-941
| Species                | Type strain, isolated from                                      | Reference | gltA     | rpoB     | 16S rRNA | ftsZ     |
|-----------------------|------------------------------------------------------------------|-----------|----------|----------|----------|----------|
| *B. alsatica*         | IBS 382, rabbit (Oryctolagus cuniculus)                         | (1)       | AF204273 | AF165987 | AJ002139 | AF467673 |
| *B. australis*        | Aust/NH1, kangaroo (Macropus giganteus)                        | (2)       | NC_020300| NC_020300| DG538394 | NC_020300|
| *B. bacilliformis*    | KC583, unknown origin                                         | (3)       | YP_088907| AF165988 | NR_044743| AB292602 |
| *B. birtlesii*        | IBS 325, mouse (Apodemus spp.)                                 | (4)       | AF204272| AB196425 | NR_025051| AF467762 |
| *B. bovis*            | 91–4, domestic cow                                            | (5)       | AF293394 | AY166581 | NR_025121| AGWA01000007|
| *B. capreoli*         | IBS 193, roe deer (Capreolus capreolus)                        | (5)       | AF293392 | AB290188 | NR_025120| AB290192 |
| *B. chomelii*         | A828, domestic cow                                            | (6)       | AF293392 | AB290189 | NR_025120| AB290193 |
| *B. clarridgeiae*     | Houston-2, cat                                                | (7)       | U84386   | AF165990 | AB292603 | AF41018  |
| *B. coopersplainsensis* | AUST/NH20, rat (Rattus leucopus)                             | (8)       | EU111803 | EU111792 | EU111759 | EU111781 |
| *B. dioxiae*          | R18, field vole (Mictorus agrestus)                            | (9)       | Z70017   | AF165991 | NR_023968| AF467754 |
| *B. elizabethae*      | F9251, human                                                   | (10)      | Z70009   | AF165992 | NR_025889| AF467760 |
| *B. grahamii*         | V2, bank vole (Myodes glareolus)                               | (11)      | Z70016   | AF165993 | NR_023966| AF467753 |
| *B. japonica*         | Fuji 18–1, mouse (Apodemus argenteus)                          | (12)      | AB242289 | AB242288 | AB440632 | AB440633 |
| *B. koehlerae*        | C-29, cat                                                      | (13)      | AF176091 | AY166580 | NR_024932| AF467755 |
| *B. melophagi*        | K-2C, sheep ked                                               | (14)      | AY172476 | EF605288 | AIMOA01000004| EF605286|
| *B. phoeoensis*       | 16120, rat (Rattus norvegicus)                                 | (15)      | AY515126 | AY515132 | AY515119 | AY515135 |
| *B. queenslandensis*  | Aust/NH12, rat (Melomys sp.)                                  | (16)      | EU111798 | EU111767 | EU111754 | EU111776 |
| *B. quintana*         | Fuller, human                                                 | (16)      | Z70014   | AF165994 | NR_044748| AB292605 |
| *B. rattiassiliensis* | 15908, rat (Rattus norvegicus)                                 | (17)      | AY515124 | AY515130 | AY515120 | AY515133 |
| *B. rochalimae*       | ATCC BAA-1498, human                                           | (17)      | DQ683195 | DQ683198 | FN645466 | FN645461 |
| *B. schoenbuchensis*  | R1, roe deer (Capreolus capreolus)                            | (18)      | AJ278183 | AY167409 | AJ278187 | AF467765 |
| *B. sibatica*         | Fuji 23–1, mouse (Apodemus species)                            | (19)      | AB242287 | AB242292 | AB440636 | AB440637 |
| *B. taylorii*         | M6, mouse (Apodemus spp.)                                     | (19)      | Z70013   | AF165995 | NR_023967| AF467756 |
| *B. taylorii*         | Sb944nv, ground squirrel (Spermophilus beecheyi)               | (24)      | AF470616 | AB292596 | AB292597 | AB292598 |
| *B. vinsonii*         | OK-94–513, human                                              | (21)      | AF214557 | AY166582 | AF214558 | AF467758 |
| *B. vinsonii*         | 93-CO1, dog                                                   | (22)      | U26075   | AF165989 | L35052   | AF467764 |
| *B. washoensis*       | Sb944nv, ground squirrel (Spermophilus beecheyi)               | (24)      | AF470616 | AB292596 | AB292597 | AB292598 |
| *B. vinsonii*         | 506, rat (Rattus norvegicus)                                  | (20)      | AJ005494 | AF165996 | AM260525 | AF467759 |
| *B. vinsonii*         | BAA-42, human                                                 | (23)      | Z70015   | AF165997 | NR_037056| AF467757 |
| *B. vinsonii*         | Baker, vole (species unknown)                                | (23)      | Z70015   | AF165997 | NR_037056| AF467757 |
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Technical Appendix Figure 1. Performance of the Ion Torrent Personal Genome Machine. A) Loading density of the chip (average 31%). Twenty-five percent of the loaded beads were polyclonal and were distracted from further analysis together with beads that gave low quality reads (18% of the loaded beads). Approximately 200,000 good quality sequences were obtained with 58% of the loaded beads. B) Read length histogram of the bat fecal metagenome. Sequences <50 bp (dashed line) were not used in the BLASTN/GenBank homology search-based assignments.
Technical Appendix Figure 2. Transmission electron micrographs of the bat Bartonella isolates. *B. mayotimonensis* strain 1157/3 (A), *B. mayotimonensis* strain 1160/1T (B) and *B. naantaliensis* sp. nov. strain 2574/1T (C). Original magnification × 12,000. Scale bars = 500 nm.