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

Ville Veikkolainen,[1] Eero J. Vesterinen,[1] Thomas M. Lilley, and Arto T. Pulliainen

A plethora of pathogenic viruses colonize bats. However, bat bacterial flora and its zoonotic threat remain ill defined. In a study initially conducted as a quantitative metagenomic analysis of the fecal bacterial flora of the Daubenton's bat in Finland, we unexpectedly detected DNA of several hemotrophic and ectoparasite-transmitted bacterial genera, including *Bartonella*. *Bartonella* spp. also were either detected or isolated from the peripheral blood of Daubenton's, northern, and whiskered bats and were detected in the ectoparasites of Daubenton's, northern, and Brandt's bats. The blood isolates belong to the *Candidatus*-status species B. mayotimonensis, a recently identified etiologic agent of endocarditis in humans, and a new *Bartonella* species (*B. naantaliensis* sp. nov.). Phylogenetic analysis of bat-colonizing *Bartonella* spp. throughout the world demonstrates a distinct *B. mayotimonensis* cluster in the Northern Hemisphere. The findings of this field study highlight bats as potent reservoirs of human bacterial pathogens.

The 1,100 species of bats (*1*) constitute ≈20% of known mammalian species and are outnumbered only by animals in the order Rodentia. Bats play a vital role in natural ecosystems in arthropod suppression, seed dispersal, and pollination. Modern-day economies also benefit from these voracious predators of crop and forest pests (*2*). However, bats have been implicated as reservoir hosts for viral human pathogens, such as paramyxoviruses (*3*) and rabies virus and related lyssaviruses (*4*). Compelling evidence also indicates that bats carry asymptomatically some of the most deadly viruses, including Marburg (*5*) and Ebola (*6*) viruses. Whether bats carry clinically significant bacterial pathogens is unknown.

Author affiliations: University of Turku, Turku, Finland (V. Veikkolainen, E.J. Vesterinen, T.M. Lilley, A.T. Pulliainen); and University of Helsinki, Helsinki, Finland (A.T. Pulliainen)

DOI: http://dx.doi.org/10.3201/eid2006.130956

The development of next-generation sequencing techniques has revolutionized biological science. It is now possible—and cost-friendly—to gain access to massive amounts of qualitative and quantitative sequencing data in a short time without a priori knowledge of the sequence (*7*). Most bacteria do not grow on laboratory media, and next-generation sequencing technologies have proven useful for studying bacterial species diversity and dynamics, even in complex systems like the gut (*8*). Our initial objective in 2010 and 2011 was to conduct a quantitative metagenomic analysis of the fecal bacterial flora of the Daubenton's bat (*Myotis daubentonii*) in Finland. Unexpectedly, we found that the fecal material contained DNA of several hemotrophic and ectoparasite-transmitted bacterial genera, such as *Bartonella*. This DNA may originate either from bleeding into the intestine or from the insect prey of the bats that includes the abundant bloodfeeding bat ectoparasites. Therefore, the study further focused on detecting and isolating *Bartonella* spp. from peripheral blood and ectoparasites of several bat species in Finland in 2012.

Materials and Methods

Bartonella spp. nucleotide sequences have been deposited in GenBank under accession nos. KF003115–KF003145. The metagenomic reads are stored at the National Center for Biotechnology Information Sequence Read Archive under BioProject SRP023235 (accession nos. experiment: SRX286839, run: SRR868695). We have described the detailed protocols, including bat sampling for peripheral blood, fecal droppings, and ectoparasites; metagenomic analysis of fecal DNA; isolation of *Bartonella* from peripheral blood; extraction of DNA from bat blood, ectoparasites, and *Bartonella* isolates; *Bartonella* and ectoparasite PCR analyses; transmission electron

[1]These authors contributed equally to this article.

microscopy; and nucleotide sequence and phylogenetic analyses in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/6/13-0956-Techapp1.pdf).

Results

Quantitative Metagenomic Analysis of DNA from Bat Feces

We obtained ≈200,000 high-quality sequences (average length 167 bp) from DNA sequencing of fecal material from a Daubenton's bat (online Technical Appendix Figure 1). Sequences (≥50 bp) were assigned on the basis of best E-value BLASTN scores (http://blast.ncbi.nlm.nih.gov/blast.cgi) in GenBank. The most abundant non-metazoan sequence matches were with bacteria. The genera *Leuconostoc*, *Enterobacter*, *Lactococcus*, and *Chlamydia* dominated (Figure 1). Surprisingly, the fecal material also contained DNA of the ectoparasite-transmitted genera, such as the hemotrophic bartonellae (*9*). It was thought that this DNA originated either from bleeding into the intestine or from the insect prey of the bats that includes the abundant bloodfeeding bat ectoparasites. PCR verified the presence of *Bartonella* DNA in the bat fecal material. The transfer messenger RNA gene (*ssrA*) (*10*) could be amplified and was sequenced from the fecal material of 1 Daubenton's bat, 1 northern bat (*Eptesicus nilssonii*), and 1 Brandt's bat (*Myotis brandtii*) (no. 2771, no. 2788, and no. 2786, respectively; online Technical Appendix Table 1). The obtained 218-bp *ssrA* sequences were 100% identical. The closest matches in GenBank, with a similarity score of 94.8% (183/193 bp), were *B. tamiae* Th339 (GenBank accession no. JN029780) and Th307 strains (GenBank accession no. JN029778) isolated from 2 humans in Thailand (*11*).

Candidatus Status Species B. mayotimonensis and Novel *Bartonella* Species

Bats belonging to the 4 most prevalent bat species in Finland were captured in August and September 2012 at 3 locations in southwestern Finland (online Technical Appendix Table 1). Culturing of peripheral blood samples of 5 Daubenton's bats and 1 northern bat yielded distinct colonies. The isolates were identified as *Bartonella* spp. by sequencing a PCR-amplified 485-bp fragment containing the hypervariable regions V6–V8 of the 16S rRNA gene. Overall health of the bats as analyzed by body condition indexing was not affected by the *Bartonella* infection (online Technical Appendix Table 1).

The 16S rRNA gene sequences are highly conserved within the genus *Bartonella* and thus not robust in differentiating species (*12*). Therefore, we sequenced PCR-amplified fragments of the RNA polymerase b-subunit gene (*rpoB*), citrate synthase gene (*gltA*), filamenting temperature-sensitive mutant Z gene (*ftsZ*), VirB type IV secretion system VirB4 component gene (*virB4*), hypervariable region 2 of the 16S-23S rRNA intergenic spacer region (ISR), and *ssrA* (online Technical Appendix Table 2). Sequencing of *rpoB* was first conducted on all 28 clonal isolates. Three distinct *rpoB* alleles were identified (online Technical Appendix Table 1). The multilocus sequence analysis (MLSA) was completed on 1 *rpoB*-1 allele isolate (clone 3, bat no. 1157, referred to hereafter as 1157/3), 1 *rpoB*-2 allele isolate (clone 1, bat no. 2574, referred to hereafter as 2574/1), and 1 *rpoB*-3 allele isolate (clone 1, bat no. 1160, referred to hereafter as 1160/1). Thin-section transmission electron micrographs of these isolates are shown in the online Technical Appendix Figure 2. No major pili or fimbriae-like structures were detected on the surface of the rod-shaped bacteria.

Results of BLASTN homology searches performed in January 2013 are shown in online Technical Appendix Table 3. ISR is a robust species discriminatory marker within the genus *Bartonella* (*13*,*14*). ISR of the strain 2574/1 did not have any hits, whereas ISR of strains 1157/3 and 1160/1 had on1y 1 hit in GenBank *Candidatus* B. mayotimonensis (*15*), with high sequence similarity scores. Sequence analyses of the other MLSA markers (online Technical Appendix Table 3) further indicate that isolates 1157/3 and 1160/1 belong to the *Candidatus*-status species B. mayotimonensis and that strain 2574/1 belongs to a new *Bartonella* species. Indeed, the lowest pairwise genetic distance values with the concatenated *rpoB*, *gltA*, 16S rRNA, and *ftsZ* sequence fragments of the bat strains 1157/3 and 1160/1 in the genus *Bartonella* were 0.040 and 0.038, respectively, with *Candidatus* B. mayotimonensis (online Technical Appendix Table 4). Because the distance value 0.05 is the recommended cutoff value for species delineation (*16*), the bat isolates 1157/3 and 1160/1 classify as strains of the *Candidatus*-status species B. mayotimonensis. The bat strain 2574/1 belongs to a new *Bartonella* species because the lowest genetic distance value in the genus *Bartonella* was 0.070 with *B. washoensis*, above the 0.05 cutoff value (*16*).

Figure 2 shows the phylogenetic position of the bat *Bartonella* isolates based on comparisons of concatenated sequences of *rpoB*, *gltA*, 16S rRNA and *ftsZ*, available for *Candidatus* B. mayotimonensis (*15*) and all type strains of the *Bartonella* species (online Technical Appendix Table 5). The neighbor-joining and maximum-likelihood trees demonstrate that bat isolates 1157/3 and 1160/1 cluster with *Candidatus* B. mayotimonensis with high bootstrap values in a distinct phylogenetic position. The new *Bartonella* species (strain 2754/1) clearly diverges from the other bat isolates.

Bat Ectoparasite Flies and Fleas as Vectors for Transmitting *Bartonella*

We sequenced a PCR-amplified fragment of the mitochondrial cytochrome c oxidase subunit I (*17*) and also

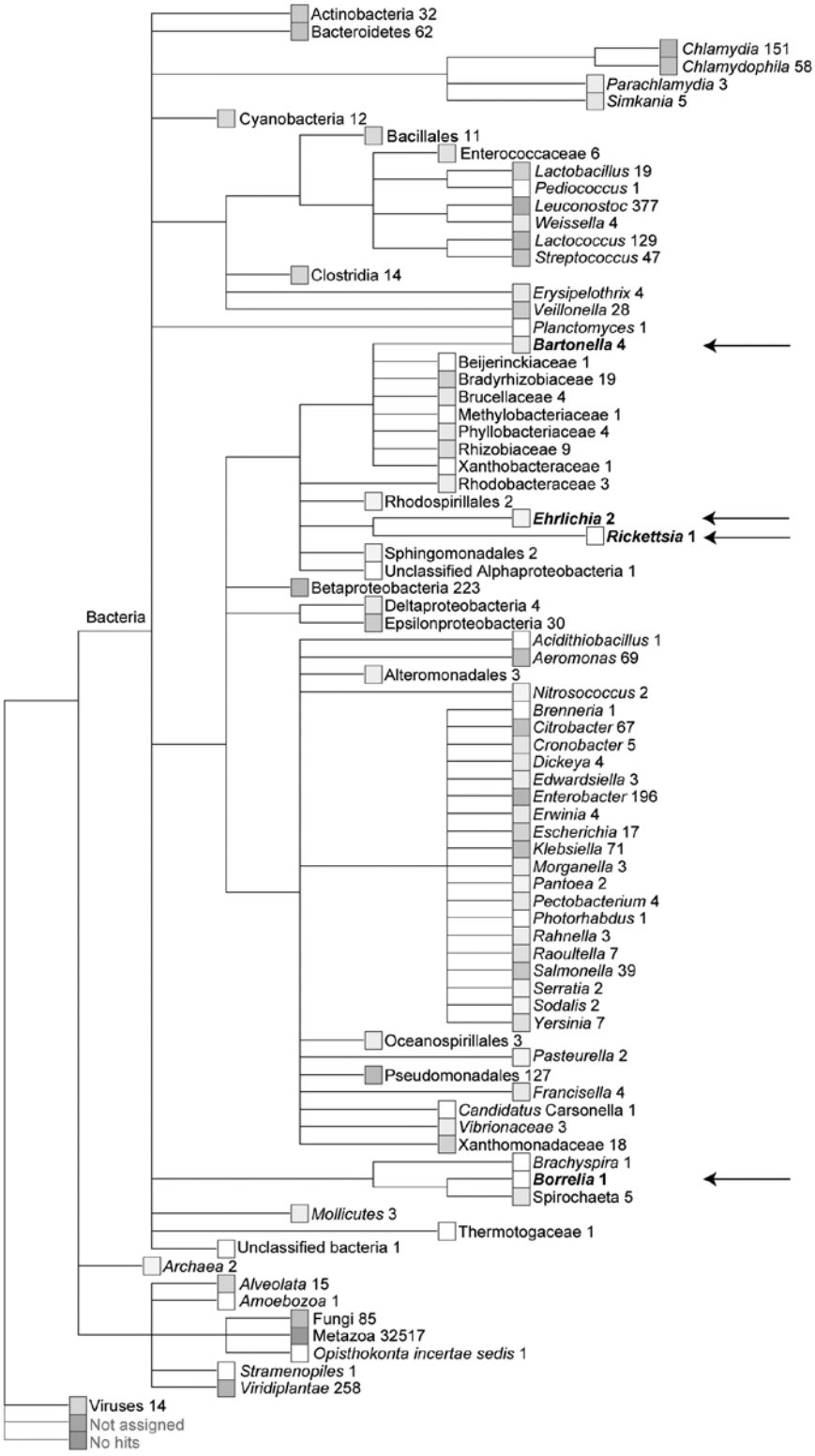

Figure 1. Quantitative metagenomic analysis of the fecal DNA of the Daubenton's bat. The sequences (≥50 bp) were assigned on the basis of best E-value BLASTN scores (http://blast.ncbi.nlm.nih.gov/blast.cgi) in GenBank. Numbers refer to the amount of sequences assigned to a given taxon. No hits refers to sequences that had no similarity to any sequences in GenBank. Not assigned refers to sequences that had similarity in GenBank but they could not be reliably assigned to any organism. Arrows mark the ectoparasite-transmitted bacterial genera, which unexpectedly were detected in the bat fecal DNA preparation.

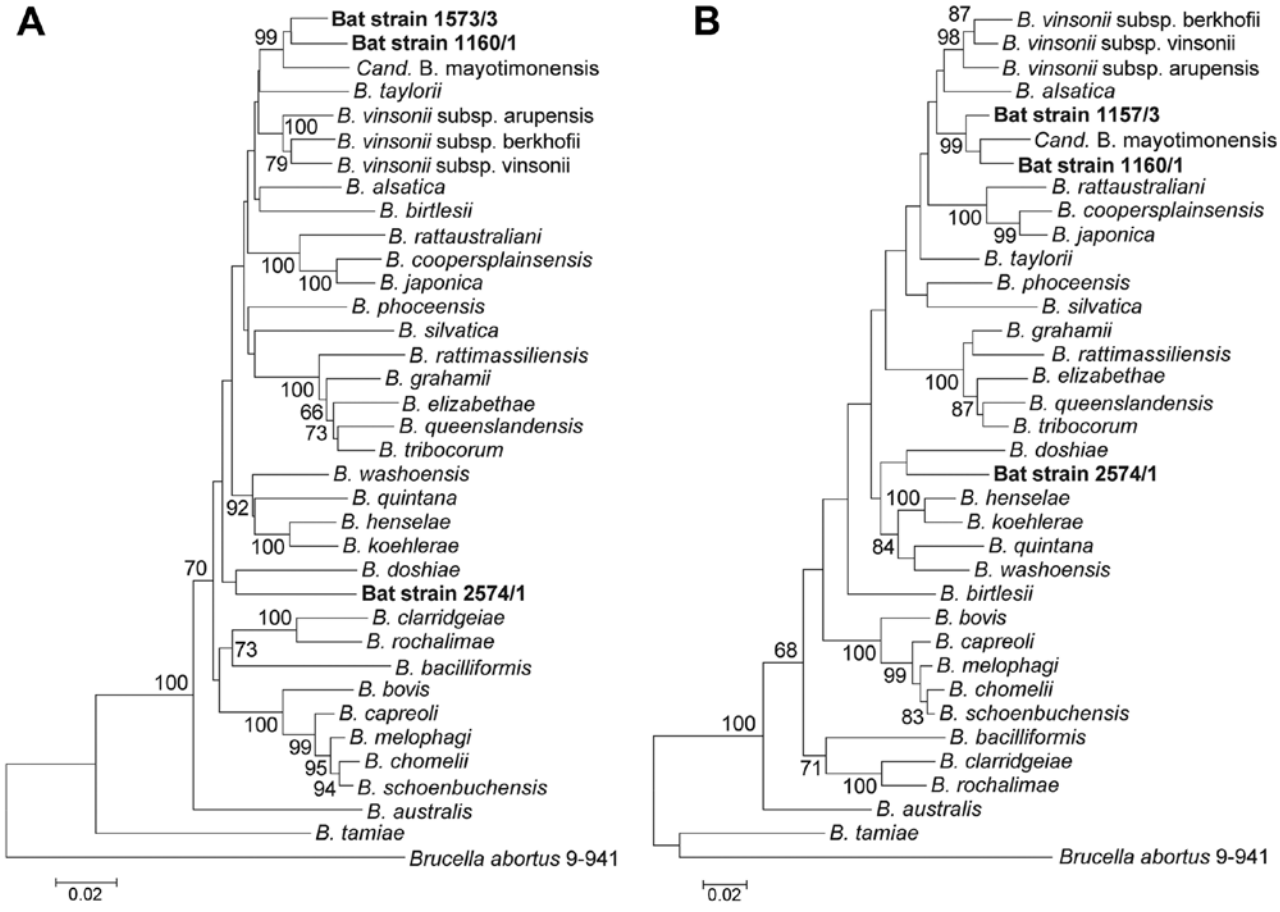

Figure 2. Phylogenetic positions of the bat blood isolates among members of the genus *Bartonella*. Neighbor-joining (A) and maximum-likelihood (B) trees are based on the alignment of concatenated sequences of 4 multilocus sequence analysis markers (*rpoB*, *gltA*, 16S rRNA, and *ftsZ*). Sequence information from the type strains of all known *Bartonella* species and from the *Candidatus* B. mayotimonensis human strain was included into the analysis (online Technical Appendix Table 5, wwwnc.cdc.gov/EID/article/20/6/13-0956-Techapp1.pdf). Numbers on branches indicate bootstrap support values derived from 1,000 tree replicas. Bootstrap values >60 are shown. Scale bars indicate nucleotide substitutions per site.

used visual inspection to identify the ectoparasites of 18 bats (online Technical Appendix Table 1). Ectoparasite DNA preparations of 2 fleas and 10 flies were analyzed with a PCR protocol targeting the *Bartonella rpoB*. The blood isolate *rpoB* alleles 1 and 2 were detected in samples from 1 flea and 2 flies, respectively (online Technical Appendix Table 1). In addition, 2 novel *rpoB* alleles were detected. The *rpoB*-5 allele detected in a fly sample is distantly related to the currently known *Bartonella rpoB* sequences. The highest BLASTN sequence identity score with the *rpoB*-4 allele detected in a flea sample, and from 1 blood DNA preparation of a culture-negative whiskered bat (no. 1156, online Technical Appendix Table 1), was 97.8% (397/406 bp) with the corresponding fragment (FJ376736) of *Candidatus* B. mayotimonensis. This is a higher value than with the *rpoB*-1 and *rpoB*-2 alleles. Moreover, a partial 338-bp *gltA* fragment could be amplified from the *rpoB*-4–positive flea sample. The highest BLASTN sequence identity score with *Candidatus* B. mayotimonensis was 93.2% (315/338 bp), which is higher than with the isolates 1157/3 (92.0%, 311/338 bp) and 1160/1 (92.3%, 312/338 bp). The data further support the conclusion that bats are reservoir hosts of *B. mayotimonensis* and indicate that the bat flies and fleas transmit *Bartonella* spp. to new hosts.

Phylogenetic Analysis of *Bartonella* spp. that Colonize Bats Worldwide

A maximum composite likelihood–based neighbor-joining tree (Figure 3) was constructed on the basis of 253-bp *gltA* sequences obtained from *Bartonella* that infect bats in the United Kingdom (*18*), Kenya (*19*), Guatemala (*20*), Taiwan (*21*), and Peru (*22*). The 5 *Bartonella*-like bacteria detected in minced heart tissues in the United

Kingdom (*18*), the *B. mayotimonensis* isolates from Finland, and the strain detected in 1 bat flea in Finland clustered in a distinct phylogenetic position away from the bat isolates and strains of the Southern Hemisphere. The new *Bartonella* species (strain 2574/1) does not belong to the Northern Hemisphere *B. mayotimonensis* cluster. Remarkably, the 253-bp *gltA* fragment of 1 of the *Bartonella*-like bacteria detected in minced heart tissue of a common noctule (*Nyctalus noctule*) (Cornwall-M451, AJ871615) yielded a 95.3% (241/253 bp) sequence identity score, compared with the corresponding fragment (FJ376732) of *Candidatus* B. mayotimonensis. This value is significantly higher than those obtained with corresponding *gltA* fragments of Finland bat isolates 1157/3 (92.9%, 235/253) or 1160/1 (94.1%, 238/253). The UK *gltA* data further support the conclusion that bats are reservoir hosts of *B. mayotimonensis*. Most importantly, bats appear to be reservoir hosts of *B. mayotimonensis* only in the Northern Hemisphere.

Bartonella naantaliensis (naan.tali´en.sis. N.L. fem. adj. n. *naantaliensis* of or belonging to Naantali) is the name proposed to highlight the municipality where the bat was trapped from which the type strain was isolated. The type strain is 2574/1. Its partial 16S rRNA gene nucleotide sequence is deposited in GenBank (accession no. KF003116).

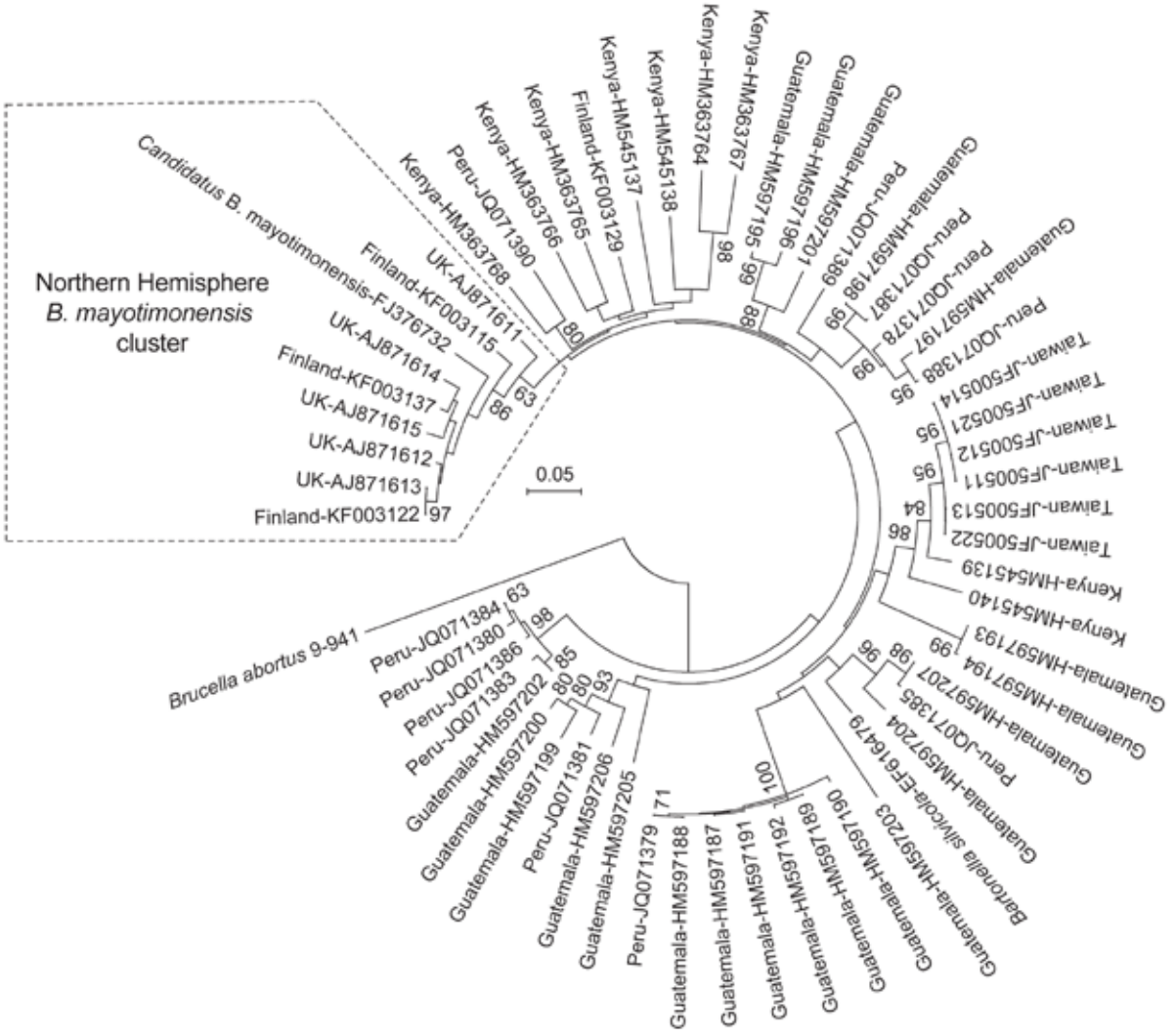

Figure 3. Phylogenetic analysis of bat-colonizing *Bartonella* spp. found worldwide demonstrates a distinct *B. mayotimonensis* cluster in the Northern Hemisphere. Maximum composite likelihood–based neighbor-joining tree is based on the alignment of the *gltA* multilocus sequence analysis marker. Information from *Bartonella gltA* sequences from bat blood isolates or from minced tissues of bats or from bat ectoparasites was included in the analysis. GenBank accession numbers of the sequences are shown after the country of origin. Numbers on branches indicate bootstrap support values derived from 1,000 tree replicas. Bootstrap values >60 are shown. Scale bar indicates nucleotide substitutions per site.

Discussion

Bartonella spp. are facultative intracellular bacteria that typically cause long-lasting hemotrophic bacteremia in their mammalian reservoir hosts, such as rodents (*9*). The relapsing bacteremia can last weeks, months, or even years, thereby favoring transmission by bloodfeeding arthropods. In recent years, increasing numbers of *Bartonella* spp. have been implicated as zoonotic human pathogens. A frequent symptom is endocarditis, usually suspected in cases in which conventional culture-based diagnostics fail. The most prevalent endocarditis-causing species are *B. quintana* (*23*,*24*) and *B. henselae* (*25*), but *B. elizabethae* (*26*), *B. alsatica* (*27*), *B. koehlerae* (*28*), *B. vinsonii* subsp. *berkhoffii* (*29*), and *B. vinsonii* subsp. *arupensis* (*30*) also have been detected or isolated. Recently, a new type of *Bartonella* was detected in a resected aortic valve tissue of a human endocarditis patient (*15*). A species name, *Candidatus* B. mayotimonensis was proposed because a pure microbiological culture was not obtained. The reservoir host in nature also remained elusive. As part of a study designed to characterize the microbiome of bats, bacteria that belong to the *Candidatus*-status species B. mayotimonensis were either detected or isolated from peripheral blood samples and the ectoparasites of bats. In addition, a new *Bartonella* species (strain 2574/1) was isolated from the blood and detected from the ectoparasites.

The ad hoc committee to reevaluate the species definition in bacteriology has proposed that descriptions of novel species could be based solely on gene sequence analyses (*31*). In the current study, 6 genes, including the robust *Bartonella* spp. discriminatory marker, the ISR, were used (*13*,*14*). It is remarkable that ISR of the 2574/1 isolate did not have any hits, whereas ISRs of 1157/3 and 1160/1 isolates had only 1 hit in GenBank, *Candidatus* B. mayotimonensis. If *gltA* shares <96.0% and *rpoB* <95.4% nt sequence similarity with those of the validated species, the newly encountered *Bartonella* strain can be considered a new species (*32*). According to these criteria, which were proposed in 2003 when half of the currently known species were known, the bat isolate 2574/1 is a new *Bartonella* species. The bat isolates 1157/3 and 1160/1 belong to the *Candidatus*-status species B. mayotimonensis on the basis of the *rpoB* sequences but would belong to a new *Bartonella* species on the basis of the *gltA* sequences. Because the species classification gave contradictory results, sequence analyses of other MLSA markers and phylogenetic analyses were performed. In addition, we used 4 concatenated MLSA markers to determine pairwise genetic distance values to the known members of the genus. The bat isolate 1157/3 and 1160/1 *ftsZ* sequences had a significantly higher sequence similarity with *ftsZ* of *Candidatus* B. mayotimonesis than with any other type strain sequence. The neighbor-joining and maximum-likelihood phylogenetic trees with the concatenated *rpoB*, *gltA*, 16S rRNA and *ftsZ* sequences both demonstrated that the bat isolates 1157/3 and 1160/1 cluster with *Candidatus* B. mayotimonensis with high bootstrap values in a distinct phylogenetic position. Moreover, the genetic distance values demonstrate that the bat isolates 1157/3 and 1160/1 classify as strains of the *Candidatus*-status species B. mayotimonensis. We propose that the bat isolate 1160/1 is the type strain of *B. mayotimonensis*.

Findings of the study raised an interesting question: how could *Bartonella* spp., or any other hemotrophic bacterium, be transmitted from the bat into the human host? Daubenton's bats prefer to roost in abandoned woodpecker cavities and bird boxes, whereas the other bat species are often found in the attics of houses in close proximity to humans. Given that *Bartonella* spp. are hemotrophic, transmission through bat bite and saliva is not considered likely. Moreover, at Turku University Central Hospital, which is responsible for a population base of 500,000, only 2 or 3 patients per year are admitted with a bat bite (J. Oksi, pers. comm.). These numbers probably reflect the frequency of bat bites in most countries of the Northern Hemisphere. We propose that fecal droppings of blood-fed bat ectoparasites might transmit *Bartonella* spp. into the human host, assisted by superficial scratching or tissue trauma of the skin. The presence of viable bacteria in feces of body lice (*Pediculus humanus*) that have been feeding on *B. quintana*–infected rabbits is well documented (*33*,*34*). Similar observations have been reported for the feces of experimentally infected cat fleas (*Ctenocephalides felis*) (*35*,*36*). Most importantly, intradermal injection of feces from fleas that had fed on a *B. henselae*–infected cat led to bacteremia in a pathogen-free cat (*37*). Ectoparasite bite–mediated transmission is also possible, but the bat bugs (*Cimex* spp.) known to also feed on humans were not analyzed in the current study.

The reported metagenomic analysis of bat fecal material indicates that bats are reservoir hosts for several pathogenic bacterial genera. No comprehensive study has been published on the bacterial flora of bats in light of its zoonotic threat to humans. The major research focus has been on viruses, and several deadly viruses have been detected or isolated (*3*–*6*). One of the main conclusions from these studies is that bats tolerate their deadly companions relatively well, a feature that has been discussed in the context of long evolutionary history of bats (*38*). Bats are also highly mobile and long-lived, ideal as pathogen reservoirs. Metagenomics-driven approaches should be continued to assess the pathogenic potential of bacteria that colonize bats.

Acknowledgment

We gratefully thank Olaf Thalmann for help in the laboratory.

This work was supported by grant 8149 and grant 9222 from the Turku University Foundation to A.T.P. and by personal grant from Emil Aaltonen Foundation and grant 8621 from Turku University Foundation to E.J.V. We acknowledge CSC–IT Center for Science Ltd. for the allocation of computational resources.

Dr Veikkolainen is a postdoctoral research fellow of the Pulliainen laboratory. His research interests include the dynamics of bat microbiome and bat immunology, with special emphasis on the recognition and control of bacterial colonization.

Mr Vesterinen is a PhD student at the Department of Biology, University of Turku. His research interests include food web dynamics and biological interactions, and he has established a laboratory dedicated to fecal analysis in the Laboratory of Genetics, University of Turku.

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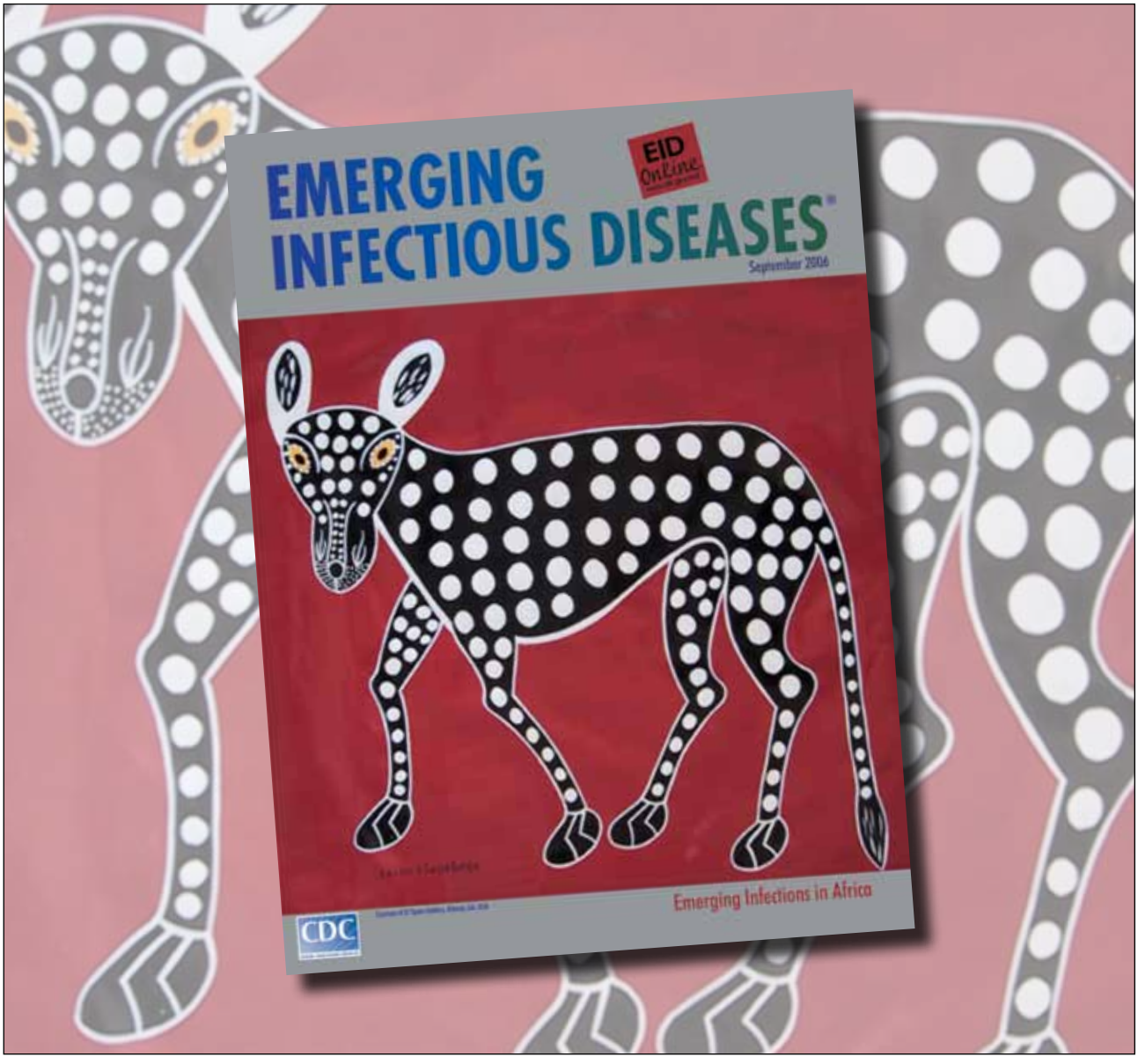
Address for correspondence: Arto T. Pulliainen, Division of General Microbiology, Department of Biosciences, University of Helsinki, P.O. Box 56 (Viikinkaari 9), Biocenter 1, FI 00790, Helsinki, Finland, email: arto.pulliainen@helsinki.fi



Article DOI: http://dx.doi.org/10.3201/eid2006.130956

Bats as Reservoir Hosts of Hhuman 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 (*1*), 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% CO_2 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 rcf, 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.

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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 brandtii	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 brandtii	2011 Jul 31	60° 26′ 54” N, 22° 06′ 29” E	2791, 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 brandtii	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	1158, F, adult, 9.6 g, 39.5 mm	0.243	–	–	–	–
Myotis brandtii	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) *Nycteribia kolenatii* (n = 3)	*rpoB-5* –
Myotis daubentonii	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 mystacinus	2012 Aug 25	59° 55′ 34” N, 22° 24′ 51” E	1163, F, adult, 6.6 g, 35.4 mm	0.186	–	–	–	–
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	–	–	*Nycteribia kolenatii* (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	*Nycteribia kolenatii* (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	*Nycteribia kolenatii* (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.

Technical Appendix Table 2. Oligonucleotide primers used in this study

Oligo	Target genetic marker, oligo orientation	Sequence 5′→3′	Reference
Bartonella spp.			
fD1	16S rRNA gene, forward	AGAGTTTGATCCTGGCTCAG	(*1*)
rP2	16S rRNA gene, reverse	ACGGCTACCTTGTTACGACTT	(*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	GGGGACCAGCTCATGGTGG	(*3*)
BhCS.1137n	GltA gene, reverse	AATGCAAAAAGAACAGTAAACA	(*3*)
prAPT0244	RpoB gene, forward	GATGTGCATCCTACGCATTATGG	(*4*)
prAPT0245	RpoB gene, reverse	AATGGTGCCTCAGCACGTATAAG	(*4*)
prAPT0257	FtsZ gene, forward	GCCTTCAAGGAGTTGATTTTGTTGTTGCCAAT	This study
prAPT0258	FtsZ gene, reverse	ACGACCCATTTCATGCATAACAGAAC	This study
ssrA-F	SsrA gene, forward	GCTATGGTAATAAATGGACAATGAAATAA	(*5*)
ssrA-R	SsrA gene, reverse	GCTTCTGTTGCCAGGTG	(*5*)
prPE23	VirB4 gene, forward	GGTTGCTTTATATTCTCACATC	(*6*)
prPE24	VirB4 gene, reverse	GAAGTTGCGCCCACCATG	(*6*)
Ectoparasites			
ZBJ-ArtF1c	Mitochondrial cytochrome c oxidase subunit I (COI)	AGATATTGGAACWTTATATTTTATTTTTGG	(*7*)
ZBJ-ArtR2c	Mitochondrial cytochrome c oxidase subunit I (COI)	WACTAATCAATTWCCAAATCCTCC	(*7*)

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Technical Appendix Table 3. Results of the BLASTN homology searches performed in January 2013

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		
			Closest	Second closest	Third closest
1157/3	16S rRNA (485 bp)	KF003116	Nondisc†, 100% (485/485)	Nondisc, 99.8% (484/485)	Nondisc, 99.6% (483/485)
	ITS (259 bp)	KF003117	*Candidatus* B. mayotimonensis, 85.7% (239/279) FJ376735	None	None
	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% (553/595) 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	*B. japonica*, 100% (485/485) AB440632	Nondisc, 99.8% (484/485)	Nondisc, 99.6% (483/485)
	ITS (265 bp)	KF003124	*Candidatus* B. mayotimonensis, 83.3% (235/282) FJ376735	None	None
	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	Nondisc, 100% (485/485)	Nondisc, 99.8% (484/485)	Nondisc, 99.6% (483/485)
	ITS (163 bp)	KF003131	None	None	None
	rpoB (406 bp)	KF003132	*B. quintana*, 91.9% (372/405) AF165994	Nondisc, 91.1% (370/406)	Nondisc, 90.9% (369/406)
	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	*B. vinsonii* subsp. vinsonii, 88.5% (452/511) AF467757	Nondisc, 88.3% (451/511)	*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% (239/253) JN029777	Nondisc, 93.3% (236/253)

*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.

Technical Appendix Table 4. Pairwise genetic distance values of the concatenated *rpoB*, *gltA*, 16S rRNA and *ftsZ* sequence fragments. Lowest genetic distance values of the bat strains compared with the *Brucella abortus* outgroup 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	
1	*B. alsatica*																																					**1**
2	*B. australis*	0.109																																				**2**
3	*B. bacilliformis*	0.102	0.118																																			**3**
4	*B. birtlesii*	0.065	0.098	0.106																																		**4**
5	*B. bovis*	0.096	0.104	0.095	0.084																																	**5**
6	*B. capreoli*	0.090	0.097	0.097	0.067	0.045																																**6**
7	*B. chomelii*	0.099	0.106	0.104	0.079	0.045	0.021																															**7**
8	*B. clarridgeiae*	0.096	0.106	0.096	0.097	0.096	0.092	0.100																														**8**
9	*B. coopersplainsensis*	0.070	0.113	0.112	0.086	0.112	0.101	0.111	0.107																													**9**
10	*B. doshiae*	0.090	0.107	0.109	0.084	0.097	0.084	0.096	0.099	0.100																												**10**
11	*B. elizabethae*	0.084	0.127	0.110	0.092	0.099	0.099	0.103	0.102	0.097	0.097																											**11**
12	*B. grahamii*	0.076	0.118	0.109	0.084	0.094	0.087	0.095	0.098	0.090	0.088	0.042																										**12**
13	*B. henselae*	0.070	0.106	0.102	0.077	0.084	0.086	0.093	0.099	0.081	0.080	0.091	0.080																									**13**
14	*B. japonica*	0.074	0.111	0.109	0.085	0.104	0.095	0.105	0.102	0.027	0.097	0.099	0.088	0.080																								**14**
15	*B. koehlerae*	0.070	0.107	0.099	0.081	0.083	0.086	0.093	0.094	0.084	0.080	0.083	0.078	0.031	0.081																							**15**
16	*B. melophagi*	0.095	0.100	0.095	0.074	0.040	0.017	0.014	0.096	0.106	0.092	0.099	0.090	0.087	0.099	0.086																						**16**
17	*B. phoceensis*	0.064	0.107	0.096	0.082	0.092	0.084	0.092	0.095	0.073	0.092	0.080	0.075	0.076	0.074	0.067	0.086																					**17**
18	*B. queenslandensis*	0.082	0.119	0.105	0.089	0.101	0.092	0.101	0.099	0.098	0.091	0.040	0.039	0.085	0.093	0.082	0.095	0.089																				**18**
19	*B. quintana*	0.077	0.109	0.111	0.087	0.095	0.090	0.102	0.093	0.091	0.088	0.094	0.085	0.056	0.087	0.059	0.096	0.078	0.088																			**19**
20	*B. rattaustraliani*	0.079	0.113	0.115	0.083	0.110	0.106	0.108	0.108	0.054	0.095	0.097	0.091	0.082	0.054	0.089	0.104	0.082	0.101	0.093																		**20**
21	*B. rattimassiliensis*	0.083	0.130	0.117	0.091	0.099	0.093	0.103	0.111	0.099	0.098	0.056	0.045	0.088	0.097	0.090	0.099	0.087	0.053	0.093	0.101																	**21**
22	*B. rochalimae*	0.094	0.111	0.095	0.099	0.089	0.086	0.094	0.045	0.107	0.101	0.097	0.096	0.088	0.101	0.095	0.092	0.097	0.094	0.093	0.107	0.104																**22**
23	*B. schoenbuchensis*	0.100	0.103	0.099	0.079	0.039	0.016	0.011	0.098	0.111	0.093	0.102	0.095	0.091	0.104	0.090	0.012	0.087	0.098	0.098	0.110	0.100	0.091															**23**
24	*B. silvatica*	0.082	0.125	0.121	0.094	0.099	0.093	0.101	0.104	0.089	0.102	0.091	0.087	0.090	0.088	0.096	0.098	0.078	0.098	0.090	0.092	0.093	0.103	0.097														**24**
25	*B. tamiae*	0.153	0.160	0.168	0.158	0.163	0.155	0.158	0.166	0.156	0.155	0.159	0.162	0.155	0.154	0.155	0.159	0.143	0.156	0.150	0.161	0.161	0.158	0.156	0.157													**25**
26	*B. taylorii*	0.061	0.117	0.099	0.068	0.092	0.079	0.090	0.095	0.078	0.082	0.088	0.082	0.071	0.077	0.070	0.085	0.065	0.087	0.069	0.081	0.090	0.095	0.087	0.081	0.152												**26**
27	*B. tribocorum*	0.072	0.115	0.102	0.078	0.096	0.090	0.102	0.095	0.088	0.088	0.035	0.033	0.078	0.086	0.076	0.098	0.079	0.030	0.081	0.092	0.053	0.088	0.098	0.093	0.157	0.078											**27**
28	*B. vinsonii* subsp. arupensis	0.054	0.110	0.100	0.068	0.094	0.087	0.093	0.090	0.068	0.078	0.082	0.075	0.068	0.070	0.068	0.089	0.062	0.080	0.064	0.074	0.084	0.092	0.092	0.080	0.150	0.054	0.075										**28**
29	*B. vinsonii* subsp. berkhoffii	0.052	0.106	0.100	0.068	0.096	0.090	0.094	0.092	0.070	0.083	0.081	0.076	0.068	0.072	0.069	0.093	0.065	0.078	0.069	0.072	0.084	0.095	0.097	0.081	0.151	0.059	0.072	0.035									**29**
30	*B. vinsonii* subsp. vinsonii	0.048	0.107	0.099	0.064	0.090	0.081	0.088	0.095	0.072	0.076	0.083	0.074	0.068	0.072	0.069	0.083	0.064	0.078	0.068	0.076	0.082	0.093	0.086	0.082	0.155	0.050	0.072	0.030	0.028								**30**
31	*B. washoensis*	0.073	0.107	0.106	0.077	0.090	0.085	0.095	0.094	0.081	0.077	0.080	0.076	0.053	0.081	0.054	0.088	0.071	0.081	0.055	0.084	0.084	0.090	0.095	0.088	0.151	0.067	0.075	0.057	0.061	0.061							**31**
32	*Candidatus* B. mayotimonensis	0.057	0.109	0.100	0.075	0.100	0.093	0.100	0.099	0.076	0.090	0.086	0.086	0.076	0.076	0.073	0.096	0.067	0.086	0.075	0.076	0.090	0.103	0.099	0.085	0.159	0.059	0.080	0.055	0.050	0.056	0.072						**32**
33	Bat strain 1157/3	0.050	0.109	0.103	0.061	0.086	0.081	0.091	0.089	0.068	0.084	0.078	0.075	0.063	0.065	0.065	0.083	0.064	0.082	0.068	0.064	0.080	0.093	0.089	0.078	0.161	0.051	0.070	0.044	0.048	0.046	0.057	**0.040**					**33**
34	Bat strain 1160/1	0.061	0.103	0.103	0.073	0.097	0.089	0.099	0.102	0.076	0.085	0.084	0.079	0.076	0.070	0.076	0.092	0.066	0.083	0.081	0.071	0.087	0.097	0.096	0.085	0.156	0.057	0.075	0.056	0.056	0.058	0.070	**0.038**	0.030				**34**
35	Bat strain 2574/1	0.089	0.099	0.106	0.084	0.086	0.079	0.094	0.097	0.102	0.079	0.100	0.091	0.077	0.099	0.073	0.087	0.087	0.095	0.079	0.097	0.098	0.091	0.088	0.107	0.163	0.089	0.086	0.083	0.080	0.080	**0.070**	0.088	0.081	0.087			**35**
36	*Brucella abortus* 9–941	0.243	0.246	0.245	0.255	0.260	0.247	0.256	0.252	0.256	0.254	0.236	0.235	0.247	0.245	0.249	0.249	0.243	0.236	0.237	0.251	0.239	0.245	0.254	0.250	0.231	0.248	0.244	0.244	0.236	0.245	0.239	0.243	0.240	0.252	0.263		**36**

Technical Appendix Table 5. *Bartonella* spp. type strain sequences used in the multilocus sequence and phylogenetic analyses

Species	Type strain, isolated from	Reference	GenBank accession no.			
			gltA	*rpoB*	16S rRNA	*ftsZ*
B. alsatica	IBS 382, rabbit (*Oryctolagus cuniculus)*	(*1*)	AF204273	AF165987	AJ002139	AF467763
B. australis	Aust/NH1, kangaroo (*Macropus giganteus*)	(*2*)	NC_020300	NC_020300	DQ538394	NC_020300
B. bacilliformis	KC583, unknown origin	(*3*)	YP_988907	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*)	AY254308	AB290189	NR_025736	AB290193
B. clarridgeiae	Houston-2, cat	(*7*)	U84386	AF165990	AB292603	AF141018
B. coopersplainsensis	AUST/NH20, rat (*Rattus leucopus*)	(*8*)	EU111803	EU111792	EU111759	EU111781
B. doshiae	R18, field vole (*Migrotus agrestis*)	(*9*)	Z70017	AF165991	NR_029368	AF467754
B. elizabethae	F9251, human	(*10*)	Z70009	AF165992	NR_025889	AF467760
B. grahamii	V2, bank vole (*Myodes glareolus*)	(*9*)	Z70016	AF165993	NR_029366	AF467753
B. henselae	Houston-1, human	(*11*)	CAF27442	AF171070	NC_005956	AF061746
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*)	AY724768	EF605288	AIMA01000004	EF605286
B. phoceensis	16120, rat (*Rattus norvegicus*)	(*15*)	AY515126	AY515132	AY515119	AY515135
B. queenslandensis	Aust/NH12, rat (*Melomys* sp.)	(*8*)	EU111798	EU111787	EU111754	EU111776
B. quintana	Fuller, human	(*16*)	Z70014	AF165994	NR_044748	AB292605
B. rattimassiliensis	15908, rat (*Rattus norvegicus*)	(*15*)	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. silvatica	Fuji 23–1, mouse (*Apodemus speciosus*)	(*12*)	AB242287	AB242292	AB440636	AB440637
B. tamiae	Th239, human	(*19*)	DQ395177	EF091855	AIMB01000009	DQ395178
B. taylorii	M6, mouse (*Apodemus* spp.)	(*9*)	Z70013	AF165995	NR_029367	AF467756
B. tribocorum	IBS 506, rat (*Rattus norvegicus*)	(*20*)	AJ005494	AF165996	AM260525	AF467759
B. vinsonii subsp. arupensis	OK-94–513, human	(*21*)	AF214557	AY166582	AF214558	AF467758
B. vinsonii subsp. berkhofii	93-CO1, dog	(*22*)	U28075	AF165989	L35052	AF467764
B. vinsonii subsp. vinsonii	Baker, vole (species unknown)	(*23*)	Z70015	AF165997	NR_037056	AF467757
B. washoensis	Sb944nv, ground squirrel (*Spermophilus beecheyi*)	(*24*)	AF470616	AB292596	AB292597	AB292598

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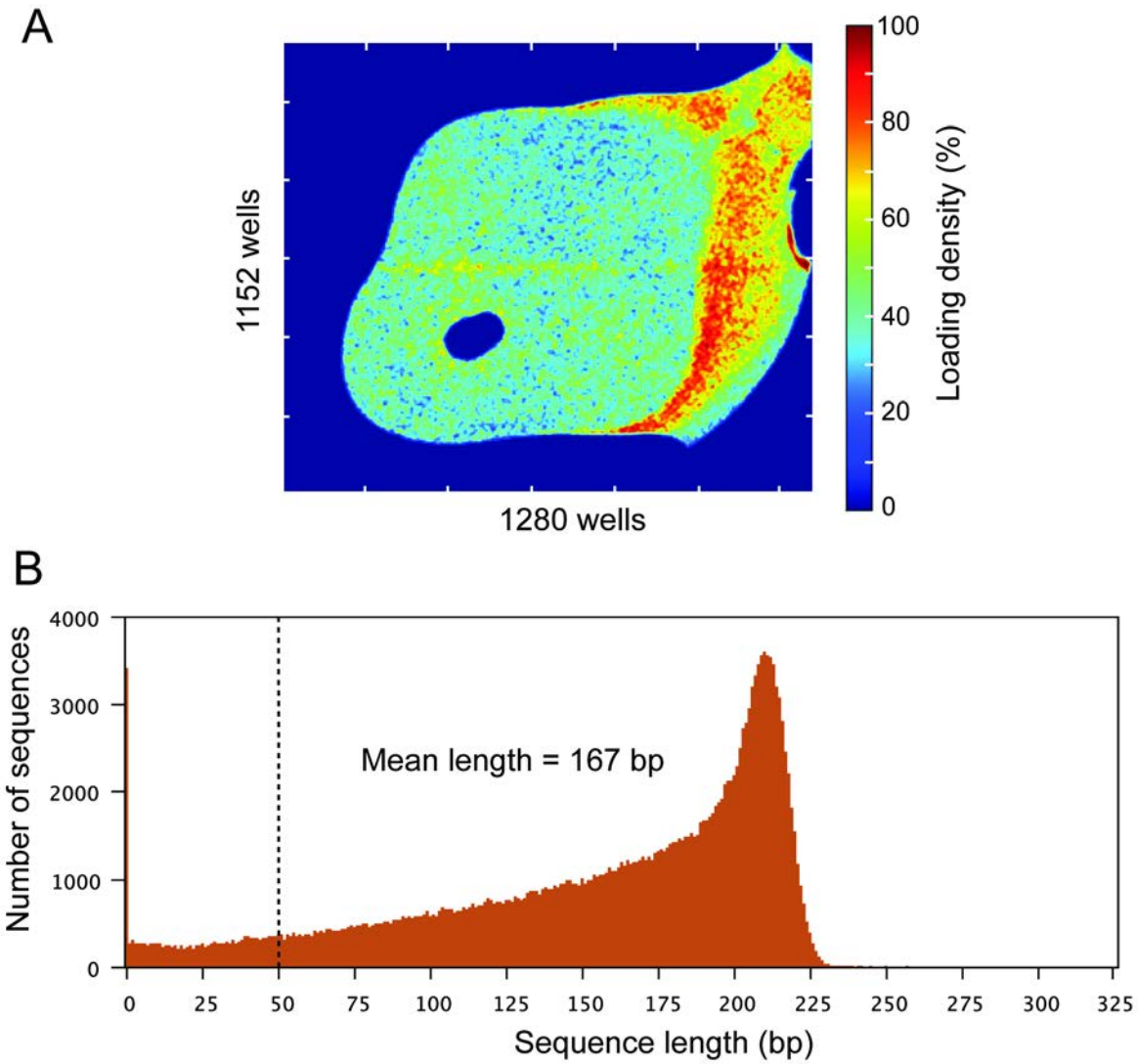
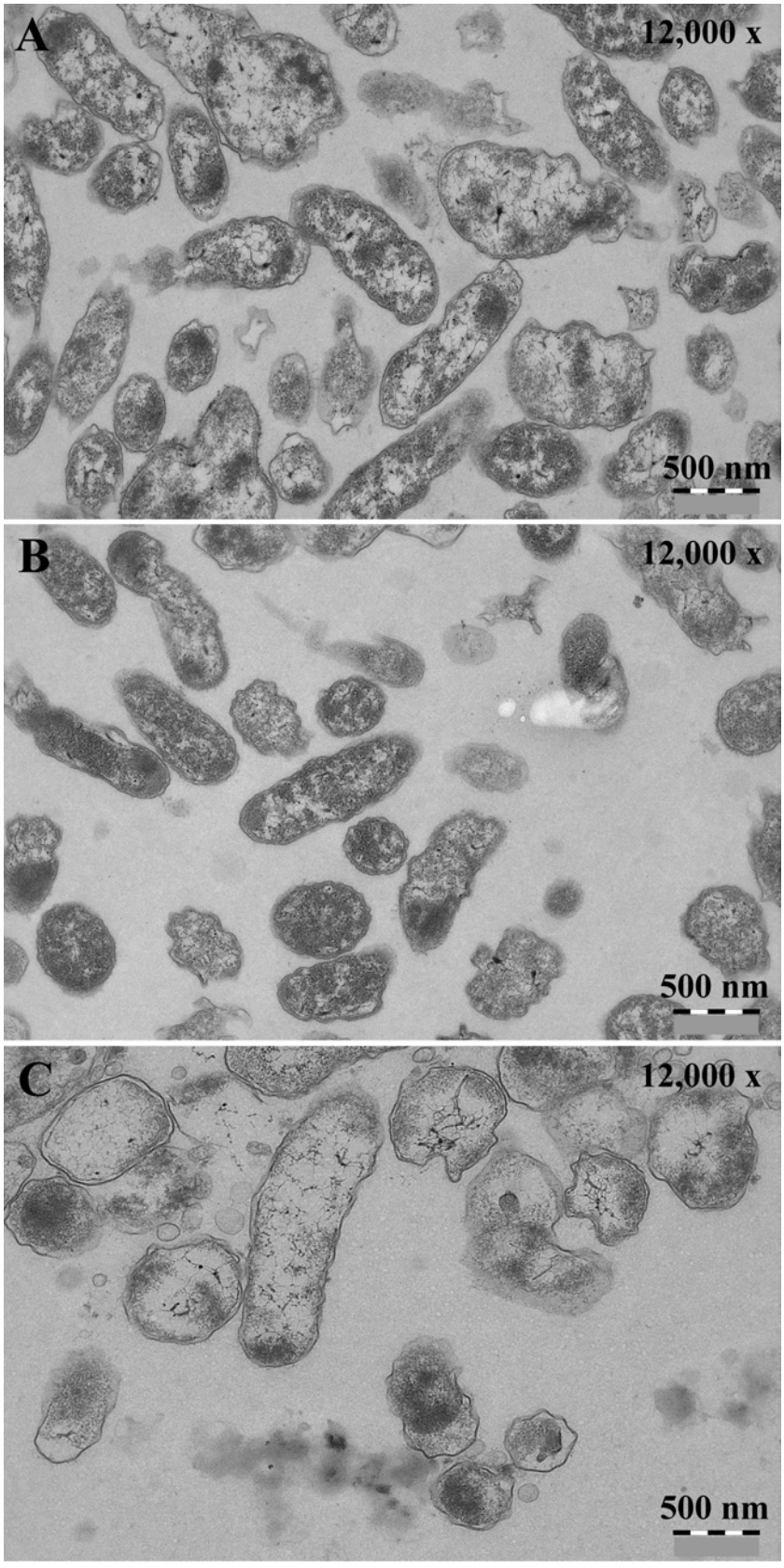


Technical Appendix Figure 1. Performance of the Ion Torrent Personal Genome Machine. A) Loading density of the chip (average 31%). Twenty-five percent of the loaded beads were polyclonal and were distracted from further analysis together with beads that gave low quality reads (18% of the loaded beads). 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.