INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Hashemi Shahraki *et al.*, *Int J Syst Evol Microbiol*
DOI 10.1099/ijsem.0.002103

MICROBIOLOGY SOCIETY

# *Mycobacterium aquaticum* sp. nov., a rapidly growing species isolated from haemodialysis water

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**Abstract**

The characterization of five Iranian isolates, four from hospital haemodialysis water and one from the sputum of a patient, led to the detection of a novel mycobacterium species. The strains were characterized by mucoid colonies developing in 3–5 days at temperatures ranging from 25 to 37 °C. The biochemical test pattern was unremarkable while the HPLC profile of mycolic acids resembled that of *Mycobacterium fortuitum*. The sequences of three major housekeeping genes (16S rRNA, *hsp65* and *rpoB*) were unique and differed from those of any other mycobacterium. *Mycobacterium brisbanense*, which is the species that shared the highest 16S rRNA gene sequence similarity (99.03 %), was distinct, as shown by the average nucleotide identity and by the genome to genome distance values (91.05 and 43.10 %, respectively). The strains are thus considered to represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium aquaticum* sp. nov. is proposed. The type strain is RW6$^T$ (=DSM 104277$^T$=CIP111198$^T$).

Water is the most important reservoir for non-tuberculous mycobacteria (NTM) [1]. While the isolation of NTM from environmental stagnant waters is expected, the presence of NTM in plumbing remains, despite a considerable number of reports [2–4], underestimated. We report here a previously unrecognized mycobacterium isolated from hospital water systems in Iran.

Two isolates (Rw6$^T$ and Rw7) were grown in 2014 from haemodialysis water in one Iranian general hospital (Ahwaz, Khuzestan province). One year later an additional strain (SD72) was isolated from the sputum of a woman suffering from asthma. She had no symptoms suggesting mycobacterial disease and the isolation was not considered clinically significant. In 2015, two isolates (SD53 and SD55) were detected in haemodialysis water in another Iranian hospital (Karaj, Alborz province) [5].

The isolates grew large smooth colonies on blood agar in 3–4 days both at 25 and at 37 °C, but not at 42 °C. Growth was achieved also on McConkey agar without crystal violet and on media containing isoniazid (1 μg ml$^{-1}$) and thiacetazone (10 μg ml$^{-1}$) but not in the presence of *p*-nitrobenzoic acid (400 μg ml$^{-1}$). The strains, which remained unpigmented after light exposure, displayed only minimal biochemical activities; they were catalase- (>45 mm) positive and were able also to reduce tellurite, but lacked urease, were unable to hydrolyse Tween 80 and to reduce nitrates while catalase was inactive at 68 °C [6]. As expected, due to the large number of mycobacterial species, biochemical and cultural features were unsuitable for differentiating the test strains from many other unpigmented rapid growers.

The five isolates presented very homogeneous antimicrobial patterns with MICs [7] within the range of susceptibility for the majority of the drugs recommended for rapidly growing mycobacteria (Table 1).

The HPLC profile of cell-wall mycolic acids [8] was determined with cells grown on Middlebrook 7H10 agar. Following saponification, extraction and derivatization, the mycolic acids were separated using a gradient of methanol

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**Keywords:** *Mycobacterium aquaticum*; *Mycobacterium fortuitum*; *Mycobacterium brisbanense*; average nucleotide identity.
**Abbreviations:** ANI, average nucleotide identity; DDH, DNA–DNA hybridization; NTM, non-tuberculous mycobacteria.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65* and *rpoB* genes sequences of strain RW6$^T$ are KY392537, KY392538 and KY392539, respectively.
Two supplementary figures are available with the online Supplementary Material.

**Table 1.** MICs of antimicrobial drugs potentially active against rapidly growing mycobacteria

| Drug | Strain | | | | | Interpretation* |
|---|---|---|---|---|---|---|
| | RW6$^T$ | RW7 | SD72 | SD55 | SD53 | |
| Trimethoprim/sulfamethoxazole | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 | 0.5/9.5 | S |
| Linezolid | 4 | 4 | 2 | 4 | 4 | S |
| Ciprofloxacin | 1 | 0.5 | 0.5 | 1 | 0.5 | S |
| Imipenem | 64 | 8 | 8 | 64 | 64 | I-R |
| Moxifloxacin | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | S |
| Cefoxitin | 32 | 8 | 32 | 32 | 32 | S-I |
| Amikacin | 8 | 4 | 4 | 8 | 8 | S |
| Doxycycline | 4 | 2 | 2 | 4 | 4 | I |
| Minocycline | 4 | 2 | 2 | 4 | 4 | I |
| Tigecycline | 1 | 0.5 | 0.12 | 1 | 0.25 | (S)† |
| Tobramycin | >16 | >16 | >16 | 16 | 16 | R |
| Clarithromycin | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | S |

*S, susceptible; I, intermediate; R, resistant.

†Tentative interpretation.

and 2-propanol as recommended by the Sherlock Mycobacteria Identification System (SMIS; MIDI). The pattern of the strains was almost indistinguishable from that of *Mycobacterium fortuitum/peregrinum* (Fig. 1) as certified by the Sherlock software (similarity index 0.833).

dsDNA sequences were determined for three major housekeeping genes (16S rRNA, *hsp65* and *rpoB*) using BigDye Terminator chemistry on an ABI 3730 DNA sequencer (Applied Biosystems). In all the regions mentioned above the five test strains presented identical sequences. In the 16S rRNA gene (1518 bp) the most closely related species were *Mycobacterium brisbanense* (14 bp mismatches, 99.03 % similarity) and *Mycobacterium cosmeticum* (99.01 %). In the 442 pb of the hypervariable region of the *hsp65* gene [9], the sequence of the test strains differed only by 2 bp from *Mycobacterium farcinogenes* (99.5 %). In the same genetic region the restriction patterns produced by *Bst*EII and *Hae*III enzymes [10] were 231, 116, 79 and 139, 123, 58, 52, respectively, corresponding to that of *M. fortuitum* type 2 (http://app.chuv.ch/prasite/index.html). In the 723 bp region [11] of the *rpoB* gene the only species showing a significant similarity (95.7 %) was *Mycobacterium wolinskyi*.

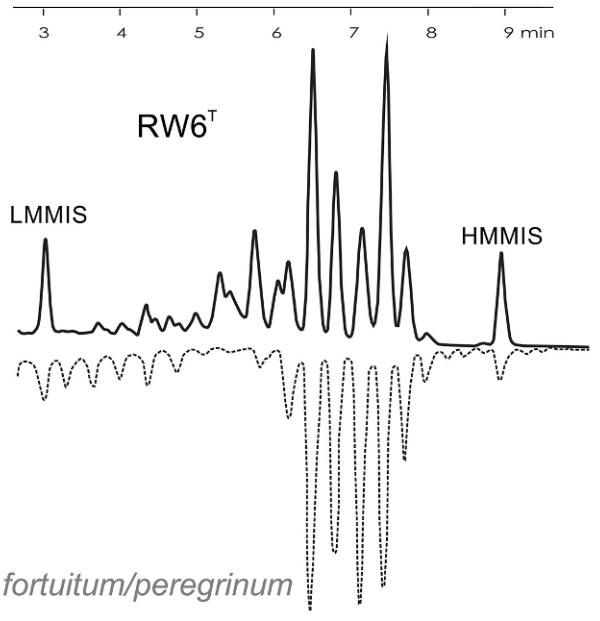


**Fig. 1.** Representative mycolic acid pattern of strain RW6$^T$ compared with the reference profile of *M. fortuitum/pregrinum* (Sherlock software). LMMIS, low-molecular-mass internal standard; HMMIS, high-molecular-mass internal standard.

The quite high similarity between the 16S rRNA gene sequences of the test strains, *M. brisbanense* and *M. cosmeticum* made determination of the average nucleotide identity (ANI) between them necessary to confirm their status as independent species [12]. For this, paired-end libraries were prepared from two strains, RW6$^T$ and SD53, representative of the proposed novel species, using a Nextera XT DNA Sample Preparation kit and Nextera XT Index kit (Illumina) according to the manufacturer's protocol. Libraries were then sequenced on an Illumina MiniSeq platform. Reads were quality trimmed and assembled using SPAdes ver. 3.9.0 software [13]. The ANI was calculated among the genomes above and the genomes of *M. brisbanense* JCM 15654$^T$ and *M. cosmeticum* DSM 44829$^T$ (available in GenBank) using the OrthoANI calculator ver. 0.93 [14]. As expected the ANI between strains RW6$^T$ and SD53 was close to 100 % while those concerning *M. brisbanense* and *M. cosmeticum* were clearly below 95–96 %, the cutoff for species demarcation (Table 2). To assess the ANI results the genomes above were also compared using the Genome to Genome Distance Calculator (http://ggdc.dsmz.de/distcalc2.php), which is the equivalent *in silico* of the DNA–DNA

**Table 2.** Values of ANI and DDH-equivalent (%) between *M. brisbanense* JCM 15654$^T$, *M. fortuitum* DSM 46621$^T$, *M. cosmeticum* DSM 44829$^T$, and strains RW6$^T$ and SD53

| | RW6$^T$ | | SD53 | | *M. brisbanense* JCM 15654$^T$ | | *M. fortuitum* DSM 46621$^T$ | |
|---|---|---|---|---|---|---|---|---|
| | ANI | DDH | ANI | DDH | ANI | DDH | ANI | DDH |
| SD53 | 99.99 | 99.90 | | | | | | |
| *M. brisbanense* JCM 15654$^T$ | 91.05 | 43.10 | 91.06 | 43.10 | | | | |
| *M. fortuitum* DSM 46621$^T$ | 79.62 | 22.90 | 79.62 | 22.90 | 79.56 | 22.90 | | |
| *M. cosmeticum* DSM 44829$^T$ | 77.63 | 21.10 | 77.67 | 21.10 | 77.95 | 21.70 | 77.45 | 21.00 |

hybridization (DDH) test. The distance between the genomes of both *M. brisbanense* and *M. cosmeticum* and those of strains RW6$^T$ and SD53 corresponded to DDH values clearly <70 % (Table 2) and confirmed the status of independent species.

Phylogenetic trees were reconstructed, with 1000 bootstrap replicates, by the neighbour-joining method [15], using MEGA 6.06 software [16]. In the phylogenetic reconstruction based on 16S rRNA gene sequences, the test strains occupied a position very close to *M. brisbanense* (Fig. 2). In contrast, in the tree based on *hsp65* the closest species were *Mycobacterium conceptionense* and *Mycobacterium senegalense* (Fig. S1, available in the online Supplementary Material), while in that based on *rpoB* it was *Mycobacterium phocaicum* (Fig. S2). In the tree inferred from the concatenated sequences of 16S rRNA, *rpoB* and *hsp65* genes (2520 bp) the position of the test strains was quite distant from any other *Mycobacterium* species (Fig. 3).

Based on the data presented, the strains are considered to represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium aquaticum* sp. nov. is proposed.

## DESCRIPTION OF *MYCOBACTERIUM AQUATICUM* SP. NOV.

*Mycobacterium aquaticum* (a.qua'ti.cum. L. neut. adj. *aquaticum* isolated from water).

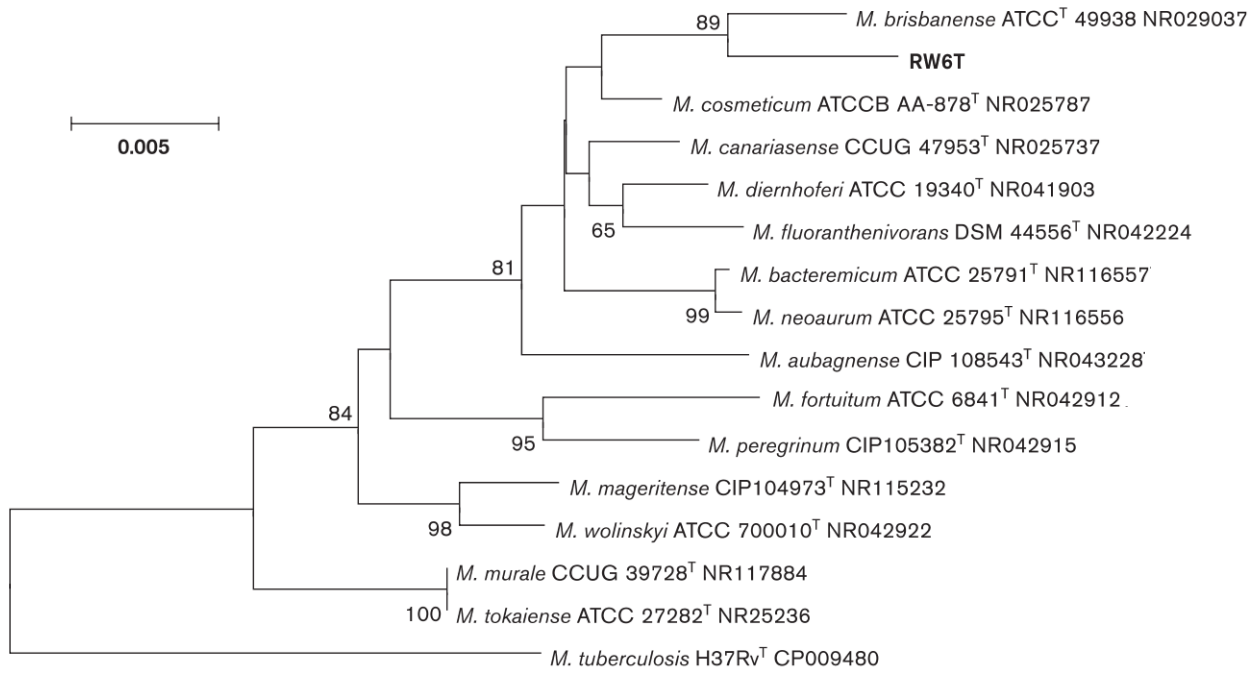


**Fig. 2.** Phylogenetic tree based on 16S rRNA gene sequences, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 % are given at nodes. Bar, 0.005 substitutions per nucleotide position. RW6$^T$ is representative of the five test strains, which had identical sequences in this gene.

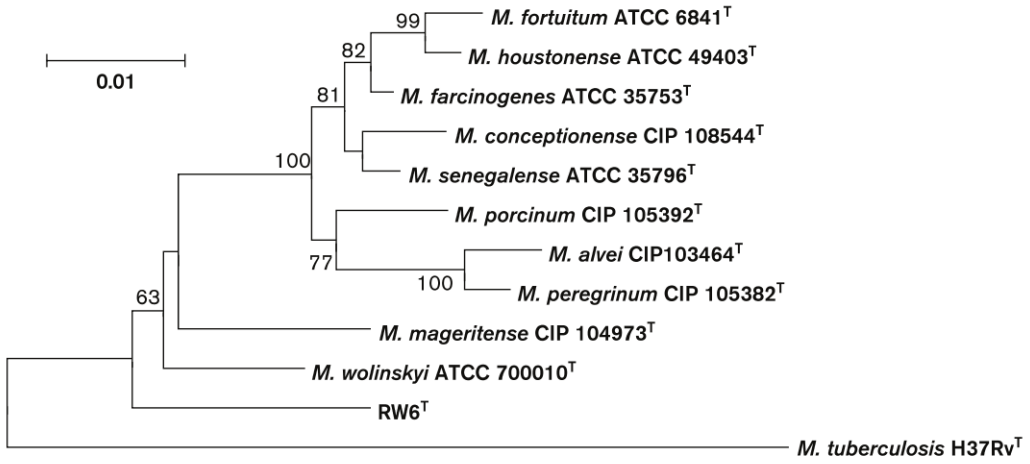


**Fig. 3.** Phylogenetic tree based on concatenated sequences of 16S rRNA, *hsp65* and *rpoB* genes, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 % are given at nodes. Bar, 0.01 substitutions per nucleotide position.

Cells are acid-fast, non-motile and non-spore-forming. Mucoid, unpigmented colonies develop in 3–5 days at temperatures ranging from 25 to 37 °C. Catalase- (>45 mm of foam) positive and able to reduce tellurite; negative for urease, Tween 80 hydrolysis and nitrate reductase. Susceptibility is shown for the large majority of the drugs recommended for tests of rapidly growing species. The HPLC pattern of mycolic acids is similar to that observed for *M. fortuitum*. The sequences of the 16S rRNA, *hsp65* and *rpoB* genes, which provide the method of choice for their identification, differ from any other mycobacterium species.

The type strain is RW6$^T$ (=DSM 104277$^T$=CIP 111198$^T$). The size of the genome of the type strain is one of the largest in the genus *Mycobacterium*, 7 927 623 bp, and includes 7614 coding DNA sequences; its G+C content is 66.48 %.

Funding information
The authors did not receive any specific grant for this work from any funding agency.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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International Journal of Evolutionary and Systematic Microbiology

# *Mycobacterium aquae* sp. nov., a rapidly growing species isolated from hemodialysis water in Iran

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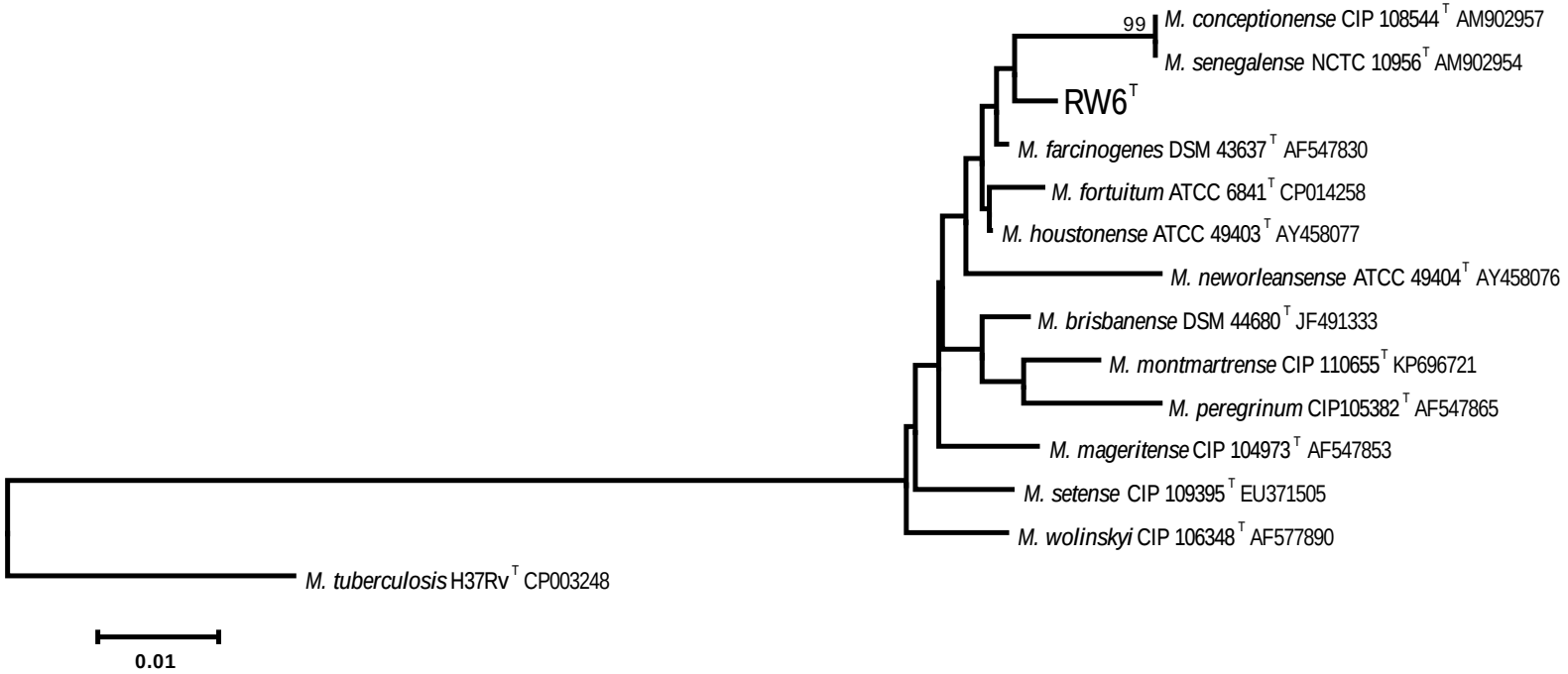
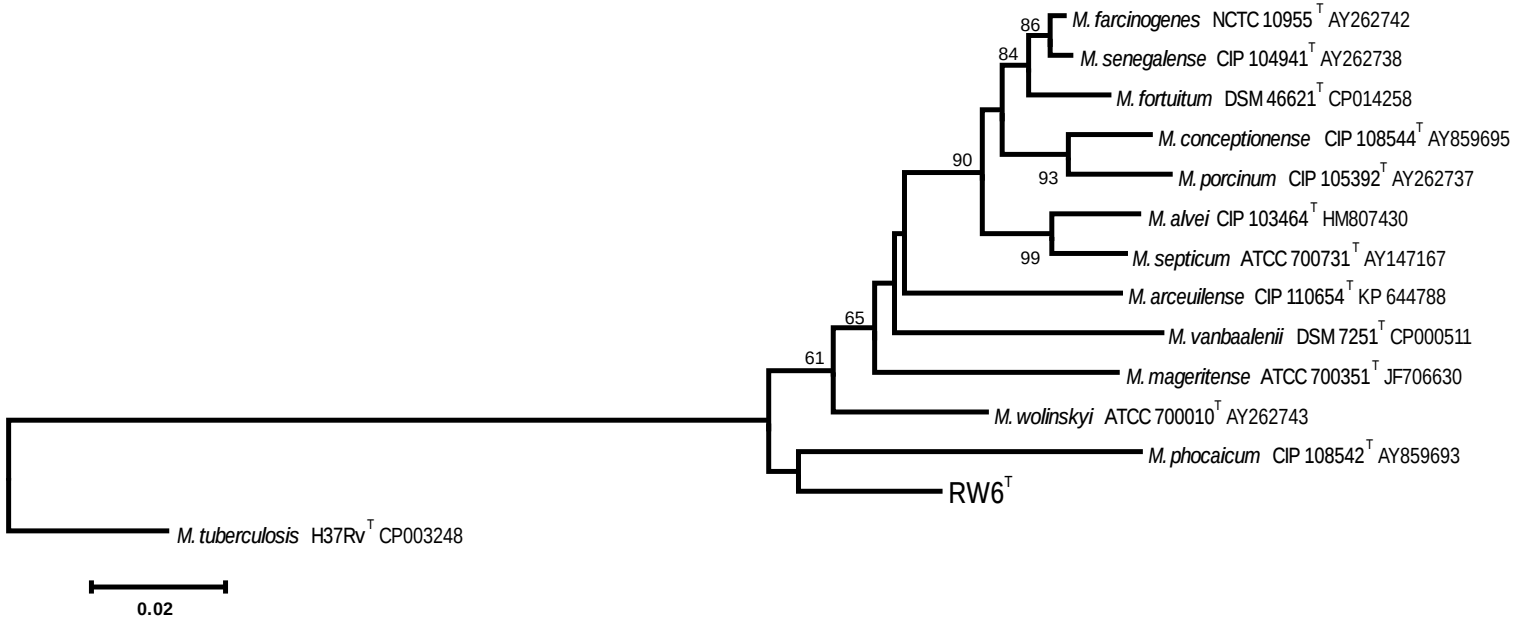



**Supplementary figure S1.** Phylogenetic tree based on *hsp65* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.




**Supplementary figure S2.** Phylogenetic tree based on *rpoB* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.02 substitutions per nucleotide position.