Mycobacterium hippocampi sp. nov., a rapidly growing scotochromogenic species isolated from a seahorse with tail rot

José Luis Balcázar [a,b,*], Miquel Planas [a], José Pintado [a]

[a] Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (CSIC), c/. Eduardo Cabello 6, 36208 Vigo, Spain.

[b] Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, Carrer Emili Grahit 101, 17003 Girona, Spain.

*** Corresponding author:** J. L. Balcázar. Tel: +34 972 183 380. Fax: +34 972 183 248. e-mail: jlbalcazar@icra.cat

Abstract

A Gram-positive, aerobic, non-motile, non-sporulating, acid-fast, and rod-shaped bacterium (BFLP-6^T), previously isolated from a seahorse (*Hippocampus guttulatus*) with tail rot, was studied using a polyphasic taxonomic approach. Growth occurred at 15–35 °C (optimum 25 °C), at pH 5.0–10.0 (optimum pH 7.0) and at NaCl concentrations between 0 and 6 % (w/v). The G+C content of DNA was 66.7 mol%. The predominant fatty acids were $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{16:1}\omega6c$. A mycolic acid pattern of alpha-mycolates and keto-mycolates was detected. Analysis of concatenated sequences (16S rRNA, *rpoB*, *ssrA* and *tuf* genes), and chemotaxonomic and phenotypic features indicated that strain BFLP-6^T represents a novel species within the genus *Mycobacterium*, for which the name *Mycobacterium hippocampi* sp. nov. is proposed. The type strain is BFLP-6^T (=DSM 45391^T =LMG 25372^T).

Keywords: *Mycobacterium hippocampi*; polyphasic taxonomic analysis; seahorse

Introduction

The development of molecular methods has facilitated the identification and classification of non-tuberculous mycobacteria that cause infectious diseases in humans and animals [7, 19]. Currently, there are 162 species and subspecies of non-tuberculous mycobacteria (http://www.bacterio.cict.fr), of which around half are considered to be potential pathogens. Among the non-tuberculous mycobacteria, *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium marinum* have been frequently associated with fish diseases [4, 5].

We have recently isolated an acid-fast bacterium from a captive seahorse (*Hippocampus guttulatus*) with tail rot, which had been maintained in captivity as previously described [14]. Based on the phylogenetic analysis, strain BFLP-6^T was preliminarily classified as "*Mycobacterium hippocampi"* [2]. In this study, we describe a polyphasic characterization and present a description of the species in accordance with the requirements established for the description of novel species in the genus *Mycobacterium* [11].

Materials and Methods

Bacterial strain and phenotypic characterization

Strain BFLP-6^T was grown on Lowenstein-Jensen medium supplemented with 1.5 % NaCl (w/v) at 25 ºC for 5 days. Acid-alcohol-fastness was determined by Ziehl-Neelsen staining. Cell morphology and motility were studied using phase-contrast microscopy and electron microscopy as previously described [3]. NaCl growth tolerance and requirements were investigated by using nutrient broth [0.5% peptone from casein, 0.3% meat extract, 0.3% yeast extract, and adjusted to pH 7.0] supplemented with various concentrations of NaCl (0–15% at intervals of 1%).

The pH range for growth was determined in marine broth (Difco) that was adjusted to various pH values with acetic acid-sodium acetate (pH 4.0-4.5, 100 mM), MES (pH 5.0-6.0, 50 mM), MOPS (pH 6.5, 50 mM), Tris (pH 7.0-9.0, 50 mM) or CHES (pH 9.5-10.0, 50 mM) buffers. The temperature range for growth was determined in marine broth incubated between 10 and 40 ºC at intervals of 5 ºC. Anaerobic growth was assessed at 25 ºC in anaerobic chambers with an H_2/CO_2 atmosphere.

Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2; oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. Some physiological characteristics were performed using API 20NE and API ZYM (bioMérieux). Cells for inoculation of the strips were grown for 5 days at 25 ºC on marine agar (Difco) and results were visually interpreted according to the manufacturer's instructions. Resistance to isoniazid (10 µg/ml), thiophene-2-carboxylic hydrazide (2.0 µg/ml), hydroxylamine (0.5 mg/ml), thiacetazone (10 µg/ml), picrate (1.0 mg/ml), ciprofloxacin (5.0 µg/ml), clarithromycin (15 µg/ml), and rifampin (5.0 µg/ml) was determined on Lowenstein-Jensen medium according to previously published standard methods [11, 20].

For base composition analysis, genomic DNA was extracted as previously described [3], and the G+C content was determined spectrophotometrically by using the thermal denaturation method as described by Mandel et al. [12].

Chemotaxonomic analyses

Whole-cell fatty acids from the isolate were extracted from biomass grown on MB medium (DSMZ Medium 924) and analysed according to the standard protocol of the Sherlock Microbial Identification System version 4.5 (MIDI). Mycolic acid analyses by thin-layer chromatography (TLC) were performed with whole cell methanolysates from freeze-dried cells as described by Springer et al. [16]. Fatty acid methyl esters were

obtained from cells after saponification, methylation and extraction as described by Schröder et al. [15].

Phylogenetic analysis

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA, *ssrA* (encoding transfer-mRNA), *rpoB* (encoding the β-subunit of RNA polymerase) and *tuf* (encoding elongation factor Tu) genes were carried out as described previously [1, 3, 13]. The sequences obtained were compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLAST program and the Eztaxon-e database [8]. Phylogenetic analysis was performed using the software MEGA version 5.0 [18]. Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-likelihood methods were determined by using bootstrap values based on 1,000 replications.

Results and Discussion

Strain BFLP-6^T was found to consist of Gram-positive-staining, aerobic, acid-alcohol-fast, non-motile and non-sporulating cells. A scanning electron micrograph revealed that strain BFLP-6^T is irregular, rod-shaped, approximately 1.2–1.4 µm in length and 0.4 µm in diameter (Fig. 1). Colonies on Lowenstein-Jensen medium were irregular, rough and orange in colour after incubation at 25 ºC for 5 days. All colonies were scotochromogenic with orange pigmentation. BFLP-6^T also showed growth on marine agar, forming orange colonies after 6 days at 25 ºC. The strain grew with 0–6 % (w/v) NaCl (optimum 2%), but not with 7.0 % NaCl. Growth was observed at pH 5.0–10.0 (optimum pH 7.0). Strain BFLP-6^T showed resistance to isoniazid, thiophene-2-carboxylic hydrazide, hydroxylamine, thiacetazone, picrate. However, the strain showed

susceptibility to ciprofloxacin, clarithromycin, and rifampin. Other phenotypic characteristics of strain BFLP-6^T are shown in Table 1.

The G+C content was calculated to be 66.7 mol%. This value is within the range for the genus *Mycobacterium* [17]. Fatty acid analysis showed straight-chain saturated and unsaturated fatty acids, as expected for a member of the genus *Mycobacterium* [9]. Major fatty acids included $C_{18:1}\omega9c$ (30.3 %), $C_{16:0}$ (21.6 %) and summed feature 2 (comprising $C_{17:1}\omega7c$; 14.6 %). Moderate amounts of $C_{16:1}\omega6c$ (9.9 %), 10-methyl $C_{18:0}$ (4.4 %) and $C_{14:0}$ (4.1 %), and smaller amounts of $C_{16:1}\omega9c$ (3.5 %), $C_{18:0}$ (2.5 %), $C_{18:2}\omega6,9c$ (1.3 %), $C_{16:1}\omega7c$ (0.7 %), $C_{15:0}$ (0.3 %), $C_{17:0}$ (0.3 %) and $C_{12:0}$ (0.2 %) were also detected. Alpha-mycolates and keto-mycolates were detected in BFLP-6^T, whereas *M. flavescens* DSM 43991^T and *M. novocastrense* DSM 44203^T showed a different mycolic acid pattern, in which alpha-mycolates, keto-mycolates and wax-ester mycolates, i.e. carboxy mycolates and 2-eicosanol and homologous alcohols, were found.

The results of the phylogenetic analysis based on the 16S rRNA gene clearly showed that strain BFLP-6^T belonged to the genus *Mycobacterium*. The closest relatives were *M. flavescens* ATCC 14474^T (98.3 % similarity), *M. goodii* ATCC 700504^T (98.0 % similarity), *M. duvalii* ATCC 43910^T and *M. novocastrense* 73^T (97.9 % similarity), and *M. gilvum* ATCC 43909^T (97.8 % similarity). The phylogenetic trees based on the neighbour-joining and maximum-likelihood methods showed that strain BFLP-6^T formed a cluster with the type strain of *Mycobacterium novocastrense* 73^T (Fig. 2). Previous studies, based on 16S rRNA gene sequence analysis, have demonstrated that some *Mycobacterium* species have a very high degree of similarity or have exactly identical sequences [6]. In order to overcome this issue, concatenated alignments from housekeeping genes have been used to define phylogenetic relationships of several

Mycobacterium species [6, 10]. In this study, the phylogenetic tree based on concatenated sequences of the 16S rRNA gene and the three housekeeping genes (*rpoB*, *ssrA* and *tuf*) showed that strain BFLP-6^T formed a long phylogenetic interspecies branch, which was clearly separated from all the other *Mycobacterium* species (Fig. 3). All independent neighbour-joining analyses based on each single gene fragments of *rpoB* (722 bp) and *ssrA* (308 bp) also showed that strain BFLP-6^T formed an independent branch, except for the *tuf* gene (Supplementary Fig. S1–S3). Therefore, the results from the current study suggest that strain BFLP-6^T is distinct from any recognized species of the genus *Mycobacterium*.

The additional information gained on the phenotypic, chemotaxonomic and phenotypic properties of strain BFLP-6^T support its description as a novel species within the genus *Mycobacterium*.

Description of *Mycobacterium hippocampi* sp. nov.

Mycobacterium hippocampi (hip.po.cam'pi. L. gen. n. *hippocampi*, of the seahorse).

Cells are irregular, rod-shaped, 0.4 × 1.2–1.4 μm, Gram-positive, nonmotile, aerobic, acid-alcohol-fast and non-sporulating. Colonies on Lowenstein-Jensen medium supplemented with 1.5% (w/v) NaCl are orange coloured, circular and 1.5–2.0 mm in diameter. Optimum growth temperature is 25 °C. No growth occurs below 15 °C or above 35 °C. Growth occurs at pH 5.0–10.0. Growth occurs at NaCl concentrations between 0 and 6 % (w/v), but not in the presence of 7 % (w/v) NaCl. Positive for catalase; glucose fermentation; arginine dihydrolase; urease; aesculin; assimilation of glucose, mannitol, potassium gluconate and malate. Negative for nitrate reduction to nitrite; oxidase; indole production; gelatine hydrolysis; *N*-acetyl-D-glucosamine; assimilation of arabinose, mannose, maltose, caprate, adipate, citrate and phenyl-acetate. API ZYM tests show activities for alkaline phosphatase, esterase (C4), esterase

lipase (C8), lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-glucosidase and *β*-glucosidase. Trypsin, *α*-chymotrypsin, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase activities are not observed. The major fatty acids are $C_{18:1}\omega 9c$, $C_{16:0}$ and summed feature 2 (comprising $C_{17:1}\omega 7c$). Mycolic acids include alpha-mycolates and keto-mycolates. The G+C content of the type strain is 66.7 mol%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB*, *ssrA* and *tuf* gene sequences of strain BFLP-6^T are FN430736, FR775976, HF912158 and HF912159, respectively. The type strain, BFLP-6^T (=DSM 45391^T =LMG 25372^T), was isolated from a seahorse with tail rot.

Acknowledgements

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Table 1. Characteristics of strain BFLP-6^T and some related *Mycobacterium* species.

Fig. 1. Scanning electron micrograph of strain BFLP-6^T showing a rod-shaped morphology (0.4 × 1.2–1.4 μm). Bar, 0.5 μm.

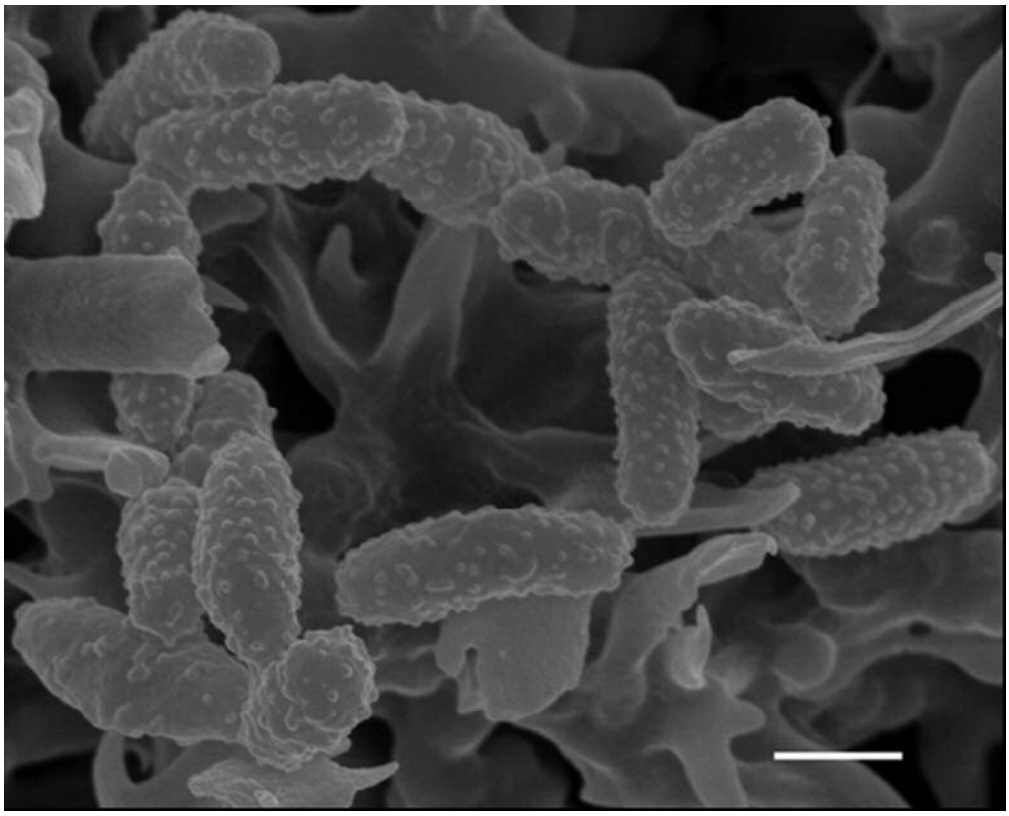
Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel bacterium (in bold) within the genus *Mycobacterium*. This tree combines the results of both the neighbour-joining (NJ) and maximum-likelihood (ML) methods. The topology shown was obtained by using the NJ method. Bootstrap values (>50%) at the nodes (NJ/ML) are expressed as a percentage of 1,000 replications. *Nocardia farcinica* IFM 10152 was used as an outgroup. Bar, 0.002 substitutions per nucleotide position.

Fig. 3. Phylogenetic tree of strain BFLP-6^T (in bold) and closely related *Mycobacterium* species based on 16S rRNA, *rpoB*, *ssrA* and *tuf* gene sequences. This tree combines the results of both the neighbour-joining (NJ) and maximum-likelihood (ML) methods. The topology shown was obtained by using the NJ method. Bootstrap percentages (>50%) based on 1,000 replications are shown at branch nodes (NJ/ML). *Nocardia farcinica* IFM 10152 was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics of strain BFLP-6^T and some related *Mycobacterium* species

Characteristic	1	2	3	4	5
Growth at 42°C	–	+	w	+	w
Nitrate reduction	–	+	+	+	+
Production of:					
Alkaline phosphatase	+	+	–	+	+
Leucine arylamidase	+	+	+	+	–
Valine arylamidase	+	+	+	+	–
Cystine arylamidase	+	+	+	+	–
α-chymotrypsin	–	–	+	–	–
β-galactosidase	–	–	–	–	+
α-glucosidase	+	–	+	+	–
β-glucosidase	+	–	–	+	–
Trypsin	–	+	+	+	–
Urease	+	–	+	+	+

Strains: 1, *Mycobacterium hippocampi* sp. nov. BFLP-6^T; 2, *M. flavescens* DSM 43991^T; 3, *M. duvalii* DSM 44244^T; 4, *M. novocastrense* DSM 44203^T; 5, *M. gilvum* DSM 44503^T. Abbreviations: +, Positive; –, negative; w, weakly positive. All data are from this study.



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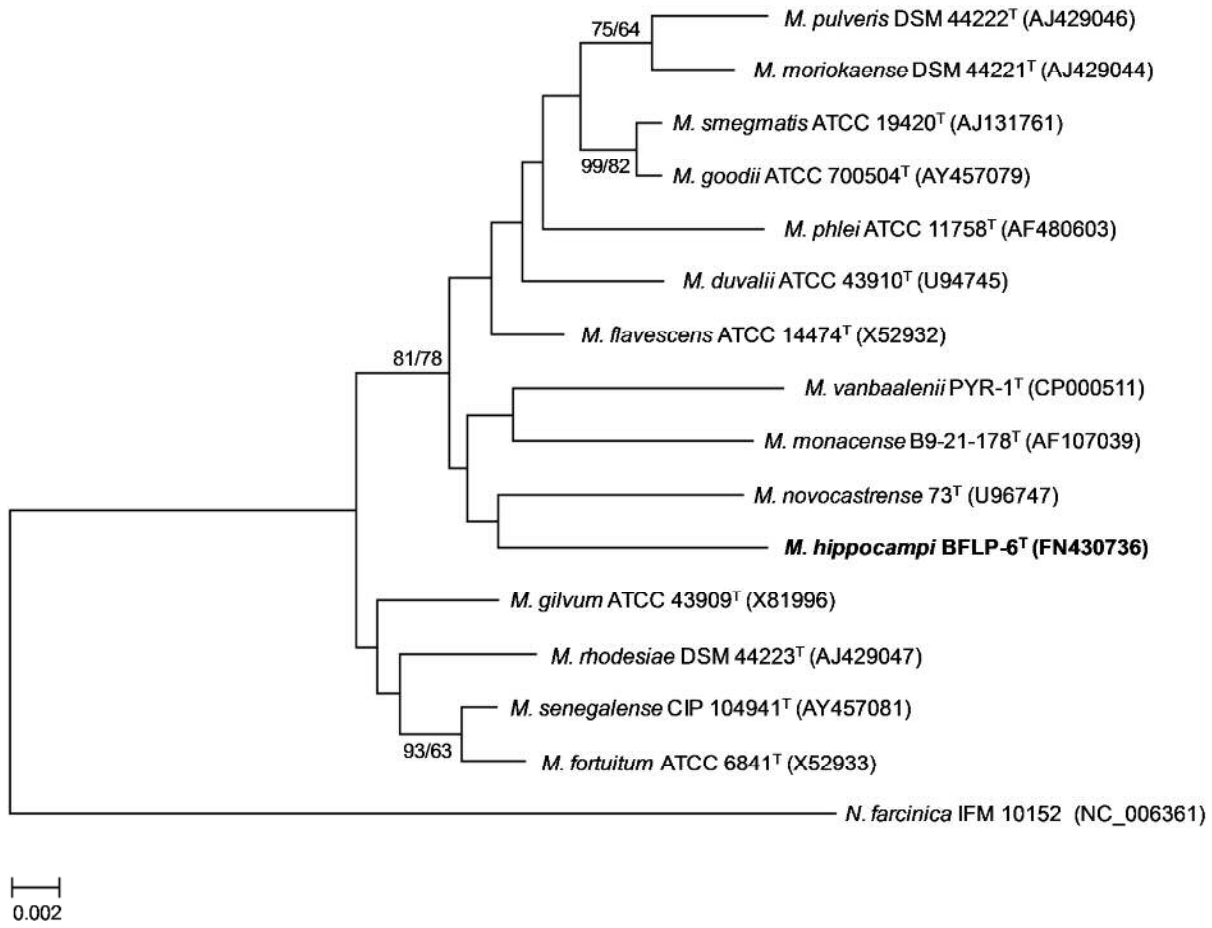

Figure 2. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the novel bacterium (in bold) within the genus *Mycobacterium*. This tree combines the results of both the neighbour-joining (NJ) and maximum-likelihood (ML) methods. The topology shown was obtained by using the NJ method. Bootstrap values (>50%) at the nodes (NJ/ML) are expressed as a percentage of 1,000 replications. *Nocardia farcinica* IFM 10152 was used as an outgroup. Bar, 0.002 substitutions per nucleotide position.

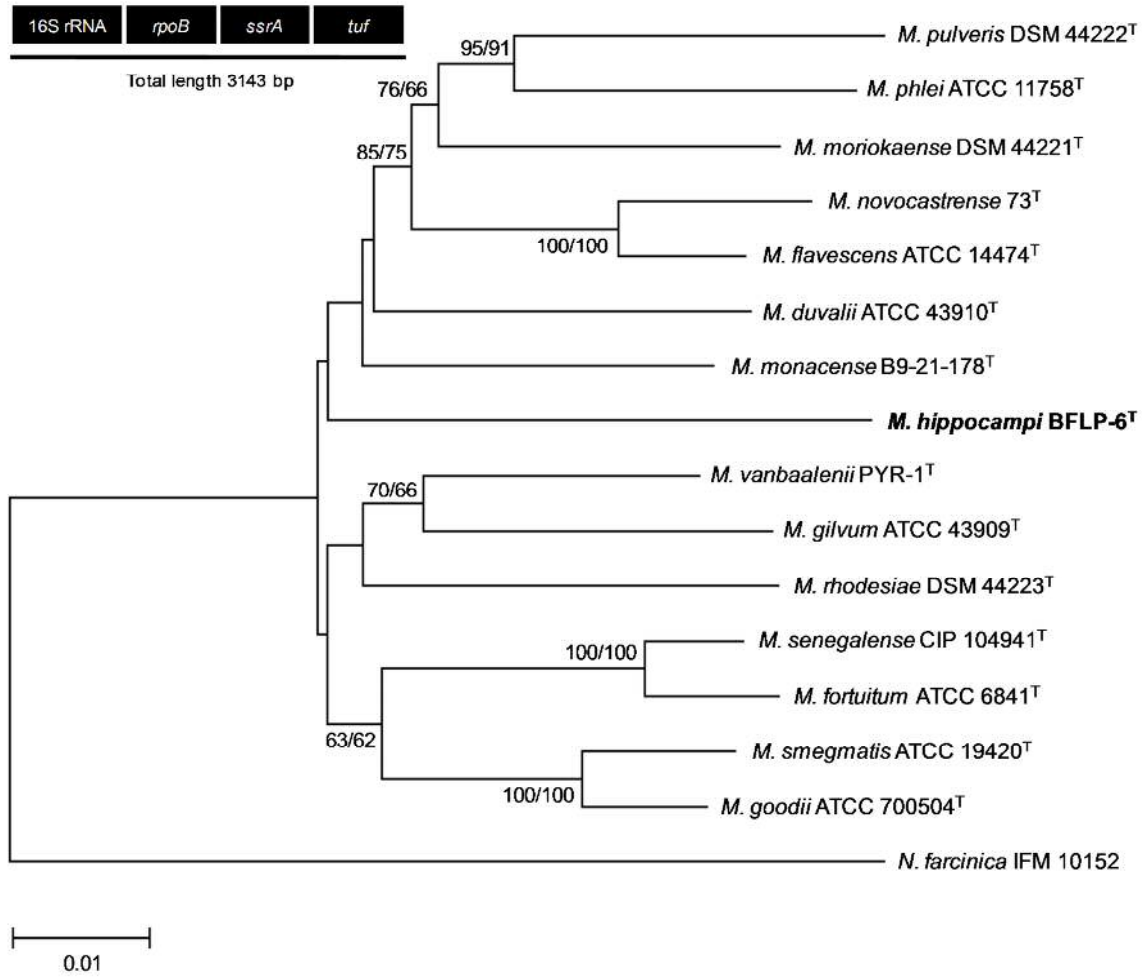

Figure 3. Phylogenetic tree of strain BFLP-6^T (in bold) and closely related *Mycobacterium* species based on 16S rRNA, *rpoB*, *ssrA* and *tuf* gene sequences. This tree combines the results of both the neighbour-joining (NJ) and maximum-likelihood (ML) methods. The topology shown was obtained by using the NJ method. Bootstrap percentages (>50%) based on 1,000 replications are shown at branch nodes (NJ/ML). *Nocardia farcinica* IFM 10152 was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

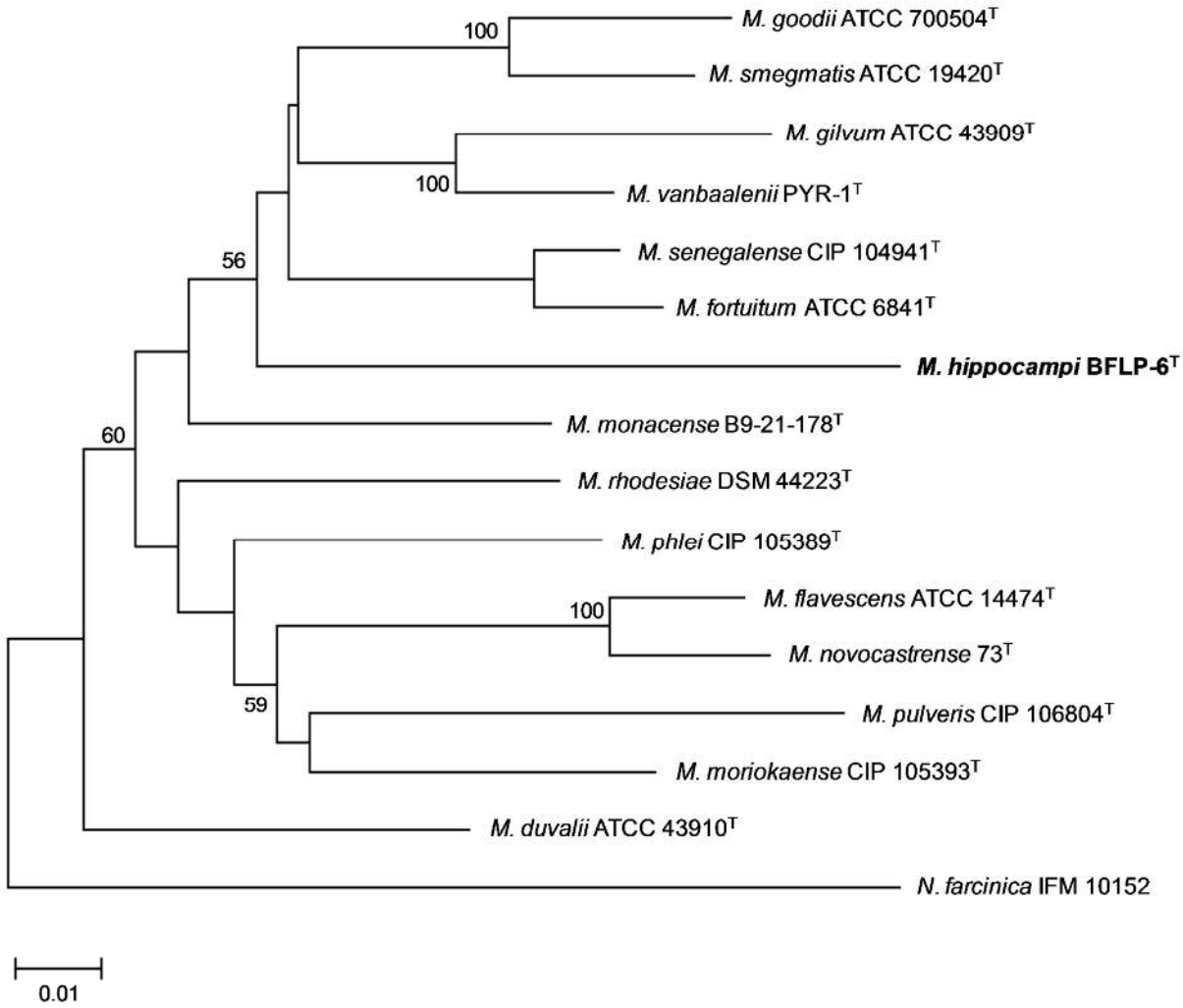

Fig. S1. Neighbour-joining phylogenetic tree based on *rpoB* gene sequences. *Nocardia farcinica* IFM 10152 was used as an outgroup. Bootstrap values (>50%) at the nodes are expressed as a percentage of 1,000 replications. Bar, 0.01 substitutions per nucleotide position.

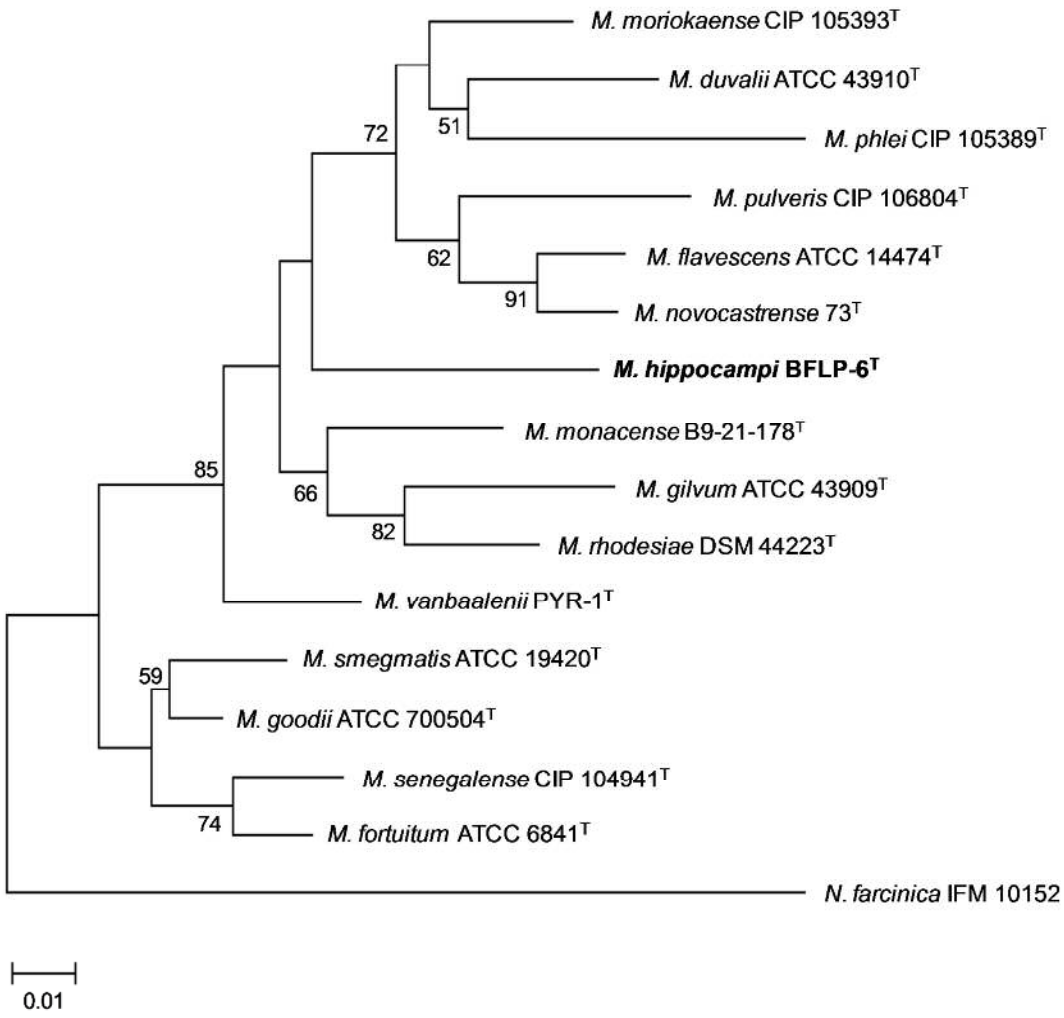

Fig. S2. Neighbour-joining phylogenetic tree based on *ssrA* gene sequences. *Nocardia farcinica* IFM 10152 was used as an outgroup. Bootstrap values (>50%) at the nodes are expressed as a percentage of 1,000 replications. Bar, 0.01 substitutions per nucleotide position.

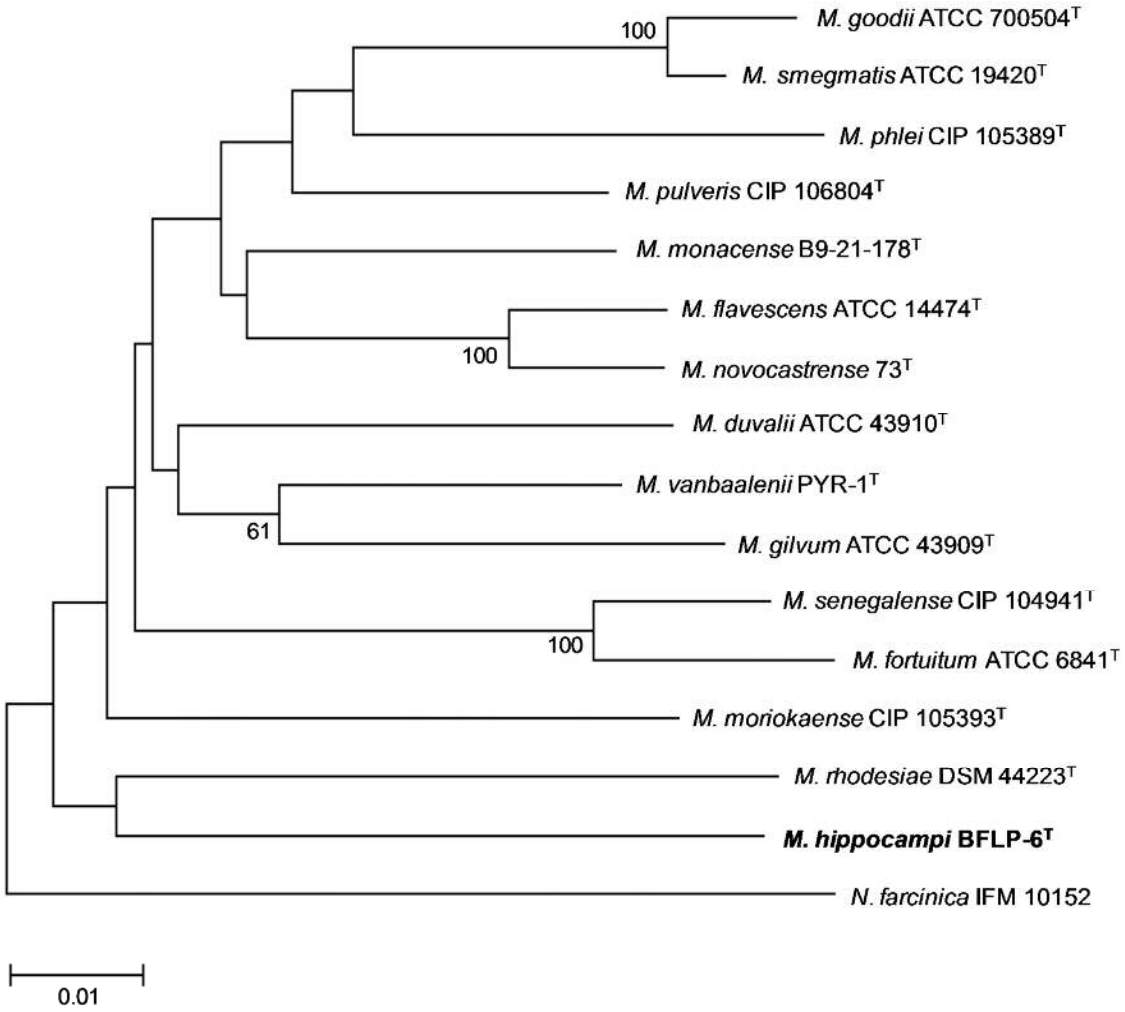

Fig. S3. Neighbour-joining phylogenetic tree based on *tuf* gene sequences. *Nocardia farcinica* IFM 10152 was used as an outgroup. Bootstrap values (>50%) at the nodes are expressed as a percentage of 1,000 replications. Bar, 0.01 substitutions per nucleotide position.