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Mycobacterium alsense sp. nov., a scotochromogenic slow grower isolated from clinical respiratory specimens

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The name '*Mycobacterium alsiense*', although reported in 2007, has not been validly published. Polyphasic characterization of three available strains of this species led us to the conclusion that they represent a distinct species within the genus *Mycobacterium*. The proposed novel species grows slowly and presents pale yellow-pigmented colonies. Differentiation from other mycobacteria is not feasible on the basis of biochemical and cultural features alone while genetic analysis, extended to eight housekeeping genes and one spacer region, reveals its clear distinction from all other mycobacteria. *Mycobacterium asiaticum* is the most closely related species on the basis of 16S rRNA gene sequences (similarity 99.3 %); the average nucleotide identity between the genomes of the two species is 80.72 %, clearly below the suggested cut-off (95–96 %). The name *Mycobacterium alsense* sp. nov. is proposed here for the novel species and replaces the name '*M. alsiense*', ex Richter *et al.* 2007, given at the time of isolation of the first strain. The type strain is TB 1906^T (=DSM 45230^T=CCUG 56586^T).

Abbreviation: ANI, average nucleotide identity.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TB 1906^T and FI-02209 are AJ938169 and KT168286, respectively; those for *hsp65* gene sequences are FM209298 and DQ381733, respectively; and those for the ITS sequences are AJ938170 and KT168286, respectively. Accession numbers for the *rpoB* gene sequences of TB 1906^T and BF431DRS are KT168292 and KT168288, respectively; those for the *gyrB* gene sequences are KT168295 and KT168296, respectively; those for the *sodA* gene sequences are KT168300 and KT168301, respectively; those for *dnaK* gene sequences are KT168303 and KT168304, respectively; those for the *secA1* gene sequences are KT168307 and KT168309, respectively; and those for the *rpoBC* gene sequences are KT168314 and KT168316, respectively. Accession numbers for the *secA1* and *rpoBC* gene sequences of strain FI-02209 are KT168308 and KT168315, respectively. The accession number for the *hsp65* gene sequence of strain BF431DRS is KT168293.

Nine supplementary figures are available with the online Supplementary Material.

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In 2007 two strains of an uncommon mycobacterium were isolated in Europe from clinical specimens and named '*Mycobacterium alsiense*' (Richter *et al.*, 2007). Since then, no other isolation has been reported and a description of the novel species has not been validly published. Following the recent isolation in Africa of an additional strain we performed an in-depth characterization of the three strains and we propose here the name *Mycobacterium alsense* sp. nov. to accommodate them.

The three mycobacterial strains were isolated from the sputum of patients with pulmonary disorders. The first strain (TB 1906^T) was repeatedly cultured, in Denmark, from a patient with bilateral lobe infiltrates; the second one (FI-02209) was grown in Italy from a patient with haemoptysis; and the third strain (BF431DRS) was isolated, in Burkina Faso, from a patient classified with drug-resistant tuberculosis on the basis of the persistent presence of acid-fast bacilli in the sputum, despite a completed cycle of first-line anti-tuberculosis treatment.

The three strains grew on solid media, both at 25 and at 37 °C, in about 3 weeks, developing tiny smooth colonies, pale yellow-pigmented, both in the light and in the dark. No growth was observed at 45 °C. All were positive at 68 °C for catalase but negative for other commonly tested biochemical features (niacin accumulation, nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, 3 day arylsulfatase, urease and *β*-glucosidase) (Kent & Kubica, 1985). Growth was seen on media supplemented with thiophene-2-carboxylic acid hydrazide (1 μg ml^{-1}), *p*-nitrobenzoic acid (500 μg ml^{-1}), thiacetazone (10 μg ml^{-1}), isoniazid (1 μg ml^{-1}) and oleate (250 μg ml^{-1}) but not on MacConkey agar without crystal violet or on Lowenstein–Jensen medium supplemented with 5 % NaCl. As expected, due to the high number of species present in the genus *Mycobacterium*, this biochemical and cultural pattern was not unique and therefore cannot be used for the differentiation from other mycobacteria with similar characteristics. Of the genetically more closely related species *Mycobacterium asiaticum* and *Mycobacterium interjectum*, only the first, which is photochromogenic and positive for Tween 80 and semi-quantitative catalase, can be easily differentiated.

With a commercial line probe assay (GenoType CM; Hain Lifesciences) (Richter *et al.*, 2006) the test strains hybridized with probes 10 and 11 and, weakly, with probe 9. The interpretation rules for this assay, recommending to take into account sharp hybridization lines only, do not provide details for such a pattern while they assign the combination of lines 9, 10 and 11 to the species *M. interjectum*.

HPLC analysis of cell-wall mycolic acids, performed as reported by the Centers for Disease Control (1996), produced a pattern characterized by a major cluster of peaks eluting after 8 min. A similar pattern is shared by a number of mycobacterial species with the closest similarity, as far as number and retention times of peaks are concerned, presented by *Mycobacterium palustre* (Fig. 1).

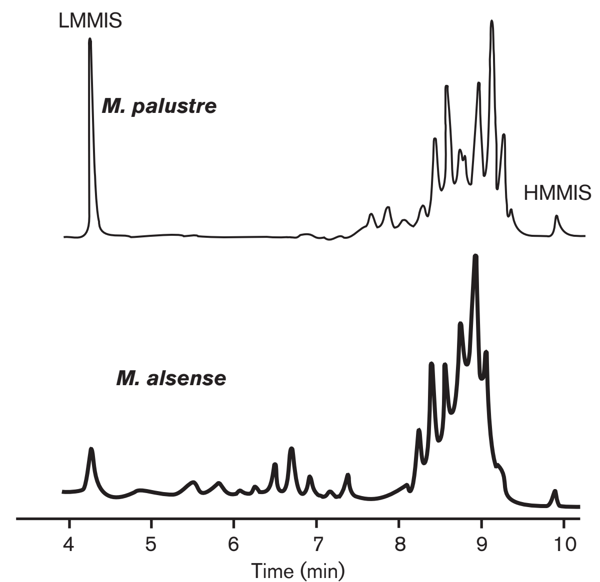

Fig. 1. Representative mycolic acid patterns of strain TB1906^T and *M. palustre* DSM 44572^T. LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard.

MICs of drugs potentially active against slowly growing mycobacteria were determined in liquid medium (Clinical & Laboratory Standards Institute, 2011) using commercially available microdilution plates (SLOMYCOI; Sensititer). The strains were susceptible to amikacin, ciprofloxacin, clarithromycin, linezolid, moxifloxacin and rifabutin. Results variable among the strains were obtained for doxycycline, ethambutol, rifampicin, streptomycin and sulfamethoxazole.

dsDNA sequences from all the strains included in the study were determined using BigDye Terminator chemistry on an ABI 3730 DNA sequencer (Applied Biosystems) following the standard protocol of the supplier. The regions investigated included the almost-complete 16S rRNA gene (Kirschner *et al.*, 1993), the complete internal transcribed spacer between 16S and 23S rRNA (ITS1) (Roth *et al.*, 1998), and parts of genes encoding 65 kDa heat-shock protein (*hsp65*) (McNabb *et al.*, 2006), RNA polymerase *β* subunit (*rpoB*) (Adékambi *et al.*, 2003), RNA polymerase *β*' subunit (*rpoBC*) (Dai *et al.*, 2011), superoxide dismutase (*sodA*) (Zolg & Philippi-Schulz, 1994), DNA gyrase *β* subunit (*gyrB*) (Gomila *et al.*, 2007), molecular chaperone DnaK (*dnaK*) and preprotein translocase subunit secA (*secA1*) (Dai *et al.*, 2011). Microheterogeneity was detected in seven genetic targets with the strains presenting the same sequence in 16S rRNA and ITS1 only (Table 1). In the almost-complete 16S rRNA gene (1497 bp), *M. asiaticum* was the most closely related species. *M. interjectum* was, by contrast, the species with the highest similarity in four of the genes mentioned above (Table 2).

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Table 1. Microheterogeneity presented by *M. alsense* sp. nov. in different genetic regions

The number of mismatches/number of sequenced base pairs (percentage similarity) is given for the other strains in comparison with the type strain, TB 1906^T.

Genetic region	Strain	
	FI-02209	BF431DRS
16S rRNA	0/1497 (100)	0/1497 (100)
ITS1	0/301 (100)	0/301 (100)
hsp65	0/371 (100)	2/371 (99.5)
rpoB	0/719 (100)	4/719 (99.4)
rpoBC	1/478 (99.8)	4/478 (99.2)
sodA	0/389 (100)	4/389 (99.0)
gyrB	0/281 (100)	10/281 (96.4)
dnaK	0/450 (100)	5/450 (98.9)
secA1	2/482 (99.6)	8/482 (98.3)

Due to high similarity (99.3 %) in the 16S rRNA gene between the proposed type strain of the novel species and that of the most closely related species (*M. asiaticum*), the whole genomes of the respective type strains were sequenced to calculate the average nucleotide identity (ANI) between them. Paired-end libraries of read length 93 bp were prepared from strain TB 1906^T and *M. asiaticum* IP108 0001^T using Nextera XT DNA Sample Preparation kit and Nextera XT Index kit (Illumina) according to the manufacturer's protocol. Libraries were then normalized to 2 nM, pooled for multiplexing in equal volumes and sequenced at 9 pM on an Illumina HiSeq 2500 platform, high output run mode, with 93 nt paired end reads to achieve a coverage >100 per base. Read tags were processed and assembled with Velvet software (Zerbino & Birney, 2008) supported by VelvetOptimizer (https://github.com/tseemann/Velvet Optimiser) for optical setting of parameters. The resulting contigs (with *N*50 values (50% average length) of 46 190 and 23 719 bp, respectively) were used to calculate the ANI between the respective core genomes, using the software available at http://enve-omics.ce.gatech.edu/ani/ (Konstantinidis & Tiedje, 2005). The resulting value was 80.72 %, clearly below the accepted cut-off 95–96 % (Kim *et al.*, 2014) (Fig. S1, available in the online Supplementary Material).

Table 2. Highest sequence similarities between *M. alsense* sp. nov. and other *Mycobacterium* species in different genetic regions

Genetic region	Closest species	Sequence similarity (%)
16S rRNA	*M. asiaticum*	99.3
ITS1	*M. interjectum*	89.9
hsp65	*M. parmense*	97.3–97.8
rpoB	*M. interjectum*	94.3
rpoBC	*M. interjectum*	93.9–94.3
sodA	*M. interjectum*	91.2–91.5
gyrB	*M. agri*	90.0–91.8
dnaK	*M. hiberniae*	92.9–93.1
secA1	*M. asiaticum*	94.4–94.9

PCR restriction analysis patterns (Telenti *et al.*, 1993) were deduced on the basis of restriction sites present in the *hsp65* gene sequences. No restriction site for *BstE*II was detected in any of the three strains. By contrast, the restriction enzyme *Hae*III produced fragments of 214 and 127 bp, a pattern not shared by any known mycobacterium (http://app.chuv.ch/prasite/index.html).

For phylogenetic analysis, the sequences of the type strains of the most closely related species were retrieved from the GenBank database, aligned using CLUSTAL W software (Thompson *et al.*, 1994) and trimmed to start and finish at the same position. The analysis was conducted for each investigated genetic region and *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. The neighbour-joining method (Saitou & Nei, 1987), supported by the MEGA 6 software (Tamura *et al.*, 2013), was used for the reconstruction of phylogenetic trees; 1000 bootstrap replications were implemented. All the phylogenetic trees confirmed that the three test strains belonged to a single, highly robust, branch. Most of the trees (Figs S2–S9) were characterized by low to moderate robustness. In four of them (Figs S2, S3, S4 and S6) the proposed novel species appeared more closely related to a member of the *Mycobacterium simiae* complex (Tortoli *et al.*, 2011). In the others (Figs S5, S7, S8 and S9) the branch including the test strains was clearly separate from other mycobacteria. The tree reconstructed on the basis of the almost-complete 16S rRNA gene sequences (Fig. 2) had 57 % of the nodes with bootstrap values >50, with *M. asiaticum* being the closest species.

A further phylogenetic tree was built by concatenating the sequences of eight of the nine investigated genetic regions in a single 4604 bp long string (Fig. 3); *gyrB* was excluded because of the low number of species for which sequences were available in GenBank. In this tree, *M. interjectum* was the species most closely related to the test strains.

On the basis of the data presented, the three strains are considered to represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium alsense* sp. nov. is proposed.

Description of *Mycobacterium alsense* sp. nov.

Mycobacterium alsense (als.en′se. N.L. neut. adj. *alsense* of, or belonging to, the isle of Als, Denmark, where the type strain was isolated).

Non-motile, non-spore-forming and acid-fast. Visible growth requires more than 2 weeks of incubation at 25–37 °C to develop. On solid media colonies are small, smooth and pale-yellow-pigmented both in the light and

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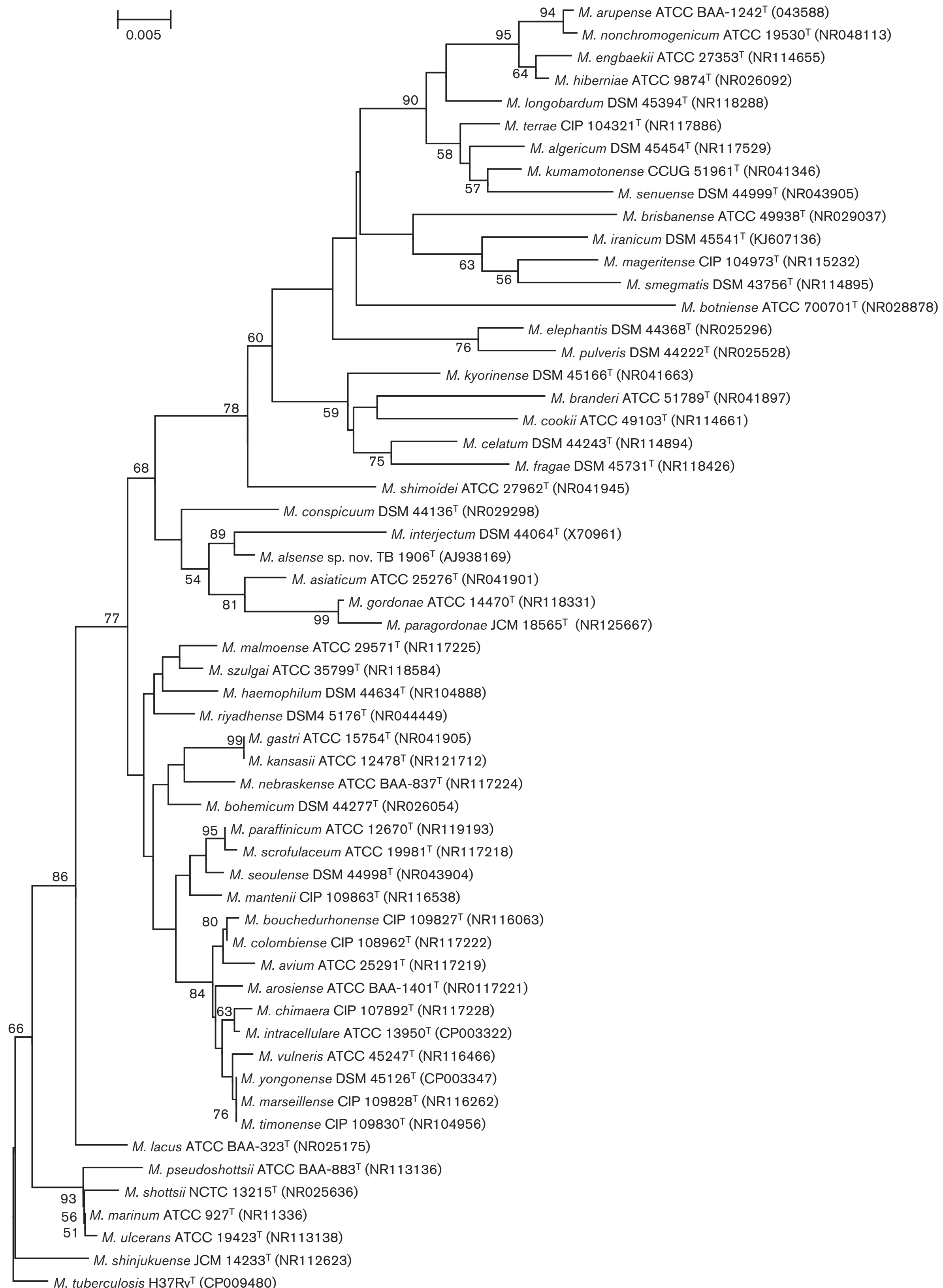

Fig. 2. Phylogenetic tree of representative species of the genus *Mycobacterium* based on 16S rRNA sequences, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.005 substitutions per nucleotide position.

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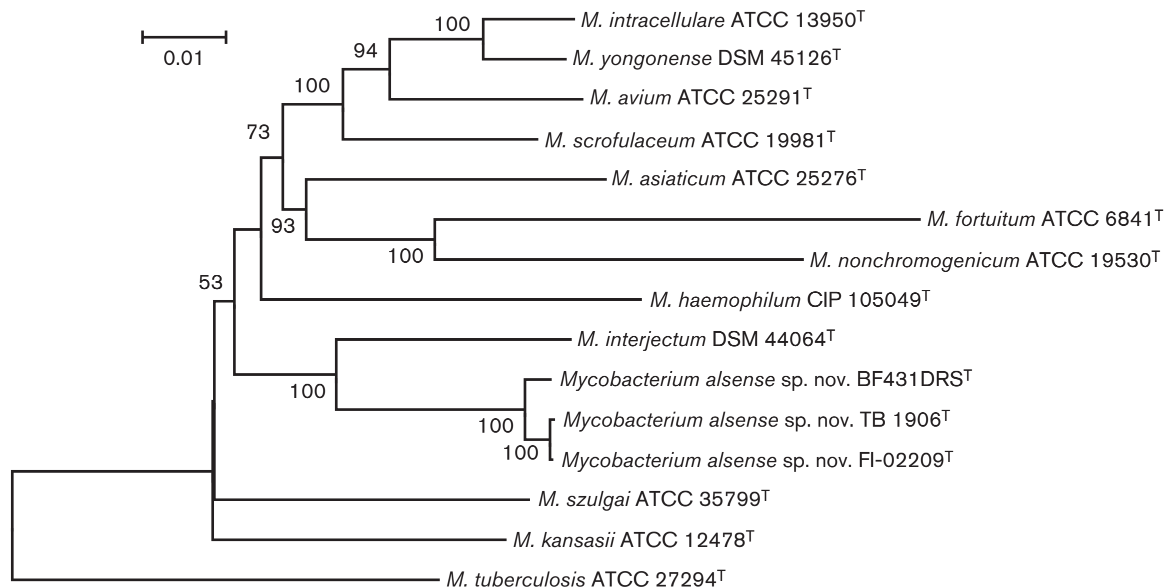

Fig. 3. Phylogenetic tree of representative species of the genus *Mycobacterium* based on concatenated sequences of 16S rRNA, ITS1, *hsp65*, *rpoB*, *rpoBC*, *secA1*, *sodA* and *dnaK* genes, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

in the dark. Heat-stable catalase test is positive while nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, 3 day arylsulfatase, urease and β-glucosidase are negative. Has a TLC pattern that is very common among mycobacteria, with α-, keto- and methoxy-mycolic acids (Richter *et al.*, 2007). The major lipid components, identified by GC/LC-MS, are $C_{16:0}$, $C_{18:1\omega9}$, tuberculostearic acid and $C_{18:0}$ (Richter *et al.*, 2007). Most closely related (99.3 % similarity) to *M. asiaticum* based on the 16S rRNA gene; the ANI (80.72 %) between their core genomes is, however, clearly supportive of the status of independent species.

The type strain, isolated from a clinical sample in Denmark, is TB 1906^T (=DSM 45230^T=CCUG 56586^T).

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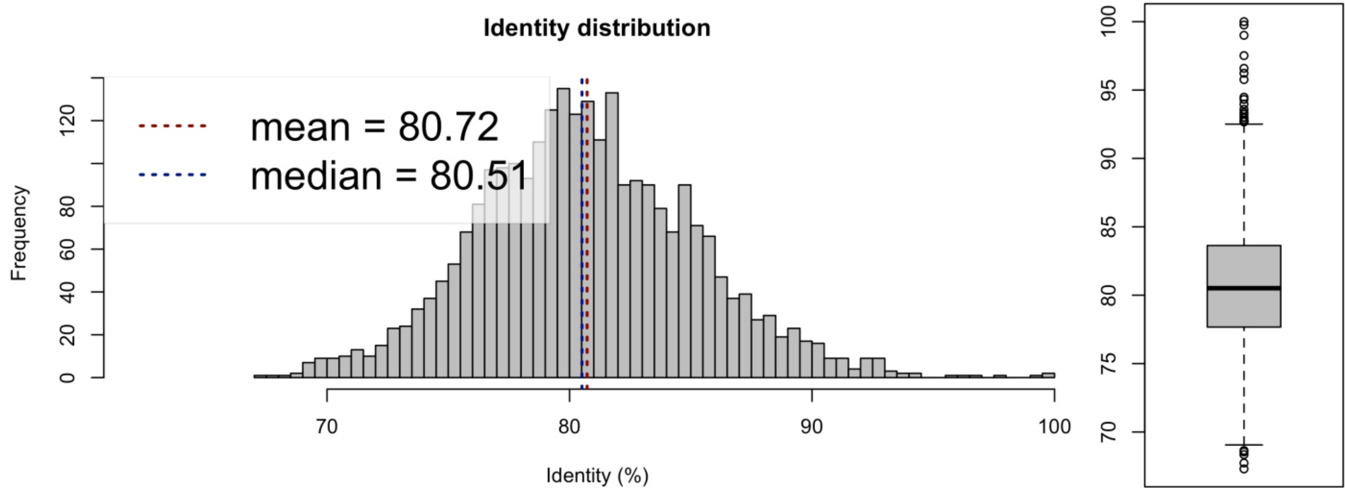
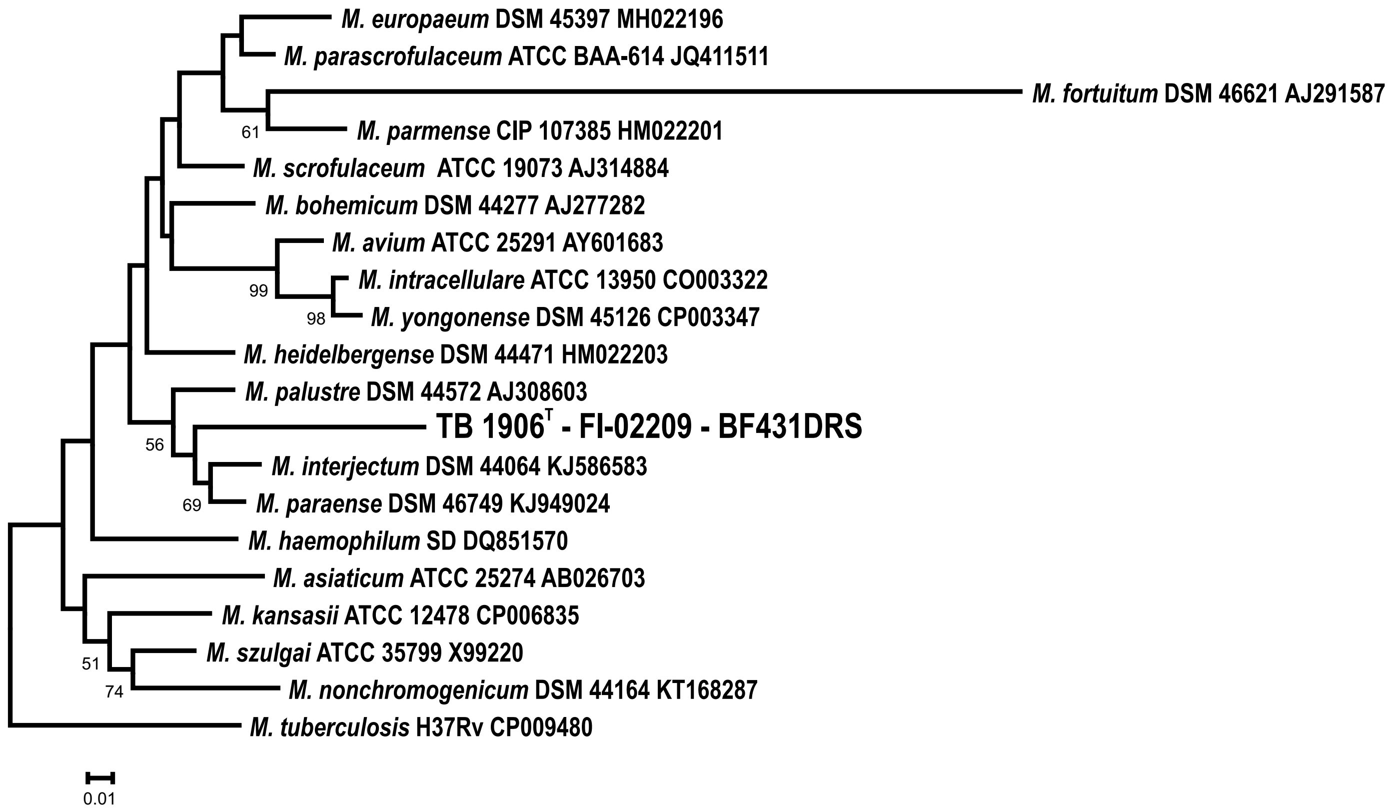


Supplementary figure S1. Average nucleotide identity between TB 1906^{T} and *Mycobacterium asiaticum*^{T}. The distribution of ANI is calculated using draft genomes obtained by assembly of short read tags.

Supplementary figure S2. Phylogenetic tree based on ITS1 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 S3. 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.

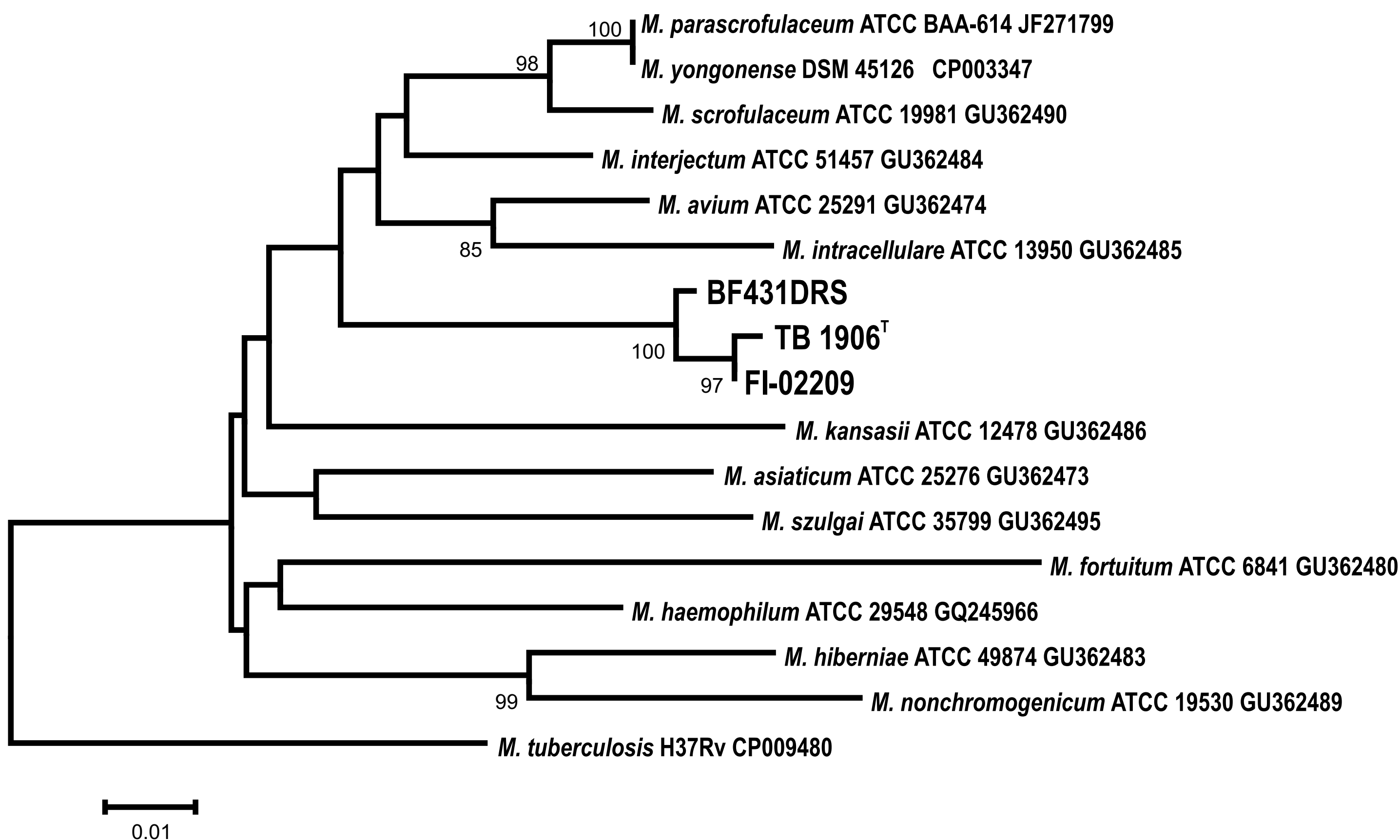
TB 1906^T - FI-02209
99
BF431DRS
71
M. parmense CIP 107385 HM022199
M. paraense DSM 46749 HM056137
M. palustre DSM 44572 AY943200
M. avium ATCC 25291 GQ153289
M. intracellulare ATCC 13950 CP003322
63
99
M. yongonense DSM 45126 CP003347
94
M. europaeum DSM 45397 HM022220
62
M. scrofulaceum ATCC 19981 AF434733
M. heidelbergense CIP 105424 AF547844
M. asiaticum DSM 44297 AF547806
M. szulgai ATCC 35799 AF434731
M. bohemicum CIP 105811 AF547811
78
M. interjectum DSM 44064 AF547846
M. parascrofulaceum ATCC BAA-614 AY337274
M. haemophilum CIP 105049 AF4547841
M. kansasii ATCC 12478 CP006835
M. agri CIP 105391 AY438080
M. obuense CIP 106803 AF547863
94
M. fortuitum DSM 46621 AY299152
56
M. hiberniae ATCC 49874 JF491297
99
M. nonchromogenicum DSM 44164 AF547861
M. tuberculosis H37Rv CP009480

0.01

Supplementary figure S4. Phylogenetic tree based on *rpoB* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

M. hiberniae ATCC 49874 JN571246
M. nonchromogenicum ATCC 19530 JN881351
M. fortuitum ATCC 6841 JF346874
M. asiaticum IP 1080001 KT168291
M. parmense CIP 107385 HM022211
M. haemophilum ATCC 29548 GQ246956
M. szulgai ATCC 29716 JN881348
M. avium ATCC 25291 GQ153306
M. intracellulare ATCC 13950 GQ153307
M. europaeum DSM 45397 HM022215
M. scrofulaceum ATCC 19981 EU591494
M. parascrofulaceum ATCC BAA-614 JQ411510
M. yongonense DSM 45126 JF738053
M. palustre DSM 44572 HM022210
M. kansasii ATCC 12478 HQ880687
M. interjectum DSM 44064 HM022207
M. paraense DSM 46749 KJ949010
TB 1906^T - FI-02209
BF431DRS
M. tuberculosis H37Rv CP009480
100
82
62
74
56
88
100
100
94
100
0.01

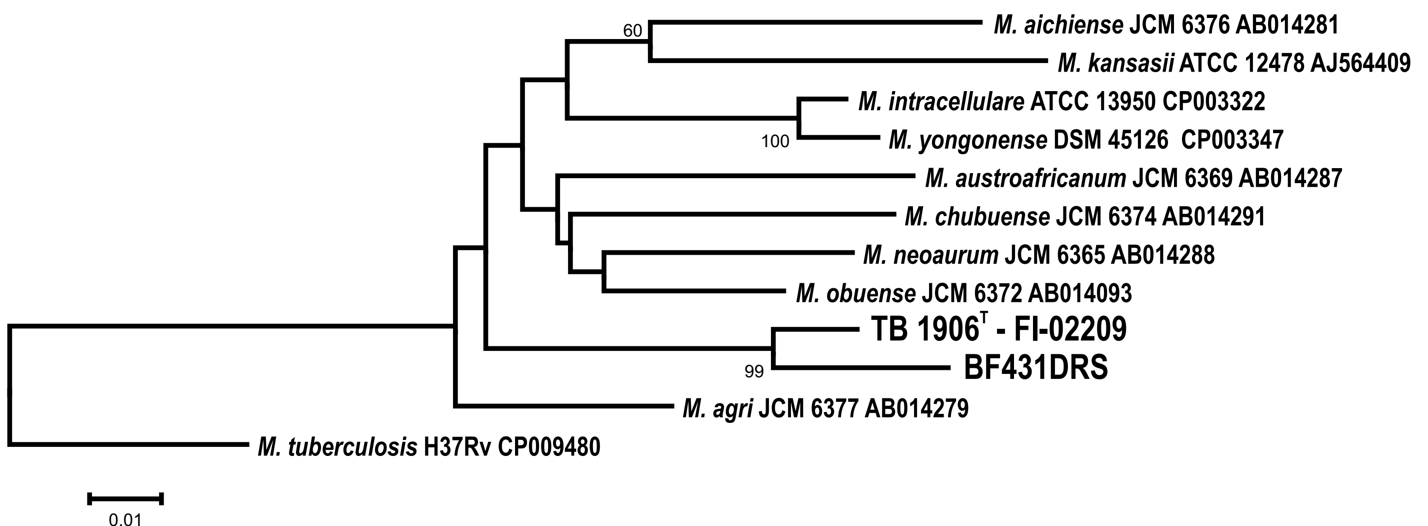
Supplementary figure S5. Phylogenetic tree based on *rpoBC* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.



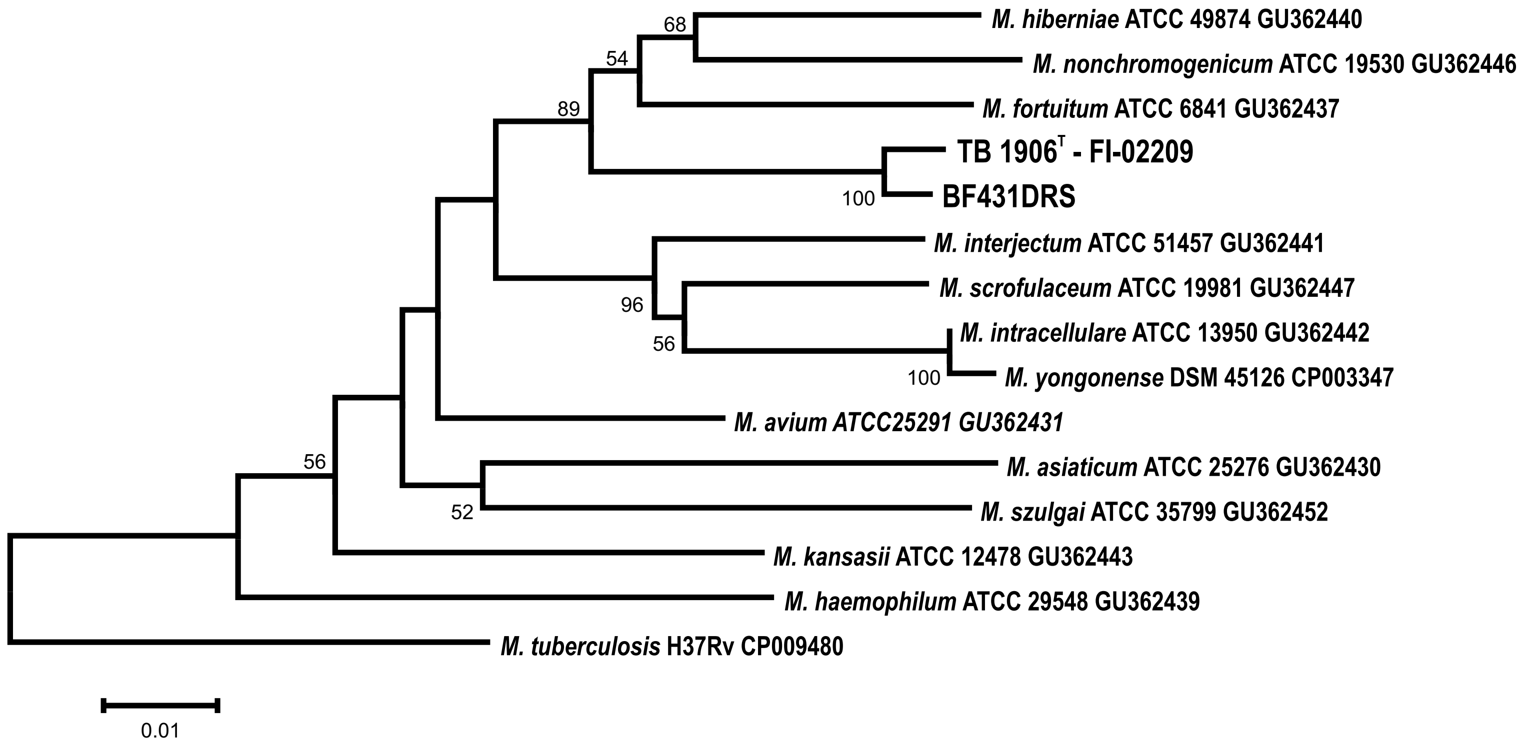
Supplementary figure S6. Phylogenetic tree based on *sodA* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

M. agri CIP 105391 AY544797
M. fortuitum CIP 104534 AY458114
M. asiaticum DSM 44297 AY544800
M. obuense CIP 106803 AY544853
M. scrofulaceum CIP 105416 AY544860
M. avium ATCC 25291 EU409983
M. intracellulare ATCC 3950 JQ411518
M. yongonense DSM 45126 JQ937023
M. hiberniae DSM 44241 AY544832
M. nonchromogenicum DSM 44164 AY544851
M. heidelbergense CIP 105424 AY544831
M. palustre DSM 44572 AY943180
M. haemophilum CIP 105049 AY544828
M. europaeum DSM 45397 HG673732
M. parascrofulaceum ATCC BAA-614 JQ411517
M. parmense CIP 107385 AY943182
M. bohemicum CIP 105811 AY544805
M. interjectum DSM 44064 AY544835
TB 1906^T - FI-02209
BF431DRS
M. kansasii strain CIP 104589 AY544838
M. szulgai CIP 104532 AY544867
M. tuberculosis H37Rv CP009480
68
61
97
100
99
99
53
98
96
95
100
83
0.01

Supplementary figure S7. Phylogenetic tree based on *gyrB* 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 S8. Phylogenetic tree based on *dnaK* 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 S9. Phylogenetic tree based on *secA1* sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.