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Mycobacterium iranicum sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents

Hasan Shojaei,[1] Charles Daley,[2] Zoe Gitti,[3] Abodolrazagh Hashemi,[1] Parvin Heidarieh,[1] Edward R. B. Moore,[4] Abass Daei Naser,[1] Cristina Russo,[5] Jakko van Ingen[6] and Enrico Tortoli[7]

Correspondence
Enrico Tortoli
e.tortoli@libero.it

[1]Isfahan University of Medical Sciences, Isfahan, Iran

[2]Department of Mycobacterial and Respiratory Infections, National Jewish Health, Denver, CO, USA

[3]Department of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine, University Hospital of Heraklion, Heraklion, Greece

[4]Department of Infectious Disease, Culture Collection University of Gothenburg (CCUG), Sahlgrenska Academy of the University of Gothenburg, Gothenburg, Sweden

[5]Virology Laboratory, Bambino Gesù Children's Hospital, Rome, Italy

[6]Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

[7]Emerging Bacterial Pathogens Unit, San Raffaele Scientific Institute, Milan, Italy

The isolation and characterization of a novel, rapidly growing, scotochromogenic mycobacterial species is reported. Eight independent strains were isolated from clinical specimens from six different countries of the world, two in Iran, two in Italy and one in each of following countries: Greece, the Netherlands, Sweden and the USA. Interestingly, two of the strains were isolated from cerebrospinal fluid. The strains were characterized by rapid growth and presented orange-pigmented scotochromogenic colonies. DNA-based analysis revealed unique sequences in the four regions investigated: the 16S rRNA gene, the rRNA gene internal transcribed spacer 1 and the genes encoding the 65 kDa heat-shock protein and the beta-subunit of RNA polymerase. The phylogenetic analysis placed the strains among the rapidly growing mycobacteria, being most closely related to *Mycobacterium gilvum*. The genotypic and phenotypic data both strongly supported the inclusion of the strains investigated here as members of a novel species within the genus *Mycobacterium*; the name *Mycobacterium iranicum* sp. nov. is proposed to indicate the isolation in Iran of the first recognized strains. The type strain is M05^T (=DSM 45541^T=CCUG 62053^T=JCM 17461^T).

Abbreviations: CFA, cellular fatty acid, ITS, internal transcribed spacer; PRA, PCR restriction analysis ; TBSA, tuberculostearic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M05^T and CCUG 52297 are HQ009482 and JQ886106, respectively; the accession numbers for the *rpoB* gene sequences of strains M05^T, FI-05198, CCUG 52297 and NLA001001296 are HQ009483, JQ886107, JQ886108 and JQ906698, respectively; the accession numbers for the ITS sequences of strains M05^T, HNTM87 and CCUG 52297 are HQ009484, HQ406789 and DQ523525, respectively; the accession numbers for the *hsp65* gene sequences of strains M05^T, FI-05198, GN10803, OPBG12013762 and CCUG 52297 are HQ009485, DQ381734, HM775984, JQ898289 and JQ898290, respectively.

Four supplementary figures and a supplementary table are available with the online version of this paper.

Among the non-tuberculous mycobacteria, the rapid growers have long been considered less important members of the genus *Mycobacterium*, as they were thought to be poorly pathogenic for humans. Only in recent years, with the recognition of their roles in a number of human infections (van Ingen *et al.*, 2009; De Groote & Huitt, 2006; Brown-Elliott & Wallace, 2002), has a revival of interest arisen, including interest in their taxonomic relationships (Adékambi & Drancourt, 2004; Schinsky *et al.*, 2004).

We analysed, using a combined phenotypic and genotypic approach, eight independent strains characterized by rapid growth and deep orange pigmentation. They were isolated from various clinical specimens in countries on three continents (America, Asia and Europe). Our results

indicate that they represent a novel species, for which the name *Mycobacterium iranicum* is proposed in recognition that attention to such micro-organisms was focused, for the first time, in Iran.

Two strains originated in different regions of Iran. Strain M05^T was isolated twice, with a time lapse of 3 weeks, from the bronchoalveolar lavage of a 60-year-old, HIV-negative, female patient suffering from chronic pulmonary disease. Strain HNTM87 was isolated three times from the hand wound of an 18-year-old male with a history of long-term steroid therapy due to renal transplantation. One strain (GN10803) originated in Greece from the cerebrospinal fluid of a 57-year-old woman; it was considered to be clinically non-relevant and the patient was not treated. One strain (NJH) originated in the USA, also from cerebrospinal fluid, in a patient affected by cancer. One strain (CCUG 52297) originated in Sweden from the sputum of a 26-year-old woman. Strain NLA001001296 was isolated from the sputum of an 89-year-old female in the Netherlands; it was considered clinically insignificant and no treatment was undertaken. Of the two Italian strains, FI-05198 was isolated from the sputum of a 74-year-old male with chronic bronchitis and a history of tuberculosis from when he was 22-years-old, and strain OPBG12013762 was isolated from the sputum of a 21-year-old woman with cystic fibrosis and Crohn's disease. Clinical and microbiological information on the strains is reported in Table 1.

The strains were inoculated onto solid egg-media incubated at different temperatures, MacConkey agar without crystal violet and 5 % (w/v) NaCl medium. Biochemical tests included 3 day arylsulfatase, pyrazinamidase and catalase activities, iron uptake, nitrate reduction, Tween 80 hydrolysis, urease production and niacin accumulation (Kent & Kubica, 1985).

The antibiotic susceptibility testing was performed by determining minimal inhibitory concentrations with the Mueller–Hinton broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

Mycolic acids were analysed as reported previously (Rhodes *et al.*, 2005). In short, colonies grown for 10 days at 37 °C on Middlebrook 7H11 agar were saponified with KOH, extracted with chloroform, derivatized according to the manufacturer's instructions (MIDI) and loaded onto an Agilent ChemStation high-performance liquid chromatograph (Agilent Technologies). Mycolic acids were separated with a gradient of methanol and 2-propanol (starting ratio 75 : 25 %, end ratio 95 : 5 %) and analysed using the MIDI Sherlock software, version 6.1 and database MICAG1 1.02.

Cellular fatty acid–fatty acid methyl ester (CFA–FAME) analyses were performed using gas chromatography (HP 5890, Hewlett Packard) and a protocol similar to that of the MIDI Sherlock MIS system (Sasser, 2001). Bacteria were cultivated for 10 days at 37 °C on Middlebrook 7H11

Table 1. Microbiological and clinical features of the patients from which the strains were isolated

F, Female; M, male; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; NA, not available; COPD, chronic obstructive pulmonary disease; TB, tuberculosis, +, positive; −, negative.

Strain	Age	Sex	Specimen type	Microscopy	Culture (positive/done)	Disease	Underlying condition	Anti-mycobacterial treatment	Outcome	Year
M05^T	60	F	BAL	+	2/2	Chronic pulmonary disease	None	Amikacin, ciprofloxacin	Improvement	2008
HNTM87	18	M	Hand wound	+	3/3	Hand wound	Renal transplantation	Amikacin	Improvement	2005
FI-05198	74	M	Sputum	NA	1/1	COPD	TB when 22-years old	NA	NA	2005
CCUG 52297	26	F	Sputum	NA	NA	NA	NA	NA	NA	2005
GN10803	57	F	CSF	−	1/1	Meningitis	None	None	NA	2008
NJH	NA	M	CSF	−	1/1	NA	Cancer	NA	NA	2010
NLA001001296	89	F	Sputum	−	1/3	Pneumonia	None	None	Improvement	2010
OPBG12013762	21	F	Sputum	−	1/3	Leukopenia	Cystic fibrosis and Crohn's disease	None	NA	2011

agar and cell biomass was removed from the medium using a plastic loop, carefully avoiding including medium in the sample; 50–100 mg cells were transferred to glass tubes. The cells were saponified by mild alkaline methanolysis and released fatty acids were methylated, followed by organic extraction. CFAs were identified and quantified; chromatographic retention times of CFA peaks were converted to equivalent chain-length (ECL) values and the percentage area for each peak was determined. The Agilent MIS FAME standard was used as a reference for identification of peaks. The relative amount of each CFA in a strain was expressed as a percentage of the total fatty acids in the profile of the strain. Further details of the methodology can be found at http://www.ccug.se/pages/CFA_method_2008.pdf.

Genomic DNA was extracted from the mycobacterial strains. PCR-amplifications and DNA sequencing were carried out for the almost complete 16S rRNA gene, the almost complete internal transcribed spacer 1 (ITS) and the hypervariable regions of the 65 kDa heat-shock protein (*hsp65*) and the beta-subunit of the RNA polymerase (*rpoB*) genes, as previously described (Shojaei *et al.*, 1997; Kirschner *et al.*, 1993; McNabb *et al.*, 2004; Adékambi *et al.*, 2003; Roth *et al.*, 1998). For each gene, the sequences were aligned with those of the most closely related mycobacterial species retrieved from the GenBank database and were analysed using CLUSTAL W (Thompson *et al.*, 1997).

Phylogenetic trees were constructed based, with 1000 bootstrap replicates, on the aforementioned alignments by the neighbour-joining method (Saitou & Nei, 1987), with total gap removal and Kimura's two-parameter substitution model (Kimura, 1980), and by the maximum-likelihood method (Felsenstein, 1981), using MEGA 5.05 software (Tamura *et al.*, 2011). An additional tree was inferred from the concatenated sequences of the 16S rRNA, *rpoB* and *hsp65* genes.

PCR restriction analysis (PRA) patterns (Telenti *et al.*, 1993) were deduced on the basis of restriction sites present in the *hsp65* sequences.

Mature colonies developed, from diluted inocula, in 4 days on Löwenstein–Jensen medium; the growth in subculture was however problematic from cultures stored at room temperature for more than a week. Growth was observed at temperatures in the range of 25–40 °C, with optimum growth at 37 °C. The strains were able to grow in the presence of 5 % NaCl (w/v) and were positive for urease, iron uptake, tellurite reduction, 3 day arylsulfatase and heat-stable (68 °C) catalase. The strains were negative for niacin accumulation, nitrate reduction, Tween 80 hydrolysis, semiquantitative catalase, pyrazinamidase and growth on MacConkey agar without crystal violet. The phenotypic characteristics differentiating the strains from closely related species are shown in Table 2.

The strains were susceptible to amikacin, cefoxitin, clarithromycin, ethambutol, minocycline, imipenem, linezolid and sulfamethoxazole (Table 3).

Analysis of mycolic acids by HPLC revealed a profile (Fig. S1, available in IJSEM Online) that was clearly different from that of any *Mycobacterium* species described to date (http://www.mycobactoscana.it/page4.htm).

Table 2. Biochemical and cultural characteristics of *M. iranicum* and members of the most closely related species

The tests were performed on the eight isolates investigated in this study and on the type strains of the other species, grown for 7 days at 37 °C on Middlebrook 7H11 agar. Taxa: 1, *M. iranicum* (all test strains); 2, *M. houstonense* ATCC 49403^T; 3, *M. poriferae* IP141490001^T; 4, *M. farcinogenes* CCUG 21047^T; 5, *M. vanbaalenii* CCUG 55853^T; 6, *M. gilvum* IP141190001^T; 7, *M. senegalense* CCUG 21001^T. +, Positive; −, negative; S, scotochromogenic; NA, not available.

Characteristics	1	2	3	4	5	6	7
Growth at:							
25 °C	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+
42 °C	−	+	−	−	−	−	+
Pigmentation	Orange (S)	−	−	Yellow (S)	Yellow (S)	Yellow (S)	−
Arysulfatase (3 days)	+	+	−	−	−	−	+
Catalase (68 °C)	+	+	+	+	+	−	+
Nitrate reduction	−	+	−	+	+	+	+
Pyrazinamidase activity	−	+	NA	+	+	+	+
Tween 80 hydrolysis	−	−	+	+	+	−	−
Niacin production	−	−	−	−	−	−	−
Urease activity	+	−	+	+	+	−	−
Semiquantitative catalase (>45 mm)	−	−	+	+	−	+	−
β-glucosidase	+	−	−	−	−	−	−
TCH* tolerance	+	+	+	+	+	+	+
Growth on MacConkey agar without crystal violet	−	+	−	−	−	+	−

*Thiophene-2-carboxylic hydrazide.

Table 3. Minimal inhibitory concentrations of five of the clinical strains (M05^T, HNTM87, GN10803, CCUG 52297 and OPBG12013762)

Drug	MIC range (mg ml^{-1})	Interpretation*
Amikacin	⩽1	S
Cefoxitin	⩽2–8	S
Ciprofloxacin	⩽0.12–2	S–I
Clarithromycin	⩽0.12–1	S
Ethambutol	⩽0.5–1	S
Imipenem	⩽1–2	S
Minocycline	0.25–0.5	S
Rifampicin	⩽0.06–4	S–I
Streptomycin	2–4	S–I
Sulfamethoxazole	1–16	S
Linezolid	⩽0.2–4	S

*S, Susceptible; I, intermediate.

The CFA profiles of strains M05^T and FI-05198 were determined and compared with those of closely related species of mycobacteria (Table 4). Strains M05^T and FI-05198 possessed similarities to *Mycobacterium parafortuitum* but were distinct from other mycobacterial species. Strains M05^T and FI-05198 exhibited iso-$C_{14:0}$ (14–12 %), $C_{17:1}\omega7c$ (9–7 %) and iso-$C_{20:0}$ (14 %), whereas most other species of mycobacteria did not have these CFAs. Strains M05^T and FI-05198 were furthermore differentiated from other species by differences in the relative amounts of some CFAs: $C_{16:1}\omega6c$, $C_{16:0}$, $C_{18:1}\omega9c$ and 10Me $C_{18:0}$ tuberculostearic acid (TBSA). These fatty acids were in fact present in significantly lower percentages of the total CFAs than observed in most other mycobacteria that were compared (Table 4).

In the almost complete 16S rRNA gene (1450 bp) seven strains had identical sequences while one (CCUG 52297) presented 13 mismatches. All eight sequences were characterized by the signature of rapidly growing mycobacteria (Stahl & Urbance, 1990) and differed from the sequences of all known *Mycobacterium* species in both hypervariable regions A and B (Kirschner *et al.*, 1993). All the strains presented a feature unique within the genus *Mycobacterium*, the presence of two 4 nt deletions starting at positions corresponding to *Escherichia coli* 16S rRNA gene sequence positions 71 and 96, respectively. The highest sequence similarity (99.9 %) was registered for the sequence of strain Myc399, an uncharacterized mycobacterium isolated from Spain (GenBank AF491284). Among the species with validly published names, the most similar were *Mycobacterium farcinogenes*, *Mycobacterium senegalense*, *Mycobacterium fortuitum* and *Mycobacterium houstonense* (98.6 %). Despite its quite low similarity with the other strains (99.1 %), strain CCUG 52297 was clearly more closely related to strain M05^T than to any other mycobacterial species, with *M. farcinogenes* (97.9 %) being the next closest hit.

Table 4. CFA profiles of strains M05^T and FI-05198 and related species of rapidly growing mycobacteria

Strains: 1, M05^T; 2, FI-05198; 3. *M. parafortuitum* CCUG 20999^T; 4, *M. wolinskyi* CCUG 47168^T; 5, *M. mageritense* CCUG 37984^T; 6, *M. senegalense* CCUG 21001^T; 7, *M. fortuitum* CCUG 20994^T; 8, *M. porcinum* CCUG 37674^T; 9, *M. farcinogenes* CCUG 21047^T; 10, *M. insubricum* CCUG 55636^T. Only CFAs with contents greater than 1.0 % of the species total CFA profile are included. ECL, Equivalent chain-length; TR, trace. All the strains were grown for 10 days at 37 °C on Middlebrook 7H11 agar.

Cell fatty acid	ECL	1	2	3	4	5	6	7	8	9	10
Unidentified	11.502	4	–	1	–	–	–	–	–	–	–
$C_{12:0}$	12.000	–	–	–	TR	–	–	–	TR	–	3
iso-$C_{14:0}$	13.524	14	12	22	–	–	–	–	–	–	–
$C_{14:0}$	14.000	7	7	4	4	12	10	15	15	11	9
$C_{15:0}$	15.000	–	–	–	TR	1	–	–	1	–	1
anteiso-$C_{16:0}$	15.717	–	TR	–	TR	–	–	–	1	2	TR
$C_{16:1}\omega9c$	15.774	–	TR	1	3	5	3	2	2	10	1
$C_{16:1}\omega7c$	15.819	2	1	–	5	7	2	3	3	–	4
$C_{16:1}\omega6c$	15.850	9	7	7	14	17	20	23	20	17	10
$C_{16:0}$	16.000	18	21	15	29	21	29	26	24	28	32
iso-$C_{17:1}\omega7c$	16.416	–	–	–	1	–	–	–	–	1	TR
$C_{17:1}\omega8c$	16.792	–	–	–	1	2	–	–	TR	–	1
$C_{17:1}\omega7c$	16.818	9	7	17	–	–	–	–	–	–	–
$C_{18:2}\omega6,9c$/anteiso-$C_{18:0}$	17.724	4	6	2	3	1	3	4	4	5	5
$C_{18:1}\omega9c$	17.769	16	18	11	24	29	25	19	19	10	25
$C_{18:0}$	18.000	1	2	1	1	–	1	2	1	1	1
TBSA 10Me $C_{18:0}$	18.392	1	2	3	11	6	8	6	8	13	5
iso-$C_{20:0}$	18.835	14	14	16	–	–	–	–	–	–	–

For the *rpoB* gene (683 bp) five strains, including M05^T, exhibited an identical sequence, FI-05198 differed at 5 bp, strain NLA001001296 at 12 bp and strain CCUG 52297 at 19 bp. All the strains clearly had different sequences (similarity <94 %) from the *rpoB* gene sequence of the most closely related species which was, for all of them, *Mycobacterium vanbaalenii* (Fig. S2).

Five sequevars of *hsp65* (401 bp) were detected, one of which was shared by four strains, including strain M05^T. Strain FI-05198 differed by 1 bp, strain GN10803 by 4 bp, strain OPBG12013762 by 6 bp and strain CCUG 52297 by 19 bp, respectively. *Mycobacterium rutilum* and *Mycobacterium komossense*, with a sequence similarity <96 %, were the more closely related species (Fig. S3).

In the ITS sequence (311 bp) six strains were identical; strain HNTM87 differed by 1 bp while strain CCUG 52297 was distinctly different (108 bp). Despite the large diversity among the sequevars, *Mycobacterium gilvum* was, although with different similarities (ranging from 75 % to 83 %), the most closely related species (Fig. S4).

Only two strains (M05^T and NJH) presented identical combinations of sequevars in the regions investigated (Table S1).

In the 16S rRNA-gene-sequence-based phylogenetic reconstruction, obtained with the neighbour joining algorithm, the test strains occupied a very ancestral position in the lineage of rapidly growing Mycobacteria (Fig. 1). This tree was, however, characterized by poor robustness with only a limited number of nodes supported by bootstrap values >50 %. Interestingly, in the trees inferred from *rpoB*, *hsp65* and ITS sequences, the various sequevars of the clinical isolates clustered with *M. gilvum* with moderate to high bootstrap support (Figs S2, S3 and S4). To improve the robustness of the tree (Devulder *et al.*, 2005; Mignard & Flandrois, 2008; Stackebrandt *et al.*, 2002) the sequences of the 16S rRNA, *rpoB* and *hsp65* genes were concatenated as a single alignment comprising 2450 bp. A more robust tree was obtained (Fig. 2) confirming the relatedness of the strains to *M. gilvum*.

Strain CCUG 52297, which was the most divergent, clustered together with the seven other test strains in each

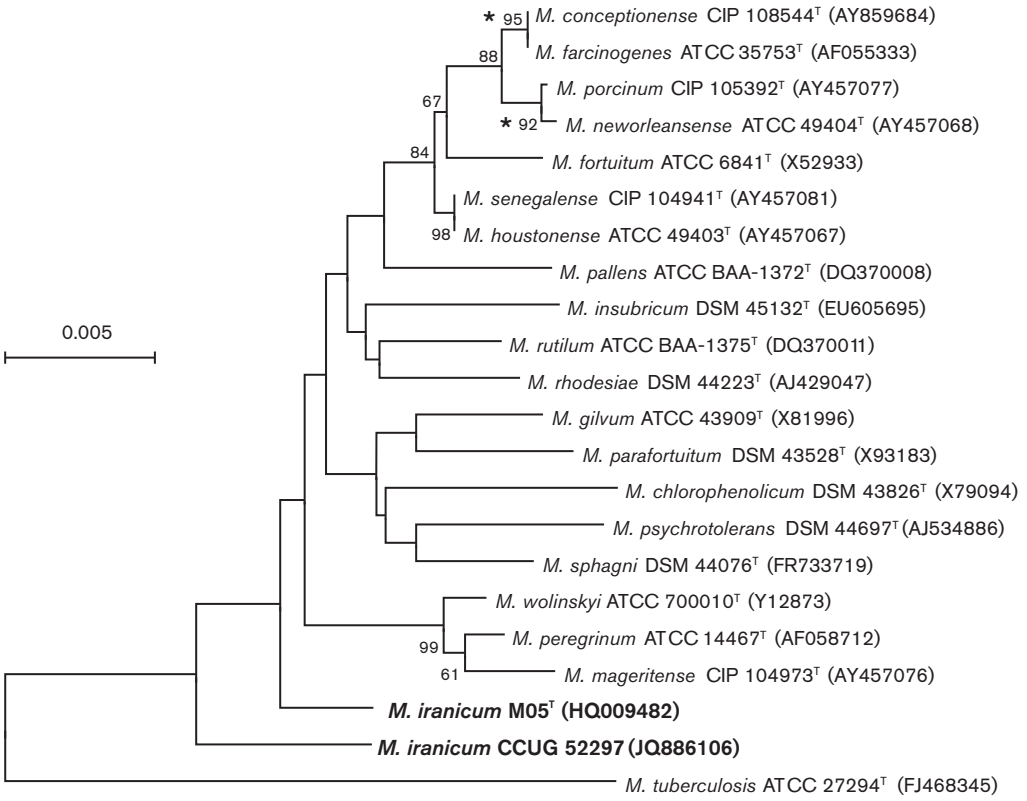

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences (1401 bp), constructed by using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50 % are given at the nodes. Bar, 0.005 substitutions per nucleotide position. The sequences of HNTM87, GN10803, NJH, NLA001001296, FI-05198 and OPBG12013762 are identical to that of M05^T. All the nodes, except the ones marked with asterisks, were confirmed in the tree constructed by using the maximum-likelihood method.

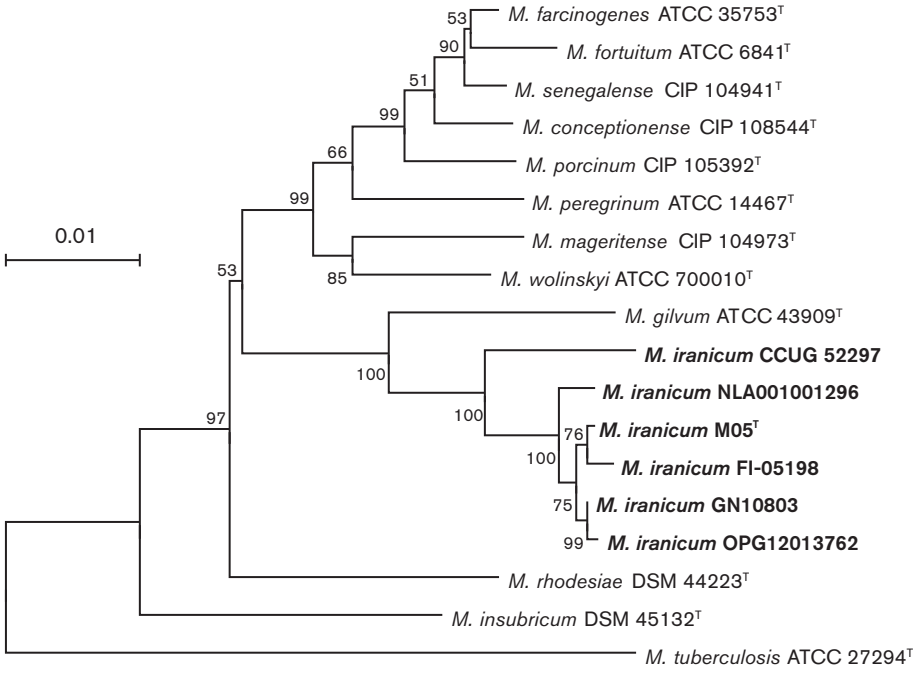

Fig. 2. Phylogenetic tree based on concatenated 16S rRNA, *rpoB* and *hsp65* genes sequences (2450 bp), constructed by using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50 % are given at the nodes. Bar, 0.01 substitutions per nucleotide position. The concatenated sequences of HNTM87 and NJH are identical to that of M05^T. All the nodes were confirmed in the tree constructed by using the maximum-likelihood method. Sequences used were the same as those in Figs 1, S2 and S3. ATCC 14467^T and CIP 105382^T, DSM 44223^T and CIP 106806^T, and ATCC 43909^T and DSM 44503^T, respectively, are synonyms.

phylogenetic tree; in all of them it was located at the root of the evolutionary path of the species (Figs 1, 2, S2, S3 and S4).

Two different PRA patterns were obtained from both *Bst*EII and *Hae*III restriction digestions of the *hsp65* gene fragments. From the combination of the patterns three different biotypes emerged (Table 5), which differed from those of known *Mycobacterium* species (http://app.chuv.ch/prasite/).

Description of *Mycobacterium iranicum* sp. nov.

Mycobacterium iranicum (i.ra′ni.cum. N.L. neut. adj. *iranicum* of or belonging to Iran, isolated in Iran).

Cells are Gram-positive, non-motile, non-spore-forming, acid–alcohol-fast bacilli. Strains grow at 37 °C on solid media producing smooth, orange and scotochromogenic colonies 1–4 mm in diameter in four days. Growth occurs at temperatures between 25 and 40 °C (with the optimum being at 37 °C) and on medium with 5 % (w/v) NaCl; no growth is obtained on MacConkey agar without crystal violet. The species is positive for urease, iron uptake, tellurite reduction, 3 day arylsulfatase and heat-stable catalase and negative for niacin production, nitrate reduction, Tween 80 hydrolysis and semiquantitative catalase. The HPLC mycolic acid pattern is unique. The gas chromatographic analysis reveals the presence of CFAs iso-$C_{14:0}$, $C_{17:1}\omega7c$ and iso-$C_{20:0}$ not present in most of the closely related mycobacteria. From the genotypic point of view, the PRA patterns of the *hsp65* are unique and the divergence from every other mycobacterium is unusually

Table 5. Patterns detected by PCR restriction analysis of *hsp65* gene sequences with enzymes *Bst*EII and *Hae*III in the test strains (only fragments >50 bp are reported)

Strain	Restriction patterns	
	***Bst*EII**	***Hae*III**
M05^T, HNTM87, FI-05198, NJH, NLA001001296, OPBG12013762	401	158–78–51
CCUG 52297	401	177–87–58
GN10803	211–190	158–78–51

high in each of the four genetic regions investigated. The two 5 nt deletions, in a 16S rRNA region highly conserved in every other mycobacterial species, add a distinctive signature to *M. iranicum* sp. nov.

The type strain is M05^T (=DSM 45541^T=CCUG 62053^T=JCM 17461^T).

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References

Adékambi, T. & Drancourt, M. (2004). Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA*, *recA* and *rpoB* gene sequencing. *Int J Syst Evol Microbiol* **54**, 2095–2105.

Adékambi, T., Colson, P. & Drancourt, M. (2003). *rpoB*-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J Clin Microbiol* **41**, 5699–5708.

Brown-Elliott, B. A. & Wallace, R. J., Jr (2002). Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev* **15**, 716–746.

CLSI (2011). Susceptibility testing of mycobacteria, nocardiae and other aerobic actinomycetes; approved standard, 2nd edn. Wayne, PA: Clinical and Laboratory Standards Institute.

De Groote, M. A. & Huitt, G. (2006). Infections due to rapidly growing mycobacteria. *Clin Infect Dis* **42**, 1756–1763.

Devulder, G., Pérouse de Montclos, M. & Flandrois, J. P. (2005). A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *Int J Syst Evol Microbiol* **55**, 293–302.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Kent, P. T. & Kubica, G. P. (1985). *Public health mycobacteriology. A guide for the level III laboratory.* Atlanta: US Department of Health and Human Services.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kirschner, P., Springer, B., Vogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F. C. & Böttger, E. C. (1993). Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J Clin Microbiol* **31**, 2882–2889.

McNabb, A., Eisler, D., Adie, K., Amos, M., Rodrigues, M., Stephens, G., Black, W. A. & Isaac-Renton, J. (2004). Assessment of partial sequencing of the 65-kilodalton heat shock protein gene (*hsp65*) for routine identification of *Mycobacterium* species isolated from clinical sources. *J Clin Microbiol* **42**, 3000–3011.

Mignard, S. & Flandrois, J. P. (2008). A seven-gene, multilocus, genus-wide approach to the phylogeny of mycobacteria using supertrees. *Int J Syst Evol Microbiol* **58**, 1432–1441.

Rhodes, M. W., Kator, H., McNabb, A., Deshayes, C., Reyrat, J. M., Brown-Elliott, B. A., Wallace, R., Jr, Trott, K. A., Parker, J. M. & other authors (2005). *Mycobacterium pseudoshottsii* sp. nov., a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *Int J Syst Evol Microbiol* **55**, 1139–1147.

Roth, A., Fischer, M., Hamid, M. E., Michalke, S., Ludwig, W. & Mauch, H. (1998). Differentiation of phylogenetically related slowly growing mycobacteria based on 16S–23S rRNA gene internal transcribed spacer sequences. *J Clin Microbiol* **36**, 139–147.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (2001). *Identification of bacteria by gas chromatography of cellular fatty acids.* MIDI Inc. http://www.microbialid.com/PDF/TechNote_101.pdf, Newark, DE.

Schinsky, M. F., Morey, R. E., Steigerwalt, A. G., Douglas, M. P., Wilson, R. W., Floyd, M. M., Butler, W. R., Daneshvar, M. I., Brown-Elliott, B. A. & other authors (2004). Taxonomic variation in the *Mycobacterium fortuitum* third biovariant complex: description of *Mycobacterium boenickei* sp. nov., *Mycobacterium houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp. nov. and recognition of *Mycobacterium porcinum* from human clinical isolates. *Int J Syst Evol Microbiol* **54**, 1653–1667.

Shojaei, H., Goodfellow, M., Magee, J. G., Freeman, R., Gould, F. K. & Brignall, C. G. (1997). *Mycobacterium novocastrense* sp. nov., a rapidly growing photochromogenic mycobacterium. *Int J Syst Bacteriol* **47**, 1205–1207.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Stahl, D. A. & Urbance, J. W. (1990). The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J Bacteriol* **172**, 116–124.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C. & Bodmer, T. (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* **31**, 175–178.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

van Ingen, J., Boeree, M. J., Dekhuijzen, P. N. & van Soolingen, D. (2009). Environmental sources of rapid growing nontuberculous mycobacteria causing disease in humans. *Clin Microbiol Infect* **15**, 888–893.

Fig. S1. Mycolic acid HPLC profile for *Mycobacterium iranicum* M05^{T}. The last peak (retention time 9.134) is the high molecular mass internal standard (HMWIS).

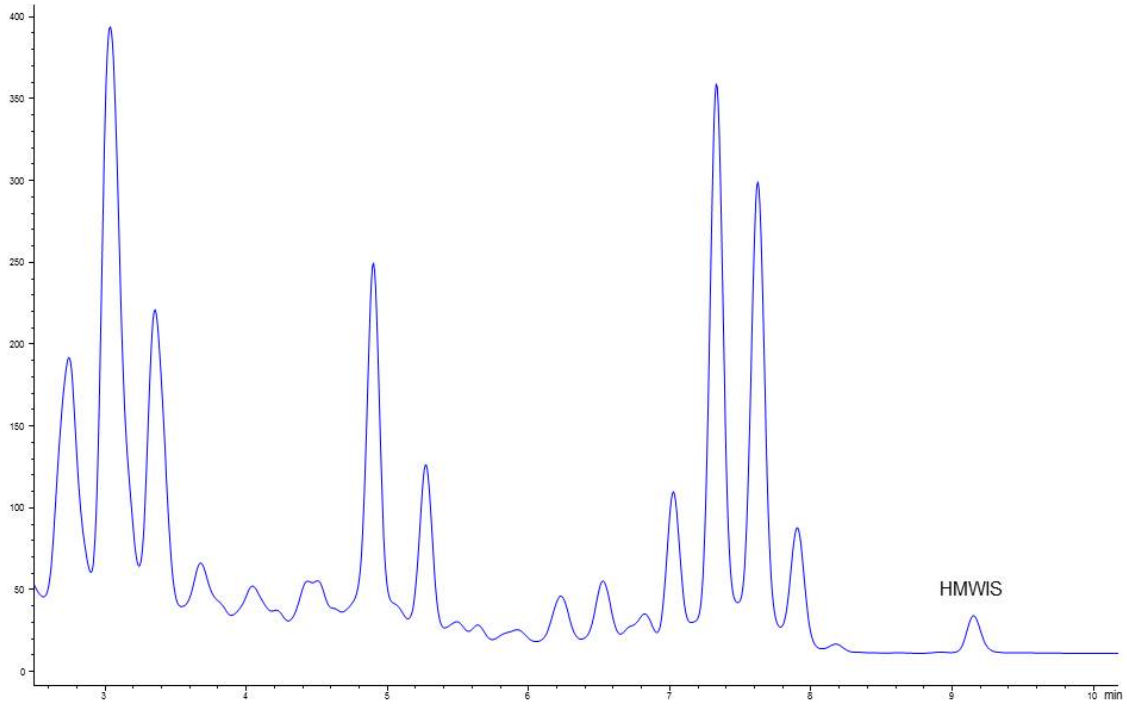
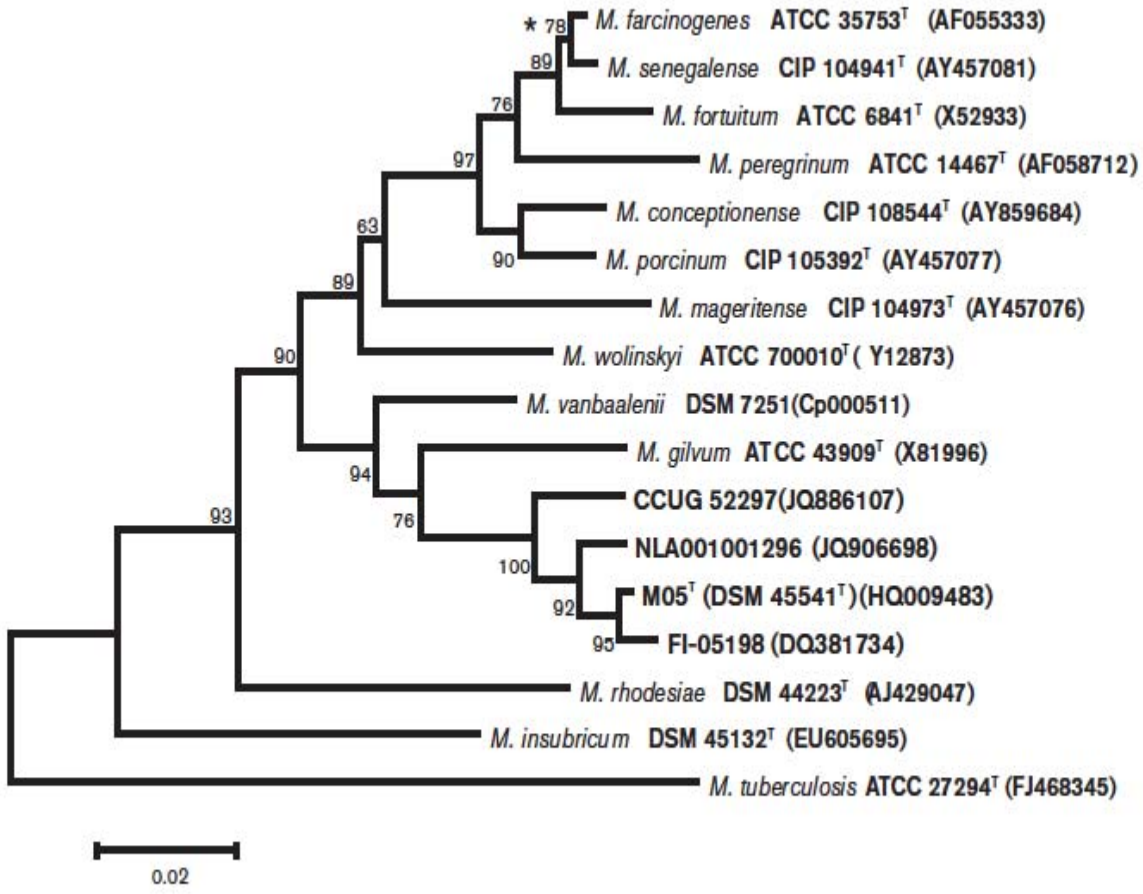


Fig. S2. Phylogenetic tree based on *rpoB* gene sequences (663 bp), constructed by using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50% are given at the nodes. Bar, 0.02 substitutions per nucleotide position. The sequences of HNTM87, GN10803, NJH and OPBG12013762 are identical to that of M05^T. All the nodes, except the one marked with an asterisk, were confirmed in the tree constructed by using the maximum-likelihood method.



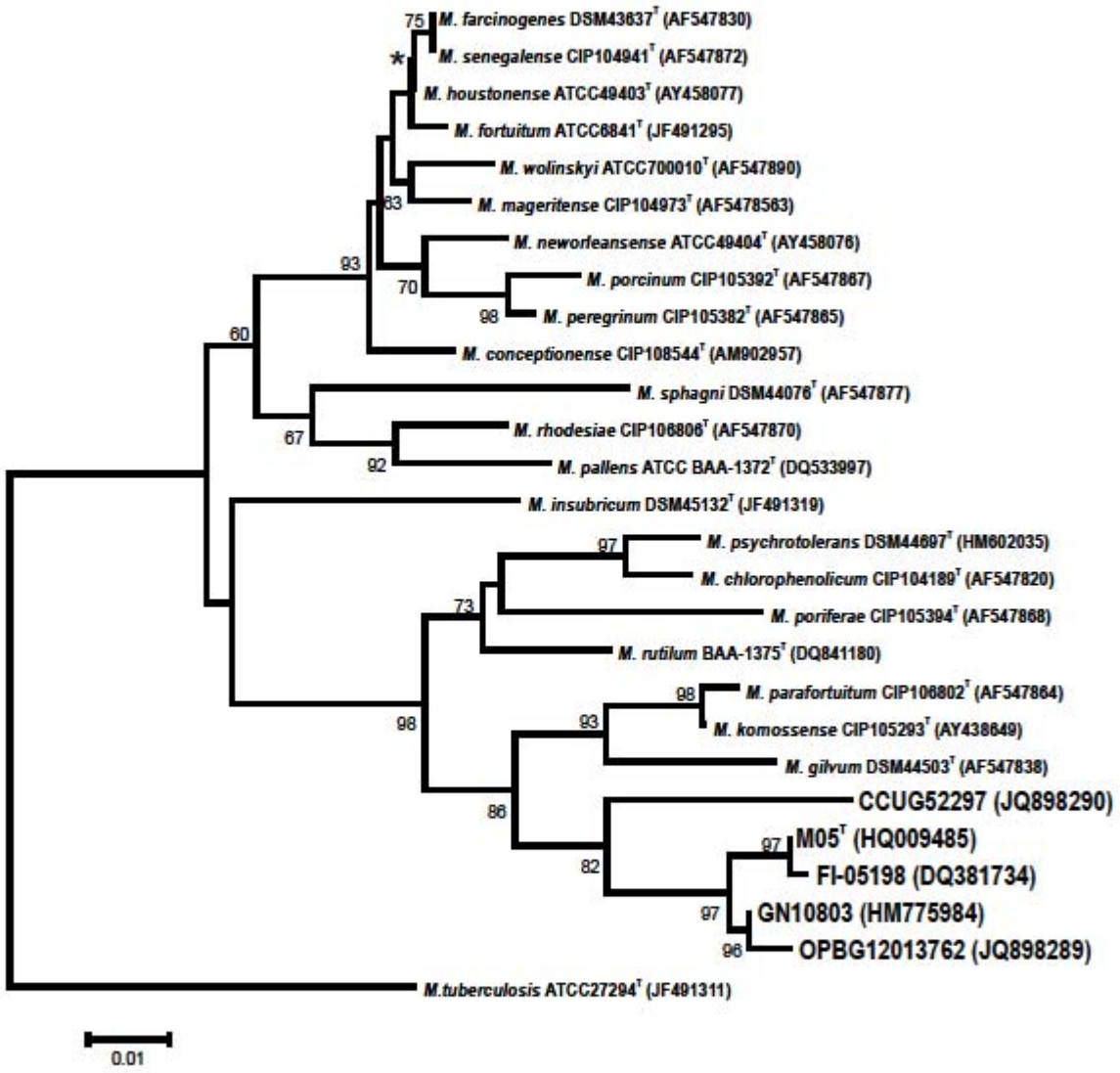


Fig. S3. Phylogenetic tree based on *hsp65* gene sequences (401 bp), constructed by using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50% are given at the nodes. Bar, 0.01 substitutions per nucleotide position. The sequences of HNTM87, NJH and NLA001001296 are identical to that of M05^T. All the nodes, but the asterisked one, were confirmed in the tree constructed by using the maximum-likelihood method.

H. Shojaei, C. Daley, Z. Gitti, A. Hashemi, P. Heidarieh, E. R. B. Moore, A. D. Naser, C. Russo, J. van Ingen & E. Tortoli (2013). *Mycobacterium iranicum* sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents. *Int J Syst Evol Microbiol* **63**, 1383–1389.

Fig. S4. Phylogenetic tree based on ITS sequences (306 bp), constructed by using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50% are given at the nodes. Bar, 0.05 substitutions per nucleotide position. The sequences of GN10803, NJH, NLA001001296, FI-05198 and ONPG12013762 are identical to that of M05^T. All the nodes were confirmed in the tree constructed by using the maximum-likelihood method.

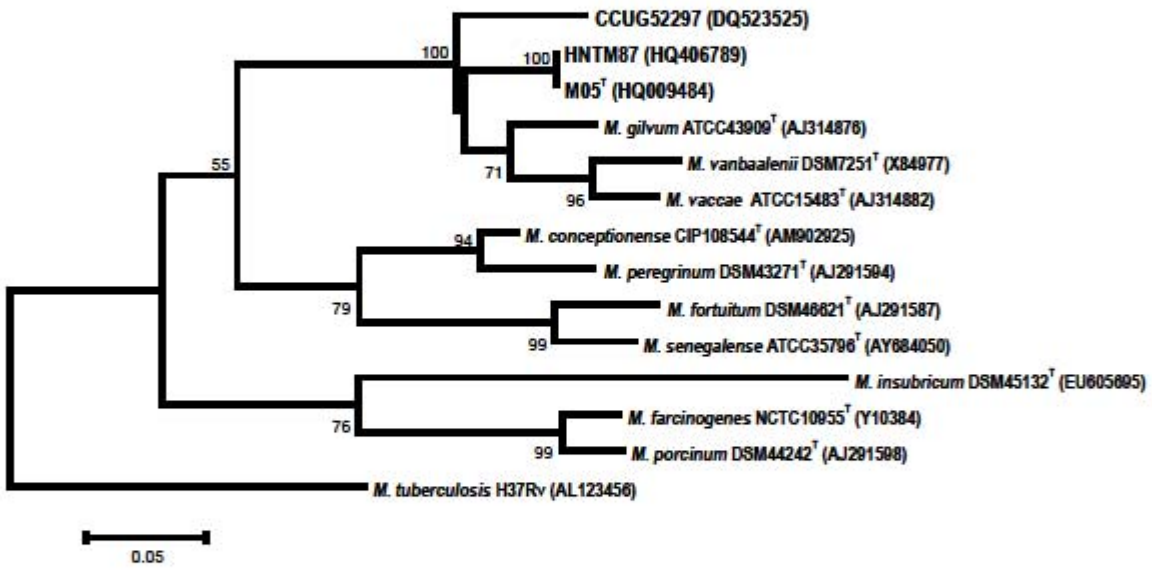


Table S1. Combinations of different sequevars, in different genetic regions, in the test strains

Sequences of other strains that are identical to those of M05^T are indicated by having M05^{T}in place of the strain designation.

Strain	**16S rRNA gene**	***rpoB* gene**	***hsp65* gene**	**ITS 1**
M05^T	M05^T	M05^T	M05^T	M05^T
CCUG 52297	CCUG 52297	CCUG 52297	CCUG 52297	CCUG 52297
GN10803	M05^T	M05^T	GN10803	M05^T
FI-05198	M05^T	FI-05198	FI-05198	M05^T
HNTM87	M05^T	M05^T	M05^T	HNTM87
NJH	M05^T	M05^T	M05^T	M05^T
NLA001001296	M05^T	NLA001001296	M05^T	M05^T
OPBG12013762	M05^T	M05^T	OPBG12013762	M05^T