Mycobacterium mantenii sp. nov., a pathogenic, slowly growing, scotochromogenic species

Jakko van Ingen,[1,2] Jerome A. Lindeboom,[3,4] Nico G. Hartwig,[5] Rina de Zwaan,[2] Enrico Tortoli,[6] P. N. Richard Dekhuijzen,[1] Martin J. Boeree[1] and Dick van Soolingen[2]

Correspondence
Jakko van Ingen
jakko.van.ingen@rivm.nl

[1]Department of Pulmonary Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

[2]National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands

[3]Department of Oral and Maxillofacial Surgery, Academic Medical Center, Amsterdam, The Netherlands

[4]Academic Center for Dentistry, University of Amsterdam, Amsterdam, The Netherlands

[5]Department of Paediatrics, Erasmus MC-Sophia, Rotterdam, The Netherlands

[6]Regional Reference Center for Mycobacteria, Microbiology and Virology Laboratory, Careggi Hospital, Florence, Italy

Slowly growing, scotochromogenic bacteria of a novel *Mycobacterium* species were isolated from lymph node samples in two children and pulmonary samples in two elderly patients from different regions in the Netherlands as well as from a surface water sample in Zambia. Its 16S rRNA gene, 16S–23S internal transcribed spacer (ITS), *hsp65* and *rpoB* gene sequences are unique in comparison with other mycobacteria. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that these micro-organisms are most closely related to *Mycobacterium scrofulaceum* ATCC 19981^T (8 differences; 0.6 % divergence). The *hsp65* sequence shows 96 % similarity to that of *Mycobacterium saskatchewanense* MB54784 and the *rpoB* sequence shows 95 % similarity to that of *Mycobacterium chimaera* CIP 107892^T. The 16S–23S ITS sequence places these micro-organisms within the *Mycobacterium avium* complex, as a novel ITS sequevar. This is not supported by analysis of the 16S rRNA, *hsp65* or *rpoB* gene sequences. Their scotochromogenicity, combined with mostly positive urease, positive semiquantitative catalase and negative tellurite reduction tests, set these isolates apart from related species. The mycolic acid patterns, obtained by HPLC, are similar to that of *Mycobacterium scrofulaceum*, though the peak heights and distribution present minor differences. We propose the name *Mycobacterium mantenii* sp. nov. for this novel species. The type strain, isolated from a lymph node biopsy sample, is strain 04-1474^T (=NLA000401474^T =CIP 109863^T =DSM 45255^T).

Abbreviations: ITS, internal transcribed spacer; MAC, *Mycobacterium avium* complex; MIC, minimal inhibitory concentration; NTM, non-tuberculous mycobacteria; TCH, thiophene-2-carboxylic hydrazide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, 16S–23S ITS, *hsp65* and *rpoB* gene sequences of strains 04-1474^T (=NLA000401474^T) and 08-224 (=NLA000800224) are respectively FJ042897 and FJ232521 (16S rRNA gene), FJ232522 and FJ426335 (16S–23S ITS), FJ232523 and FJ426336 (*hsp65*) and FJ232524 and FJ426337 (*rpoB*).

Phylogenetic trees based on concatenated 16S rRNA, *hsp65* and *rpoB* gene sequences as well as the 16S–23S ITS of the studied isolates and related *Mycobacterium* species are available as supplementary material with the online version of this paper.

Non-tuberculous mycobacteria (NTM) are common in the environment and can be opportunistic pathogens. The NTM are capable of causing a wide spectrum of clinical disease. Pulmonary NTM disease, mostly in patients with pre-existing chronic pulmonary diseases, is most common, followed by lymphadenitis in immunocompetent children and disseminated disease in immunocompromised patients (Griffith *et al.*, 2007).

Mycobacterium scrofulaceum, first described by Prissick & Masson (1956), is mainly a causative agent of paediatric cervicofacial lymphadenitis. The isolation frequency of *M. scrofulaceum* has decreased in recent decades, presumably

because of competition with *Mycobacterium avium* after chlorination of tap water became more commonplace (Griffith *et al.*, 2007). On the other hand, several scotochromogenic, slowly growing mycobacteria isolated from children with cervicofacial lymphadenitis have been elevated to separate species status. These may have previously been falsely identified as *M. scrofulaceum* (Tortoli, 2003). The International Working Group on Mycobacterial Taxonomy also found several isolates related to, but different from, *M. scrofulaceum* (Wayne *et al.*, 1996).

In this paper, we report on a group of five isolates with unique 16S rRNA gene, 16S–23S internal transcribed spacer (ITS), *hsp65* and *rpoB* sequences that represent a novel *Mycobacterium* species, related to *M. scrofulaceum*. The isolates were cultured from four patients, including two cases of paediatric cervicofacial lymphadenitis, in the Netherlands and from the Zambezi River, Zambia.

Case reports

In 2004, a previously healthy, 2 ½-year-old girl presented with an 8-week history of right submandibular erythematous swelling, 3 cm in diameter, with obvious fluctuation and a red skin. She was otherwise asymptomatic. A fine-needle aspiration from the swelling was performed. The acid-fast stain of the material obtained was positive. An *M. avium* complex (MAC) strain was detected by real-time PCR, using the 16S–23S ITS as a target, and a scotochromogenic mycobacterium grew in culture (isolate 04-1474^T). Antibiotic therapy with clarithromycin (15 mg kg^{-1}) and rifabutin (5 mg kg^{-1}) was started for a 12 week period. An open wound with persistent drainage developed and healed during therapy. After 2 years, only a small scar remained.

The second patient was a previously healthy, 18-month-old girl who presented at another hospital in the Netherlands in 2007 with a fluctuating, painless, left-sided, submandibular swelling, 4 cm in diameter, with violaceous overlying skin. She had no other symptoms. A lymph node biopsy with drainage was performed and a scotochromogenic mycobacterium was cultured from the biopsy material (isolate 07-937). Involution of the lymph node was noted during follow-up; no further therapy was necessary and after 1 year only a small scar remained.

The third patient was a 92-year-old Dutch woman who was evaluated by a respiratory physician in a regional hospital in the north of the Netherlands in 2008. Her medical history included bronchiectasis. She presented with a productive cough and slight dyspnoea. A scotochromogenic mycobacterium was isolated from one of three sputum samples (isolate 07-1794). Her symptoms and radiographic features were not suggestive of mycobacterial disease, and follow-up cultures remained negative, so this isolate was not considered clinically relevant, based on the American Thoracic Society diagnostic criteria (Griffith *et al.*, 2007).

The fourth patient was a 68-year-old Dutch male who reported with chronic cough and purulent sputum in 2008. His medical history included an α-1-antitrypsin deficiency and resulting pulmonary emphysema. A scotochromogenic mycobacterium was isolated from a bronchoalveolar lavage (isolate 08-1102). In the absence of radiographic features suggestive of pulmonary mycobacterial disease or positive follow-up cultures, this isolate was also not considered clinically relevant.

The fifth isolate was cultured from a water sample we took for other research purposes from the Zambezi river, Zambia, 150 m upstream from the Victoria Falls in 2007 (isolate 08-224).

All five isolates were subcultured on Middlebrook 7H10 and the egg-based Ogawa and Stonebrink solid media, as well in the Mycobacterial Growth Indicator Tube (MGIT) system. All media were incubated at 36 °C; Middlebrook 7H10 slants were also incubated at 25, 30 and 45 °C.

The Inno-LiPA Mycobacteria v2 reverse hybridization test was used for primary identification, according to the manufacturer's instructions. To obtain identification to the species level, we sequenced the full 16S rRNA gene and the 16S–23S ITS and partially sequenced the *hsp65* and *rpoB* genes, using previously published approaches (Springer *et al.*, 1996; Roth *et al.*, 1998; Kim *et al.*, 1999; Telenti *et al.*, 1993).

We compared the sequences obtained with the GenBank/EMBL sequence database. The full 16S rRNA gene sequences of the five isolates were aligned with those of reference strains of the closest related mycobacteria using CLUSTAL_X software (Thompson *et al.*, 1997). The resulting topology and tree, inferred by neighbour-joining and visualized using the MEGA 4.0 software package (Tamura *et al.*, 2007), were evaluated by bootstrap analyses based on 1000 resamplings (Fig. 1).

For biochemical and phenotypic identification, we investigated colony morphology, ability to grow at temperatures ranging from 25 to 45 °C, niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, 3-day arylsulfatase, urease, tellurite reduction, 68 °C and semi-quantitative catalase, growth rate, pigmentation, growth on MacConkey agar and tolerance of (μg ml^{-1}) thiophene-2-carboxylic hydrazide (TCH) (5), oleate (250), *p*-nitrobenzoic acid (500), thiacetazone (10), hydroxylamine (500) and isoniazid (1), all in Middlebrook 7H10 agar, following previously published guidelines (Kent & Kubica, 1985). HPLC was used to investigate the cell-wall mycolic acid composition according to methods reported previously (CDC, 1996). We used the HPLC mycobacterium library (http://www.MycobacToscana.it) for visual comparisons.

Susceptibility testing was performed using the Middlebrook 7H10 agar dilution method (van Klingeren *et al.*, 2007). We tested susceptibility to rifampicin, rifabutin, isoniazid, ethambutol, streptomycin, amikacin, clarithromycin, ciprofloxacin, moxifloxacin, cycloserine, prothionamide, clofazimine and linezolid.

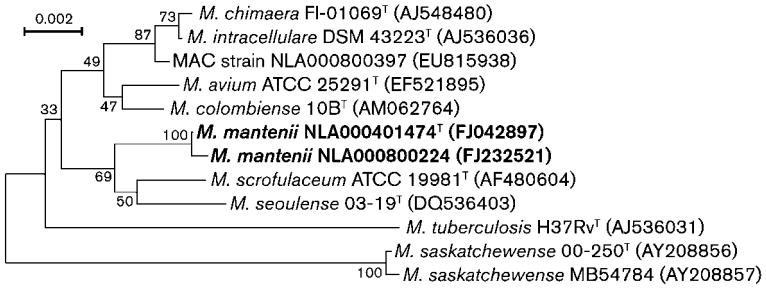

Fig. 1. Phylogenetic relationship of strains of *Mycobacterium mantenii* sp. nov. and related species of *Mycobacterium*, based on 16S rRNA gene sequences. The neighbour-joining tree was created, bootstrapped 1000 times and visualized with MEGA 4.0. Bootstrap values are indicated at the nodes. Bar, 0.002 substitutions per nucleotide position.

All five samples yielded small colonies with bright-yellow pigmentation after 3 weeks of incubation on solid media at 36 °C; growth was slower at 25 and 30 °C and no growth occurred at 45 °C.

For all five isolates, the Inno-LiPA Mycobacteria v2 reverse hybridization test revealed a reaction with the '*M. avium–intracellulare–scrofulaceum* complex' probe only. The full 16S rRNA gene sequence (1437 bp) revealed eight differences (99.4 % similarity) from the sequence of *M. scrofulaceum* ATCC 19981^T in all four clinical isolates (Table 1); the sequence of the Zambezi river isolate had a single difference from the sequences of the four clinical isolates and differed at nine positions from the sequence of *M. scrofulaceum* ATCC 19981^T. All five isolates shared the 3 bp deletion in the hypervariable region B of the 16S rRNA gene that is typical of *M. scrofulaceum* and absent from MAC species.

We recorded identical ITS, *hsp65* and *rpoB* gene sequences in all four clinical isolates; the results of comparison with sequences in GenBank are detailed in Table 1. The sequences of the Zambezi river isolate differed from the clinical isolates at 11 positions (4 %) in the 16S–23S ITS, 3 positions (1 %) in *hsp65* and 5 positions (2 %) in the *rpoB* sequence.

Based on the 1437 bp 16S rRNA gene sequence, the five isolates were most closely related to *M. scrofulaceum* and *Mycobacterium seoulense* (Table 1 and Fig. 1), which also are scotochromogenic, slowly growing mycobacteria (Prissick & Masson, 1956; Mun *et al.*, 2007). The *hsp65* sequence and, to a lesser extent, the *rpoB* sequence suggest that our isolates are related to *Mycobacterium saskatchewanense*, another scotochromogenic, slowly growing NTM (Turenne *et al.*, 2004). These observations, as well as the more distant relationship to the MAC, were confirmed in phylogenetic trees based on multisequence alignment of *hsp65* gene (Fig. 2) and *rpoB* gene (Fig. 3) sequences. In addition, we concatenated the 16S rRNA gene, *hsp65* and *rpoB* sequences and aligned these with concatenated sequences of related *Mycobacterium* species (Stackebrandt *et al.*, 2002). The resulting tree is available as Supplementary Fig. S1 in IJSEM Online.

The ITS sequence is most closely related to those of members of the MAC (Table 1 and Supplementary Fig. S2). This matches the Inno-LiPA results, which uses the ITS as

Table 1. Molecular identification results for strain 04-1474^T

Target	Length (bp)	Results of GenBank comparison		
		Similarity	Strain	Accession no.
16S rRNA gene	1437	99 % (1429/1437)	*M. scrofulaceum* ATCC 19981^T	AF480604
		99 % (1422/1439)	*M. seoulense* 03-19^T	DQ536403
		99 % (1422/1441)	*M. avium* ATCC 25291^T	EF521895
		98 % (1407/1440)	*M. saskatchewanense* 00-250^T	AY208856
16S–23S ITS	281	96 % (271/281)	*M. vulneris* RiVM 9601918 (MAC-Q)	AF315833
		96 % (270/281)	MAC strain ATCC 35847 (MAC-E)	L07852
		96 % (269/281)	*Mycobacterium* sp. RiVM 9701605 (MAC-R)	AF315834
		96 % (269/281)	MAC strain 5154-O'Connor (MAC-F)	L07853
hsp65	424	97 % (410/424)	*M. saskatchewanense* MB54784	AY208859
		97 % (410/424)	*M. triplex* CIP 106108^T	AF547882
		96 % (408/424)	*M. avium* ATCC 25291^T	AY299177
		96 % (408/424)	*M. genavense* DSM 44424^T	AF547837
rpoB	301	96 % (288/301)	*M. chimaera* CIP 107892^T	AY943187
		96 % (288/301)	*M. intracellulare* CIP 104243^T	AY544930
		96 % (282/295)	*M. saskatchewanense* 00-250^T	AY943192
		95 % (285/301)	*M. seoulense* 03-19^T	DQ536405

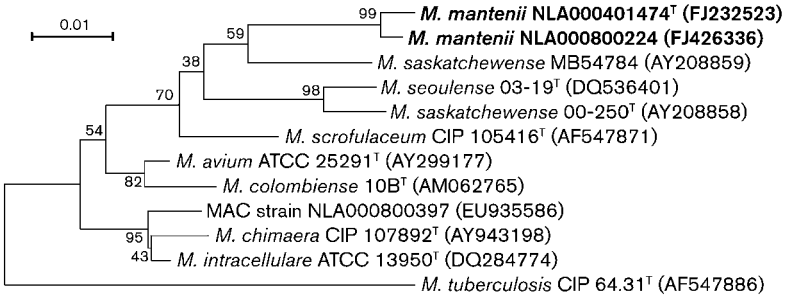

Fig. 2. Phylogenetic relationship of strains of *M. mantenii* sp. nov. and related species of *Mycobacterium*, based on *hsp65* gene sequences. See Fig. 1 for further details. Bar, 0.01 substitutions per nucleotide position.

its target. The different phylogenetic relationships arising from the different genetic targets, as well as the extent of the differences, add to our view that these isolates make up a separate species.

The five studied isolates tested negative for niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, tellurite reductase, 3 day arylsulfatase and growth on MacConkey agar, but tested positive for urease (3/5), 68 °C catalase and semiquantitative catalase. The isolates were tolerant of isoniazid, thiacetazone, *p*-nitrobenzoic acid, TCH and oleic acid, but not hydroxylamine. The negative tellurite reduction tests and mixed urease results set the isolates apart from *M. scrofulaceum*, *M. saskatchewanense* and *M. seoulense*; our isolates also differ from the latter in their negative nitrate reduction tests. Their scotochromogenicity, combined with uniformly positive semiquantitative and 68 °C catalase tests, mostly positive urease and negative tellurite reduction tests and susceptibility to hydroxylamine, set them apart from the MAC (Table 2).

The HPLC profiles of strain 04-1474^T (=NLA000401474^T) and the other clinical isolates are identical and overlap that typical of the MAC, *M. scrofulaceum* and *M. seoulense*, i.e. three clusters of peaks, one early, large cluster and two late, smaller clusters (Fig. 4), though the peak heights and distribution present minor differences. This profile differs from the single, late-emerging cluster of peaks characteristic of *M. saskatchewanense* (Turenne *et al.*, 2004). The profile of the Zambezi river isolate (isolate 08-224) is slightly divergent, with different peak heights in the first and higher peaks in the second cluster (Fig. 4). A divergent HPLC profile in environmental isolates compared with clinical isolates has also been noted for *Mycobacterium bohemicum* (Torkko *et al.*, 2001).

The clinical isolates were only susceptible *in vitro* to rifampicin, rifabutin, clarithromycin, cycloserine, clofazimine and prothionamide; minimal inhibitory concentrations (MICs) are recorded in Table 3. The drug susceptibility pattern is similar to that of *M. scrofulaceum* and the members of the MAC, except for the remarkable *in vitro* susceptibility to the rifamycins, i.e. rifampicin and rifabutin (Table 3).

Although there is a single difference in the 16S rRNA gene sequence, we consider the Zambezi river isolate and our four clinical isolates to belong to a single species, based on the minor genetic differences and overlapping biochemical and phenotypic features. Microheterogeneity in the 16S rRNA gene sequence has been described previously in the NTM (Kirschner & Böttger, 1992). The slight genetic differences may reflect the evolutionary divergence among NTM.

The five described isolates make up a novel species, phylogenetically and phenotypically related to *M. scrofulaceum*, *M. seoulense*, *M. saskatchewanense* and, albeit more distantly, the MAC. Biochemical features, however, offer little distinction in the MAC and HPLC patterns of the MAC and *M. scrofulaceum* are known to overlap (Wayne *et al.*, 1996). The 16S–23S ITS sequence places the five isolates inside the MAC, although bootstrap levels are low (Supplementary Fig. S2). This does result in misidentification as a MAC species by the Inno-Lipa assay, not unlike the identification of *M. saskatchewanense* as MAC by the Accuprobe assay (Turenne *et al.*, 2004). ITS sequences have been useful for taxonomic assignment within the MAC (Frothingham & Wilson, 1993; Tortoli *et al.*, 2004; Murcia *et al.*, 2006; van Ingen *et al.*, 2009). Our isolates comprise two distinct MAC ITS sequevars although, for a species not likely to be part of the MAC, naming them as such would be misleading.

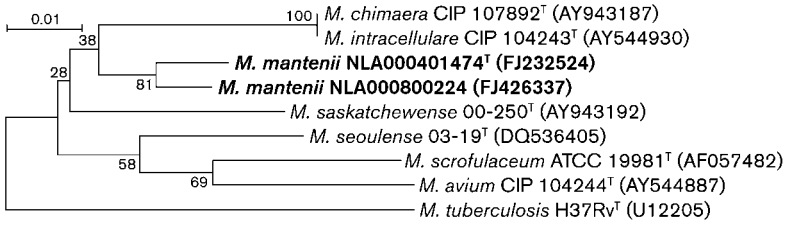

Fig. 3. Phylogenetic relationship of strains of *M. mantenii* sp. nov. and related species of *Mycobacterium*, based on *rpoB* gene sequences. See Fig. 1 for further details. Bar, 0.01 substitutions per nucleotide position.

Table 2. Biochemical features that differentiate the five novel isolates from related species

Reference species: MSC, *M. scrofulaceum*; MSE, *M. seoulense*; MSK, *M. saskatchewanense*; MAV, *M. avium*. Comparative data were extracted from Wayne & Kubica (1986), Turenne *et al.* (2004) and Mun *et al.* (2007). V, Variable; ND, no data available.

Test	04-1474^T	07-937	07-1794	08-224	08-1102	MSC	MSE	MSK	MAV
Nitrate reduction	−	−	−	−	−	−	+	−	−
68 °C catalase	+	+	+	+	+	+	+	+	V
Catalase >45 mm	+	+	+	+	+	+	ND	V	−
Tellurite reduction	−	−	−	−	−	+	V	V	+
Urease	+	−	+	−	+	+	−	−	−
Pigmentation*	SC	SC	SC	SC	SC	SC	SC	SC	NC
Growth at 45 °C	−	−	−	−	−	−	−	−	V
Tolerance of hydroxylamine†	−	−	−	−	−	ND	ND	ND	V

*SC, Scotochromogenic; NC, non-chromogenic.
†At 500 μg ml^{-1} in Middlebrook 7H10 agar.

The spectrum of human disease of the novel species, represented by the five described isolates, appears similar to that of *M. scrofulaceum*.

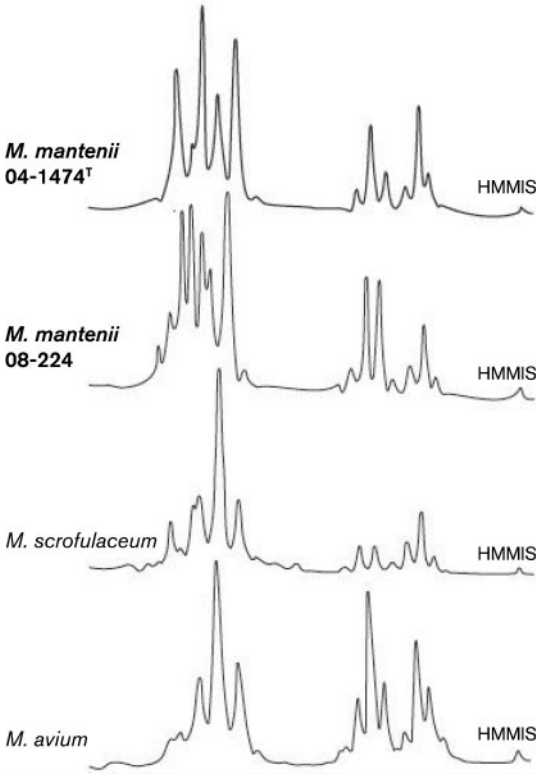

Fig. 4. Mycolic acid patterns of *M. mantenii* sp. nov. strains 04-1474^T and 08-224 (Zambezi), *M. scrofulaceum* and *M. avium* obtained by HPLC analysis. One early and one late cluster of peaks are present in all four profiles, although the peak heights and distribution are different. The Zambezi isolate is divergent, with a different peak distribution in the first cluster and a pronounced second cluster. HMMIS, High-molecular-mass internal standard.

Based on all available genetic, phenotypic and clinical data, we conclude that the five isolates make up a novel NTM species most closely related to *M. scrofulaceum* and related to, but not part of, the MAC.

Description of *Mycobacterium mantenii* sp. nov.

Mycobacterium mantenii (man.te′ni.i. N.L. gen. masc. n. *mantenii* of Manten, in honour of Dr A. Manten, microbiologist, who published the first cases of NTM disease in the Netherlands in 1957, as well as landmark reviews on the clinical relevance of NTM in the Netherlands).

The bacillus stains acid–alcohol-fast. Cells are short rods, with frequent coccid forms. No cording, spores or filaments are present. On Middlebrook 7H10, Ogawa and Stonebrink media, mature growth develops after 28 days of incubation at 25–36 °C; no growth occurs at 45 °C. Colonies are small, smooth, scotochromogenic and yellow.

Table 3. Minimum inhibitory concentrations (μg ml^{-1}) by the agar dilution method

Agent	04-1474^T	07-937	07-1794	08-1102
Isoniazid	>2	>2	>2	>2
Rifampicin	1	1	1	1
Ethambutol	10	20	10	5–10
Streptomycin	20	20	10	5
Rifabutin	⩽0.2	⩽0.2	⩽0.2	⩽0.2
Amikacin	20	>20	10	10
Ciprofloxacin	16	>16	16	16
Clarithromycin	⩽2	8	4	4
Cycloserine	20	50	10	10
Clofazimine	⩽0.5	5	⩽0.5	⩽0.5
Prothionamide	5	5	5	>20
Moxifloxacin	>2	>2	>2	>2
Linezolid	>2	>2	>2	>2

Negative for niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, tellurite reduction, 3 day arylsulfatase and growth on MacConkey agar, but positive for urease, 68 °C catalase and semiquantitative catalase. It is tolerant of isoniazid, thiacetazone, *p*-nitrobenzoic acid, TCH and oleic acid, but not hydroxylamine. Readily identifiable by its unique 16S rRNA gene, 16S–23S ITS, *hsp65* and *rpoB* gene sequences.

The type strain, recovered from a lymph node biopsy specimen, is strain 04-1474^T (=NLA000401474^T =CIP 109863^T =DSM 45255^T).

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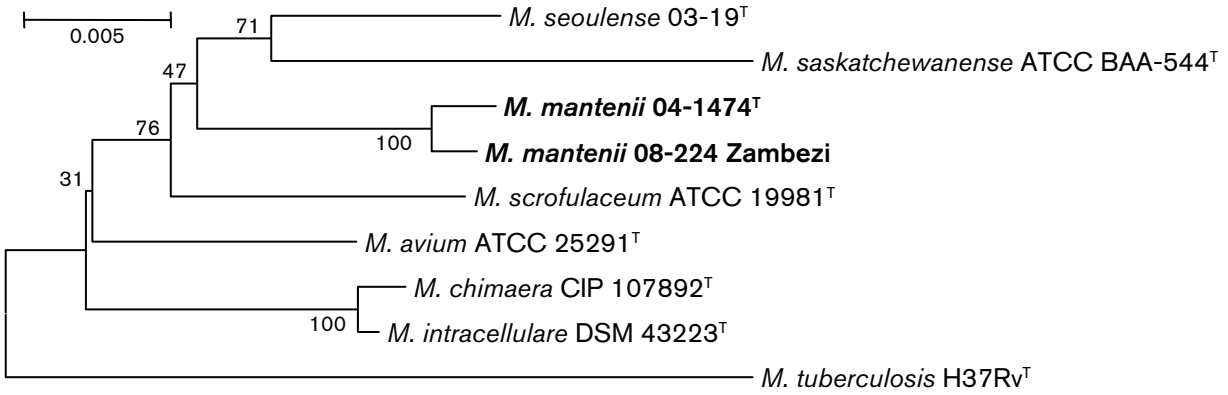
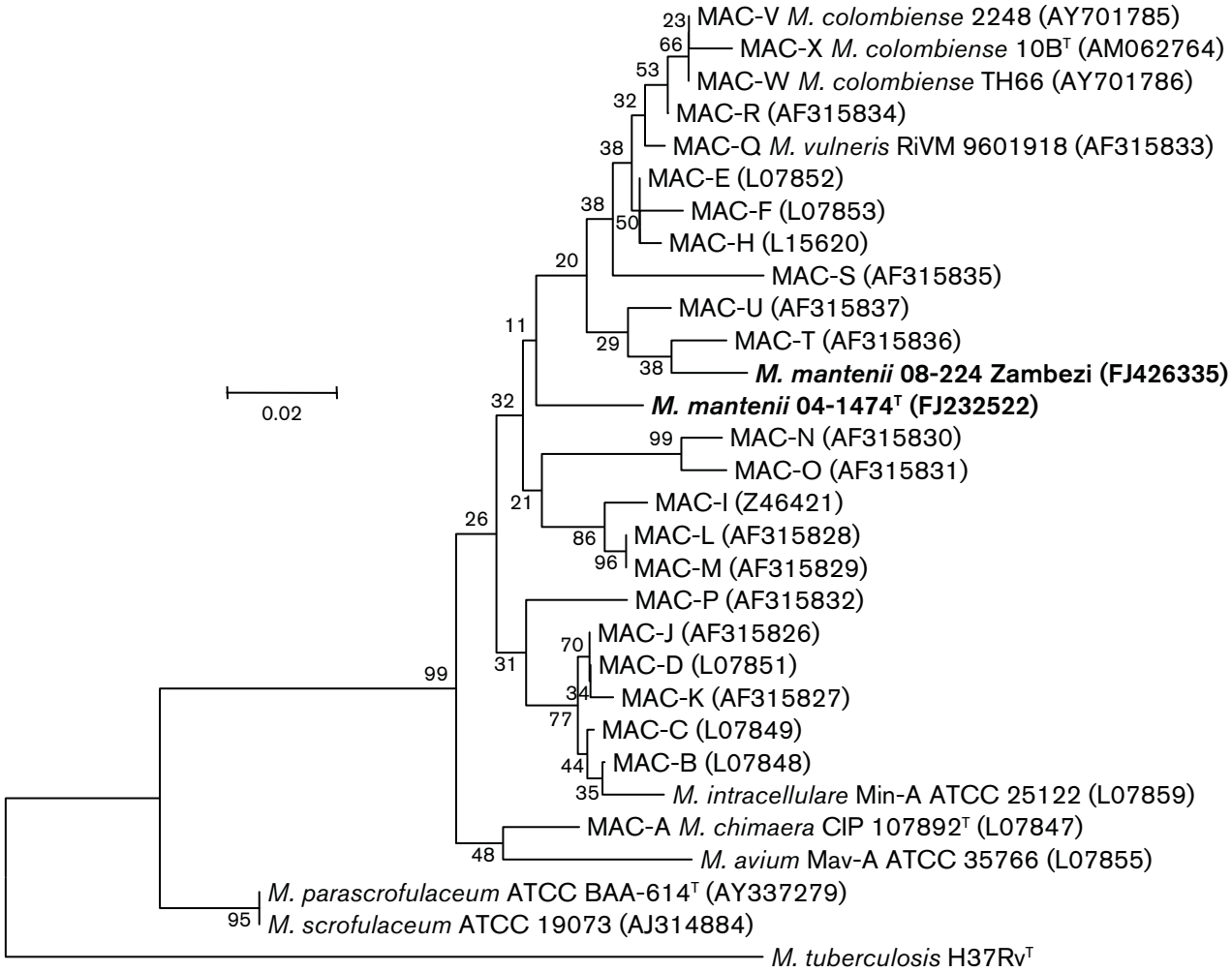


Supplementary Fig. S1. Phylogenetic relationship of strains of *Mycobacterium mantenii* sp. nov. and related species of *Mycobacterium*, based on concatenated 16S rRNA, *hsp65* and *rpoB* gene sequences. The neighbour-joining tree was created, bootstrapped 1000 times and visualized with MEGA 4.0. Bootstrap values are indicated at nodes. Bar, 0.005 substitutions per nucleotide position.



Supplementary Fig. S2. Phylogenetic relationships of strains of *M. mantenii* sp. nov. and related species of *Mycobacterium*, based on 16S–23S ITS sequences. The neighbour-joining tree was created, bootstrapped 1000 times and visualized with MEGA 4.0. Bootstrap values are indicated at nodes. Bar, 0.02 substitutions per nucleotide position.

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