

## *Mycobacterium sherrisii* sp. nov., a slow-growing non-chromogenic species

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'*Mycobacterium sherrisii*' is an undescribed species that appears to be emerging, in particular, among HIV-positive patients originating from Africa. To describe '*M. sherrisii*', to ensure that the species name is validly published and to define its phylogenetic position, we collected 11 of these strains reported in five previous studies, and subjected them to biochemical identification, cell-wall mycolic acid analysis and sequencing of multiple housekeeping genes. The bacteria formed smooth and generally non-chromogenic colonies after 2–3 weeks of subculture at 24–37 °C; photochromogenic and scotochromogenic pigmentation were exhibited by three and two strains, respectively. The strains were positive for the heat-stable catalase test, but negative in tests for hydrolysis of Tween 80, nitrate reduction,  $\beta$ -glucosidase and 3-day arylsulfatase. Mycolic acid patterns, obtained by HPLC, resembled a trimodal profile similar to those of type strains of *Mycobacterium simiae*, *Mycobacterium lentiflavum*, *Mycobacterium triplex* and *Mycobacterium genavense*. The 16S rRNA gene sequences of the 11 strains differed by 4 bp (99.7% similarity) from that of the type strain of the closest related species, *M. simiae* ATCC 25275<sup>T</sup>. Levels of internal transcribed spacer (ITS) and partial *hsp65* and *rpoB* gene sequence similarity between the two taxa were 95.8% (271/283 bp), 97.5% (391/401 bp) and 95.2% (700/735 bp), respectively. On the basis of these results, we propose the formal recognition of *Mycobacterium sherrisii* sp. nov. The type strain is 4773<sup>T</sup> (=ATCC BAA-832<sup>T</sup>=DSM 45441<sup>T</sup>).

**Abbreviations:** ITS, internal transcribed spacer; PNB, para-nitrobenzoic acid; TCH, thiophene-2-carboxylic hydrazide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences of strain 4773<sup>T</sup> are AY353699, DQ185132, AY365190 and GQ166762. Sequences of the 16S rRNA (EU883389), ITS (GU226195) and *hsp65* (DQ523524) genes have also been stored.

In 2004, a group of five *Mycobacterium* isolates related to, but different from, *Mycobacterium simiae* was first described and provisionally named as representing '*Mycobacterium sherrisii*' (Selvarangan *et al.*, 2004). Additional clinical isolates of '*M. sherrisii*' have since been recovered. Recently described strains were linked to an African source, as they were isolated from patients who were currently (Crump *et al.*, 2009) or had previously been living in Africa

(Gämperli *et al.*, 2005; Loulergue *et al.*, 2007; Tortoli *et al.*, 2007a). For two strains isolated in Italy and one in Thailand, no evident link with Africa was detected (Tortoli *et al.*, 2007b; Ballard *et al.*, 2007), while for the five strains of the first report (Selvarangan *et al.*, 2004) one was isolated in Germany and the others in three different states of the USA. From the available literature, '*M. sherrisi*' seems to be an increasingly recognized and clinically relevant non-tuberculous species of the genus *Mycobacterium*, which is encountered in HIV-positive patients in particular, who may develop invasive and disseminated '*M. sherrisi*' disease.

In the initial description of '*M. sherrisi*' (Selvarangan *et al.*, 2004), the rules of the Bacteriological Code were not fully met. To clarify '*M. sherrisi*' as a recognized species and to define its phylogenetic position based on an extended set of isolates, we collected 11 previously reported isolates with that designation. All 11 strains were subjected to biochemical identification, cell-wall mycolic acid analysis and sequencing of multiple housekeeping genes (Stackebrandt *et al.*, 2002).

We included the five strains (4733, VA2, 283416, W55 and W58) reported in the primary publication (Selvarangan *et al.*, 2004), three strains (FI-94099, FI-95229 and FI-05200) reported in Italy (Tortoli *et al.*, 2007a, b), the two strains reported from Tanzania (Crump *et al.*, 2009) and a novel isolate from Tanzania. For all 11 isolates, the full 16S rRNA gene, the 16S–23S internal transcribed spacer (ITS) region and partial *hsp65* and *rpoB* genes were sequenced by using previously published approaches (Springer *et al.*, 1996; Roth *et al.*, 1998; Telenti *et al.*, 1993; Adékambi *et al.*, 2003).

The sequences obtained were compared with those in the GenBank/EMBL/DDBJ sequence databases. The full 16S rRNA gene sequences of the 11 strains were aligned with those of the type strains of closely related mycobacteria by using CLUSTAL\_X software (Thompson *et al.*, 1997). The resulting topology and tree were inferred via the neighbour-joining method, visualized by using the MEGA 4.0 software package (Tamura *et al.*, 2007) and evaluated by bootstrap analyses based on 1000 resamplings. The 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences were concatenated (Stackebrandt *et al.*, 2002) and similarly aligned.

In addition, extensive phenotypic identifications were repeated for all 11 strains. For biochemical and phenotypic identification, the following characteristics were examined: colony morphology, ability to grow at 25–42 °C, niacin accumulation, nitrate reduction,  $\beta$ -glucosidase, Tween 80 hydrolysis, 3-day arylsulfatase, urease, tellurite reduction, heat-stable (68 °C) and semi-quantitative catalase, growth rate, pigmentation, growth on MacConkey agar, and tolerance to thiophene-2-carboxylic hydrazide (TCH; 5  $\mu\text{g ml}^{-1}$ ), oleic acid (250  $\mu\text{g ml}^{-1}$ ), para-nitrobenzoic acid (PNB; 500  $\mu\text{g ml}^{-1}$ ), thiacetazone (10  $\mu\text{g ml}^{-1}$ ), hydroxylamine (1  $\mu\text{g ml}^{-1}$ ) and isoniazid (1  $\mu\text{g ml}^{-1}$ ), all in Middlebrook 7H10 agar, following the guidelines of

Kent & Kubica (1985). The cell-wall mycolic acid composition was investigated by HPLC using methods recommended by the Centers for Disease Control and Prevention (CDC, 1996). The HPLC mycobacterium library (available online at <http://www.MycobacToscana.it>) was used for visual comparisons.

Antibiotic susceptibility testing was performed by using the broth microdilution method, as advocated by the Clinical Laboratory Standards Institute (NCCLS, 2003). The test panel included rifampicin, rifabutin, ethambutol, streptomycin, sulfamethoxazole, minocycline, clarithromycin, amikacin, ciprofloxacin, gatifloxacin, moxifloxacin and linezolid. Results are presented in Table 1.

The 11 strains formed mature colonies after 2–3 weeks of incubation on agar- as well as egg-based media at 25–37 °C; growth at 42 °C was only observed for strains W55, NLA000800396, NLA000800640 and VA2. Colonies were smooth and generally non-chromogenic, with photochromogenicity noted in strains W55 and 283416 and scotochromogenicity in the three strains from Italy (FI-94099, FI-05200 and FI-95229). In the description by Selvarangan *et al.* (2004), photochromogenicity was noted only in strain VA2, which was non-chromogenic in the current analysis. Ziehl–Neelsen staining of bacterial smears revealed acid–alcohol-fast rods, with occasional coccid forms. Biochemical identification of the 11 strains revealed uniformly positive semi-quantitative and heat-stable catalase tests as well as tolerance to isoniazid, TCH, PNB, thiacetazone and hydroxylamine. Tests for Tween hydrolysis, nitrate reduction,  $\beta$ -glucosidase and growth on MacConkey agar were consistently negative. Tests for niacin accumulation, tellurite reduction, urease, 3-day arylsulfatase and tolerance to oleic acid yielded mixed results for the 11 strains, as detailed in Table 2; all but one of the 11 strains were urease-positive. These results show

**Table 1.** Minimal inhibitory concentrations (MIC) of the 11 '*M. sherrisi*' strains

Antibiotic	MIC ( $\mu\text{g ml}^{-1}$ )	Interpretation
Ciprofloxacin	>16	Resistant
Gatifloxacin	$\geq 8$	Resistant
Moxifloxacin	2–4	Resistant
Linezolid	8–64	Resistant*
Rifampicin	4 to >8	Resistant
Rifabutin	$\leq 0.06$ –0.5	Susceptible
Sulfamethoxazole	38–152	Resistant
Minocycline	$\geq 32$	Resistant
Ethambutol	8–2	Resistant*
Clarithromycin	2–8	Susceptible†
Amikacin	4 to >64	Variable
Streptomycin	32 to >64	Resistant

\*One strain was susceptible.

†One strain was resistant.

**Table 2.** Results of biochemical and phenotypic identification of 11 '*M. sherrisii*' strains

Taxa: 1, strain 4773<sup>T</sup>; 2, VA2; 3, 283416; 4, W55; 5, W58; 6, NLA000800396; 7, NLA000800640; 8, NLA000801736; 9, FI-94099; 10, FI-95229; 11, FI-05200; 12, *Mycobacterium simiae*. Tests uniformly positive in all the strains (growth at 25–37 °C, semi-quantitative and heat-stable catalase, tolerance to isoniazid, TCH, PNB, thiacetazone and hydroxylamine) or uniformly negative in all the strains (Tween 80 hydrolysis, nitrate reduction, β-glucosidase, growth on MacConkey agar) have been omitted. +, Positive; –, negative; +/-, variable result.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Niacin	–	+	–	+	+	+	+	+	–	–	+	+/-
Tellurite reduction	+	+	+	–	+	–	+	–	+	+	+	+
3-Day arylsulfatase	–	–	–	–	–	+	–	–	–	–	+	–
Urease	+	+	+	+	+	+	+	–	+	+	+	+
Growth at 42 °C	–	+	–	+	–	+	+	–	–	–	–	–
Pigmentation*	NC	NC	PC	PC	NC	NC	NC	NC	SC	SC	SC	PC
Tolerance to oleate	–	–	–	+	–	+	+	+	+	+	–	+
Reference†	RS	RS	RS	RS	RS	JC	JC	This study	ET1	ET1	ET2	ET3

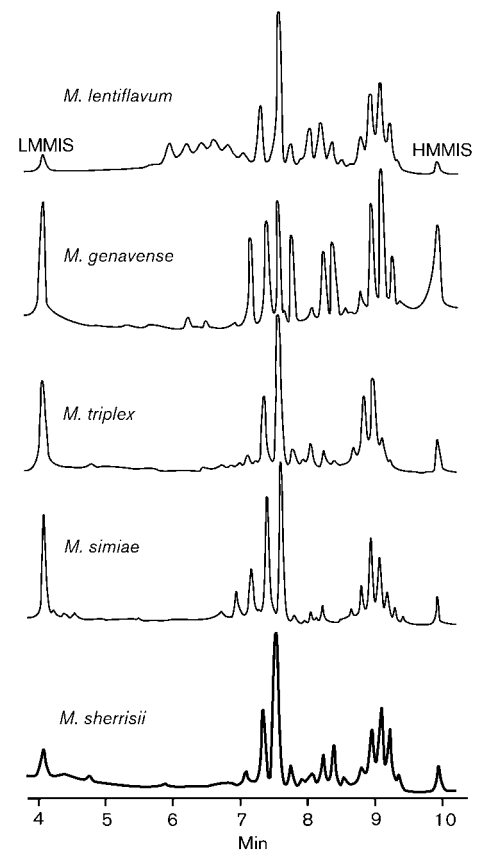
\*NC, non-chromogenic; PC, photochromogenic; SC, scotochromogenic.

†RS, Selvarangan *et al.* (2004); JC, Crump *et al.* (2009); ET1, Tortoli *et al.* (2007b); ET2, Tortoli *et al.* (2007a); ET3, Tortoli *et al.* (1997).

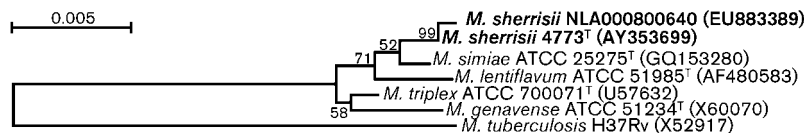
little biochemical distinction from closely related species, most notably being similar to those obtained for *Mycobacterium simiae* (Table 2). The occurrence of photochromogenic strains, a hallmark feature of *M. simiae* (Tortoli *et al.*, 1997), is especially problematic in the distinction between '*M. sherrisii*' and *M. simiae*.

HPLC of the cell-wall mycolic acid content of strain 4773<sup>T</sup> revealed three late clusters of peaks most closely related to the patterns observed for type and reference strains of *M. simiae*, *Mycobacterium lentiflavum*, *Mycobacterium triplex* and *Mycobacterium genavense* present in the HPLC mycobacterium library (Fig. 1). Minor variations in peak heights and distributions were noted but these did not allow identification to the species level.

The 16S rRNA gene sequence of strain 4773<sup>T</sup> (GenBank accession no. AY353699) differed by 4 bp from that of *M. simiae* ATCC 25275<sup>T</sup> (GQ153280; 99.7% similarity, 1459/1463 bp), by 7 bp from that of *M. triplex* ATCC 700071<sup>T</sup> (U57632; 99.5% similarity, 1453/1460 bp), by 10 bp from that of *M. lentiflavum* ATCC 51985<sup>T</sup> (AF480583; 99.3% similarity, 1445/1455 bp) and by 16 bp from that of *M. genavense* ATCC 51234<sup>T</sup> (X60070; 98.9% similarity, 1421/1437 bp). The neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences of the 11 new isolates and of those of the most closely related species, is shown in Fig. 2. The low bootstrap values observed in the phylogenetic analysis based on 16S rRNA gene sequences indicate a limitation of this approach in non-tuberculous mycobacteria; hence, an analysis of multiple concatenated sequences of housekeeping genes is preferred (Stackebrandt *et al.*, 2002). Microheterogeneity was recorded within the 16S rRNA gene, wherein strain NLA000800640 (EU883389; from Tanzania) differed by 1 bp from the remaining strains and by 5 bp from *M. simiae* ATCC 25275<sup>T</sup>. Two distinct ITS sequevars were noted; ten isolates shared the full sequence of strain 4773<sup>T</sup> (DQ185132; Msh-A), and one Tanzanian strain



**Fig. 1.** Mycolic acid patterns of strain 4773<sup>T</sup>, *M. simiae*, *M. triplex*, *M. genavense* and *M. lentiflavum* obtained by HPLC. Three late clusters of peaks are present for all five taxa, with minor differences in peak height and distribution. First (LMMIS) and last (HMMIS) peaks are the low- and high-molecular-mass internal standards.



**Fig. 2.** Neighbour-joining phylogenetic tree showing the relationship between strain 4773<sup>T</sup> and related species of the genus *Mycobacterium* based on 16S rRNA gene sequences. The tree was created, bootstrapped (1000 replications) and visualized with MEGA 4.0 (Tamura *et al.* 2007). Bootstrap values are indicated at nodes. Bar, 0.005 substitutions per nucleotide position.

(NLA000801736) had a C→T mutation at position 31 (sequevar Msh-B; GU226195). The Msh-A ITS sequence differed by 12 bp from the *M. simiae* Msi-A sequevar sequence (Y14186; 95.8% similarity, 271/283 bp), by 31 bp from that of *M. triplex* (AF214587; 89.2% similarity, 256/287 bp) and *M. genavense* (Y14183; 89.2% similarity, 255/286 bp) and by 33 bp from that of *M. lentiflavum* ATCC 51985<sup>T</sup> (AF317658; 88.6% similarity, 256/289 bp); the Msh-B sequence differed by 13 bp from the *M. simiae* Msi-A sequevar sequence (270/283 bp).

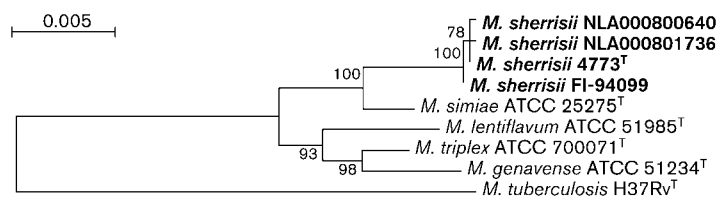
Two sequevars of the partial *hsp65* gene were noted; the five strains from Selvarangan *et al.* (2004) as well as the strains from Tanzania all shared identical sequences (AY365190); this sequence differed by 10 bp from that of *M. simiae* ATCC 25275<sup>T</sup> (GQ153292; 97.5% similarity, 391/401 bp), by 22 bp from that of *M. triplex* CIP 106108<sup>T</sup> (AF547882; 94.8% similarity, 403/425 bp) and by 25 bp from that of *M. genavense* DSM 44424 (AF547837; 94.1% similarity, 399/424 bp) and *M. lentiflavum* CIP 105465<sup>T</sup> (AF547851; 94.1% similarity, 400/425 bp). The strains from Italy (FI-94099, FI-95229 and FI-05200) had a partial *hsp65* gene sequence (DQ523524) that differed by 1 bp from that of strain 4773<sup>T</sup> and by 9 bp from that of *M. simiae* ATCC 25275<sup>T</sup> (GQ153292).

All 11 novel strains had identical *rpoB* gene sequences (GenBank accession no. GQ166762), which differed by 35 bp (95.2% similarity, 700/735 bp) from that of *M. simiae* ATCC 25275<sup>T</sup> (GQ153313), by 45 bp from that of *M. triplex* ATCC 700071<sup>T</sup> (GQ153311; 93.7% similarity,

671/716 bp) and by 53 bp from that of *M. lentiflavum* CIP 105465<sup>T</sup> (EU109300; 92.6% similarity, 663/716 bp). No comparative sequences were available in GenBank for *M. genavense*. Phylogenetic analysis based on concatenated 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences thus supported a separate species status for the 11 novel strains, with high bootstrap support (Fig. 3).

Although there was a single base-pair difference in the 16S rRNA gene sequence, it is considered that strain NLA000800640 (from Tanzania) and the remaining new isolates belong to a single species, based on the sequences in target genes other than 16S rRNA, which are identical to those of the remaining strains, as well as overlapping biochemical and phenotypic features. Microheterogeneity in the 16S rRNA gene has been previously described in non-tuberculous mycobacteria (Kirschner & Böttger, 1992; van Ingen *et al.*, 2009). The single nucleotide polymorphisms in the 16S rRNA and *hsp65* genes and ITS reflect the evolutionary divergence among non-tuberculous mycobacterial infections that are contracted from the environment.

The 11 described strains represent a separate species that is phylogenetically and phenotypically related to *M. simiae*, *M. lentiflavum*, *M. triplex* and *M. genavense*. Biochemical features offered little distinction from *M. simiae*, *M. lentiflavum* and *M. triplex* (Wayne *et al.*, 1996; Tortoli *et al.*, 1997); however, the observed growth on conventional egg- and agar-based media offered differentiation from the fastidious species *M. genavense*. Pigmentation varied



**Fig. 3.** Neighbour-joining phylogenetic tree showing the relationship between strain 4773<sup>T</sup> and related species of the genus *Mycobacterium* based on concatenated 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences. The tree was created, bootstrapped (1000 replications) and visualized with MEGA 4.0 (Tamura *et al.*, 2007). Bootstrap values are indicated at nodes. Bar, 0.005 substitutions per nucleotide position. The GenBank accession numbers of the ITS, *hsp65* and *rpoB* gene sequences of *M. simiae* ATCC 25275<sup>T</sup> are Y14186, AF547875 and GQ153313, respectively; of *M. lentiflavum* ATCC 51985<sup>T</sup> are AF317658, AF547851 and EU109300; of *M. genavense* ATCC 51234<sup>T</sup> are Y14183 and AF547837 (no *rpoB* sequences available); for *M. triplex* ATCC 700071<sup>T</sup> are Y14189, AF547882 and GQ153311; and for *M. tuberculosis* H37Rv<sup>T</sup> is NC\_000962. Accession numbers of 16S rRNA gene sequences and strain 4773<sup>T</sup> sequences are given in the text and in Fig. 2.

between strains and experiments and is probably influenced by culture conditions.

HPLC revealed a pattern of mycolic acids that closely resembled those of *M. simiae*, *M. lentiflavum*, *M. triplex* and *M. genavense*. Therefore, only genetic markers clearly indicated the separate species status of these 11 strains. The unique 16S rRNA gene sequences (Fig. 2), as well as the concatenated 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences, supported the separate species status, with high bootstrap support (Figs 2 and 3).

The spectrum of human disease caused by the studied isolates includes HIV-associated pulmonary and disseminated disease as well as pulmonary disease in HIV-negative patients, and thus resembles infections attributable to *M. simiae* and *M. genavense* (Griffith *et al.*, 2007).

Based on the available genetic, phenotypic and clinical data, it is concluded that the 11 strains previously designated as representing '*M. sherrisii*' warrant recognition as a separate species of the genus *Mycobacterium*, with the name *M. sherrisii* sp. nov.

### Description of *Mycobacterium sherrisii* sp. nov.

*Mycobacterium sherrisii* (sher.ri'si.i. N.L. gen. masc. n. *sherrisii* of Sherris, in honour of John C. Sherris for his contributions to the field of clinical microbiology).

Stains acid–alcohol-fast. Visible growth from a small inoculum requires >7 days; on Middlebrook 7H10 medium colonies are small, smooth, white and non-chromogenic; occasionally, rough colonies are noted. Photochromogenic and scotochromogenic strains can occur. Growth is fastest at 30–37 °C. Positive for semi-quantitative and heat-stable catalase tests as well as tolerance to isoniazid, TCH, PNB, thiacetazone and hydroxylamine, and negative in tests for Tween hydrolysis, nitrate reduction,  $\beta$ -glucosidase and growth on MacConkey agar. Phylogenetically, most closely related to *M. simiae* and *M. triplex*.

The type strain is 4773<sup>T</sup> (=ATCC BAA-832<sup>T</sup>=DSM 45441<sup>T</sup>); strains Va2 and W55 have been deposited as ATCC BAA-833 and ATCC BAA-834, respectively.

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