Mycobacterium europaeum sp. nov., a scotochromogenic species related to the *Mycobacterium simiae* complex

Enrico Tortoli,[1] Erik C. Böttger,[2] Anna Fabio,[3] Enevold Falsen,[4] Zoe Gitti,[5] Antonella Grottola,[3] Hans-Peter Klenk,[6] Roberta Mannino,[1] Alessandro Mariottini,[7] Massimino Messinò,[3] Monica Pecorari[3] and Fabio Rumpianesi[3]

Correspondence
Enrico Tortoli
e.tortoli@libero.it

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

[2]Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland

[3]Laboratory of Microbiology and Virology, Modena University Hospital, Modena, Italy

[4]Culture Collection University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden

[5]Clinical Microbiology Laboratory, Mycobacteriology Department, University Hospital of Crete, Herakleon, Greece

[6]DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

[7]Cytogenetics and Genetics Unit, Careggi University Hospital, Florence, Italy

Four strains isolated in the last 15 years were revealed to be identical in their 16S rRNA gene sequences to MCRO19, the sequence of which was deposited in GenBank in 1995. In a polyphasic analysis including phenotypic and genotypic features, the five strains (including MCRO19), which had been isolated in four European countries, turned out to represent a unique taxonomic entity. They are scotochromogenic slow growers and are genetically related to the group that included *Mycobacterium simiae* and 15 other species. The novel species *Mycobacterium europaeum* sp. nov. is proposed to accommodate these five strains. Strain FI-95228^T (=DSM 45397^T =CCUG 58464^T) was chosen as the type strain. In addition, a thorough revision of the phenotypic and genotypic characters of the species related to *M. simiae* was conducted which leads us to suggest the denomination of the '*Mycobacterium simiae* complex' for this group.

A unique genetic signature characterizes a limited number of slowly growing non-tuberculous mycobacteria; it is represented by a 12 nt deletion in the 16S rRNA gene starting at *Escherichia coli* position 459. About 20 years ago, when genetic sequencing of the 16S rRNA gene began to be used for the taxonomic characterization of mycobacteria, *Mycobacterium simiae* appeared to be the only slowly growing mycobacterial species presenting the aforesaid deletion (Rogall *et al.*, 1990; Kirschner *et al.*, 1993b). *Mycobacterium interjectum* (Springer *et al.*, 1993) was the first species to be newly described that was recognized to share this genetic marker with *M. simiae* and, in subsequent years, the group of *M. simiae*-like organisms has steadily extended and includes 16 species at present (Böttger *et al.*, 1993; Fanti *et al.*, 2004; Floyd *et al.*, 1996, 2000; Haas *et al.*, 1997; Karassova *et al.*, 1965; Levi *et al.*, 2003; Meier *et al.*, 1993; Pourahmad *et al.*, 2008; Selvarangan *et al.*, 2004; Springer *et al.*, 1993, 1996b; Torkko *et al.*, 2002; Tortoli *et al.*, 2005a; Turenne *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA gene and ITS of strains FI-95228^T, MCRO19 and FI-09129 are HM022196–HM022198, those for the *hsp65* gene sequences of strains CCUG 52298 and FI-95228^T are HM022219 and HM022220 and those for the *rpoB* gene sequences of strains FI-95228^T, CCUG 52298 and FI-09129 are HM 022215, HM022217 and HM022218. The following sequences were also determined: 16S rRNA and ITS sequences of *M. kubicae* CIP 106428^T (HM022200), *M. parmense* CIP 107385^T (HM022201) and *M. stomatepiae* DSM 45059^T (HM022202), ITS sequences of *M. heidelbergense* DSM 44471^T (HM022203), *M. intermedium* DSM 44049^T (HM022204) and *M. montefiorense* ATCC BAA-256^T (HM754632) and *rpoB* sequences of *M. florentinum* DSM 44852^T (HM022205), *M. genavense* FI-06288 (HM022216), *M. heidelbergense* DSM 44471^T (HM132044), *M. interjectum* DSM 44064^T (HM022207), *M. intermedium* DSM 44049^T (HM022208), *M. kubicae* CIP 106428^T (HM022206), *M. montefiorense* DSM 44602^T (HM022209), *M. palustre* DSM 44572^T (HM022210), *M. parmense* CIP 107385^T (HM022211), *M. saskatchewanense* DSM 44616^T (HM022212), *M. stomatepiae* DSM 45059^T (HM022213) and the *hsp65* sequence of *M. parmense* CIP 107385^T (HM022199).

2004a, b). Similarity of the 16S rRNA gene sequence, along with a number of shared phenotypic features, supports the hypothesis that these species belong to a homogeneous group characterized by close phylogenetic relatedness.

The characterization of several slowly growing strains presenting the genetic characteristic of *M. simiae* allowed us to propose a novel species, and provided the opportunity for a re-examination of this unique group of mycobacteria in light of a polyphasic approach including the analysis of multiple genetic targets.

Mycobacterium isolates

Four strains had been isolated independently between 1995 and 2009 in various countries of Europe (Table 1). Three of them had been grown from sputum: two (FI-95228^T and FI-09129) from Italian patients, in different Italian hospitals, and one (GN10643) from a Greek patient. Strain CCUG 52298 had been isolated in Sweden from a jaw gland of a subject with neck adenopathy. No information is available on the origins of the fifth strain, MCRO19 (Springer *et al.*, 1996a).

Biochemical and cultural tests

Major biochemical tests (Kent & Kubica, 1985) recommended for the speciation of mycobacteria were performed as described previously; they included niacin accumulation, nitrate reduction, Tween 80 hydrolysis (10 days), arylsulfatase (3 days), urease, β-glucosidase, tellurite reduction and catalase.

The five strains investigated presented identical biochemical patterns characterized by negative results for the majority of the tests performed. Tests for tellurite reduction, thermostable catalase and production of foam >45 mm in the semiquantitative catalase test were, in contrast, uniformly positive.

All the strains grew yellow, smooth, scotochromogenic colonies on solid media (Löwenstein–Jensen, Middlebrook 7H11) after about 3 weeks of incubation at 37 °C. Growth was slower at 30 °C and absent at 42 °C. Growth of the strains was not inhibited on selective media [supplemented separately with (ml^{-1}) 500 μg *p*-nitrobenzoate, 5 μg thiophene-2-carboxylic hydrazide, 10 μg thiacetazone, 500 μg hydroxylamine, 1 μg isoniazid and 250 μg oleate], while growth was not achieved on MacConkey agar without crystal violet.

The cells were characterized by acid-fastness and bacillary morphology; spores or branching production were not observed.

Lipid analyses

For identification of the mycolic acids present in the cell wall, two-dimensional TLC was carried out following the standard procedure (Minnikin *et al.*, 1975). The two strains selected for this test (FI-95228^T and GN10643) revealed the presence of α-mycolates, ketomycolates and wax esters, a pattern identical to those of *Mycobacterium parascrofulaceum* and *Mycobacterium parmense* and very close to those of *M. simiae* and related species (*Mycobacterium genavense*, *Mycobacterium heidelbergense* and *Mycobacterium intermedium*). Although the latter three species also possess α′-mycolates, they share with the test strains the presence of wax esters (carboxymycolates and 2-eicosanol homologues) and the absence of methoxymycolates.

HPLC analysis was performed as reported before. Cell-wall lipids were saponified and chloroform-extracted, derivatized to UV-absorbing esters and then separated with a gradient of methanol and methylene chloride (CDC, 1996). The five strains presented a common pattern characterized by the presence of three clusters of peaks with the first, more prominent, emerging clearly earlier than the others (Fig. 1). This pattern, which grossly resembles those of *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*, is also shared by several *M. simiae*-like species (*M. interjectum*, *M. parascrofulaceum* and *M. parmense*).

Antimicrobial susceptibility

MICs were determined using a commercially available microdilution method (MAIslow; Sensititer) including the major drugs with potential activity against slowly growing non-tuberculous mycobacteria: amikacin, streptomycin, ciprofloxacin, moxifloxacin, clarithromycin, ethambutol, linezolid, minocycline, rifampicin, rifabutin and trimethoprim-sulfamethoxazole. The strains were characterized by susceptibility to all of the antimicrobials tested except for quinolones, which were consistently ineffective. The MICs of clarithromycin and rifamycins were particularly low.

Table 1. Microbiological and patient clinical information for strains of *Mycobacterium europaeum* sp. nov.

No information was available for strain MCRO19. M, Male; F, female; TB, tuberculosis; NK, not known.

Strain	Age (years)	Sex	Microscopy	Culture	Disease	Treatment	Location	Year
FI-95228^T	81	M	+	+	Cavitary pneumopathy	NK	Italy, Florence	1995
CCUG 52298	28	F	NK	NK	Jaw gland adenopathy	NK	Sweden	2004
FI-09129	37	M	−	+	None	No	Italy, Modena	2009
GN10643	88	M	−	+	Pneumonia	Anti-TB (2 months)	Greece	2009

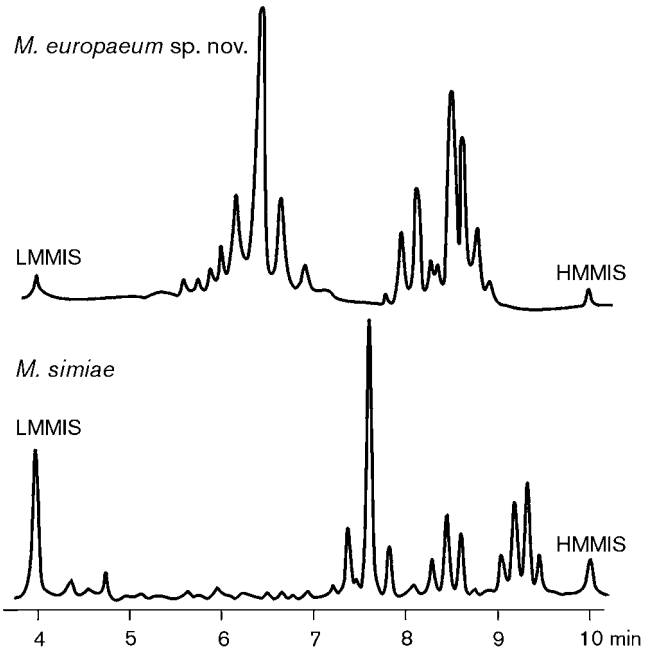

Fig. 1. Representative HPLC mycolic acid pattern of *Mycobacterium europaeum* sp. nov. FI-95228^T compared with that of *M. simiae* DSM 44165^T. LMMIS, Low molecular mass internal standard; HMMIS, high molecular mass internal standard.

Genetic sequencing

Double-strand sequences from the five strains included in the study were determined using BigDye Terminator chemistry on an AB3730 DNA sequencer (Applied Biosystems) following the standard protocol of the supplier. The regions investigated included the 16S rRNA gene (Kirschner *et al.*, 1993a), the ITS1 spacer interposed between the 16S and 23S rRNA genes (Roth *et al.*, 1998) and the hypervariable regions of the *hsp65* (McNabb *et al.*, 2004) and *rpoB* (Adékambi *et al.*, 2003) genes.

The five test strains were characterized by identical sequences in the 16S rRNA gene (1475 bp). In ITS1, three sequevars were present, with the sequence of strain FI-95228^T differing by one base from that of MCRO19 and by three bases from the sequence shared by the other three strains. The two sequevars characterizing *hsp65* differed in only one nucleotide and were shared by three and two strains, respectively. In the 711 bp region of the *rpoB* gene, three sequevars were detected, one shared by three strains (including strain FI-95228^T), which differed in two bases from the sequence of FI-09129 (similarity 99.7 %) and in 17 bases from that of CCUG 52298 (97.6 %). Once the combinations of sequevars were compared, only two strains, GN10643 and MCRO19, were identical in all the regions investigated.

To determine the similarity with known species, the sequences of the five test strains were aligned using BLAST software (Altschul *et al.*, 1997) with entries present in the GenBank database. In the 16S rRNA gene, the species presenting the highest similarity with the sequence of the test strains was *M. parascrofulaceum* (six mismatches in 1418 bp for the type strain; 99.6 % similarity).

Phylogenetic analysis

Phylogenetic analysis was conducted for all the genetic regions investigated, after trimming the sequences to start and finish at the same position; *Mycobacterium tuberculosis* ATCC 27294^T was chosen as an outgroup. The neighbour-joining method (Saitou & Nei, 1987), supported by the MEGA 4.1 software (Tamura *et al.*, 2007), was used for the construction of phylogenetic trees; 1000 bootstrap replications were implemented.

As expected, in the tree based on the 16S rRNA gene (Fig. 2), the species of the *M. simiae* group clustered together and were clearly separate from other species. Dendrograms reconstructed from ITS1, *hsp65* and *rpoB* sequences were characterized by very poor robustness (a large number of nodes presented bootstrap support <50 %), thus revealing little phylogenetic value (not shown). To try to overcome the limited confidence of the topology of these trees and to elucidate better the relatedness of the *M. simiae*-like species, the sequences of the 16S rRNA gene, ITS1, *hsp65* and *rpoB* were concatenated into a 2933 bp sequence (Devulder *et al.*, 2005; Stackebrandt *et al.*, 2002). To make this feasible, the full set of species related to *M. simiae* was completed by determining and depositing in GenBank a number of sequences for type strains that were not yet represented.

The phylogenetic relatedness among the species of the *M. simiae* group appeared much more evident once the concatenated sequences were compared (Fig. 3). Within the phylogenetic tree, two major clades were clearly recognizable. Interestingly, the variability present at the level of phenotype was in agreement with the two phylogenetic branches. The first cluster (A) included the species *Mycobacterium florentinum*, *M. stomatepiae*, *M. genavense*, *M. triplex*, *M. lentiflavum*, *M. sherrisii*, *M. simiae* and *M. montefiorense* that were characterized by a unique three-clustered HPLC mycolic acid pattern (Fig. 1) and by extreme drug-resistance (Cingolani *et al.*, 2000; Rastogi *et al.*, 1992; Tortoli, 2003, 2006; Tortoli *et al.*, 2002, 2005a) (no susceptibility data are available for *M. montefiorense* or *M. stomatepiae*). *M. parascrofulaceum* and the five strains described in this study, which constituted the second cluster (B), were characterized, in contrast, by HPLC profile similar to those of the *Mycobacterium avium* complex (Fig. 1) and susceptibility to most of the anti-mycobacterial drugs (Turenne *et al.*, 2004b). The remaining six species, found on isolated branches, were more heterogeneous at the phenotypic level.

The idea of including genetically related, not easily differentiable, mycobacteria (e.g. *M. avium* and *Mycobacterium intracellulare* or *Mycobacterium terrae* and *Mycobacterium nonchromogenicum*) in a single group, or complex, has been

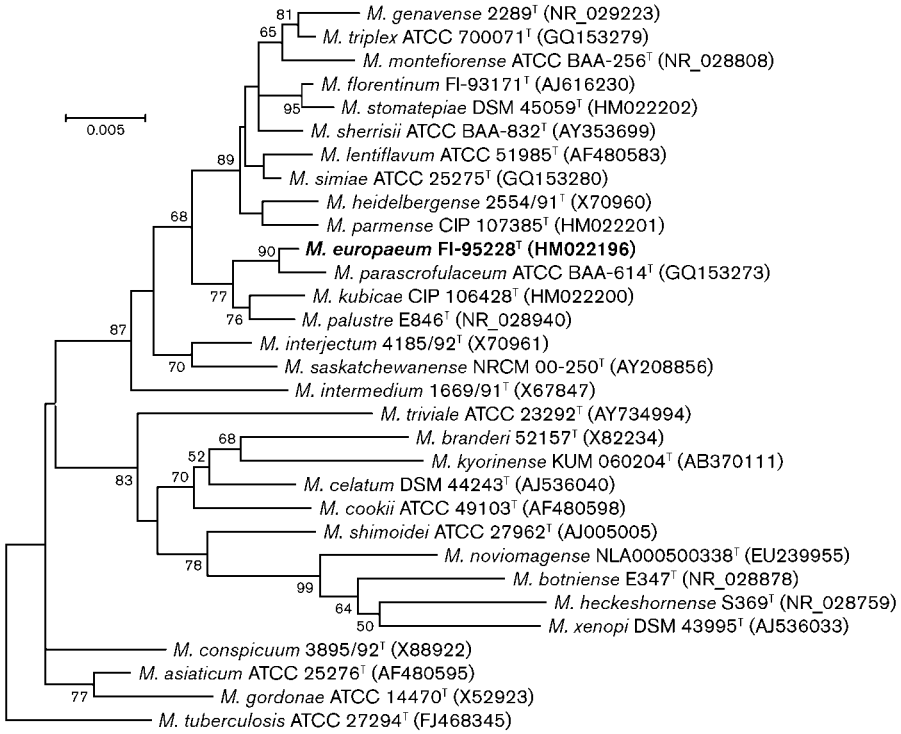

Fig. 2. Phylogenetic tree based on 16S rRNA sequences constructed using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values >50 % are given at nodes. Bar, 0.005 substitutions per nucleotide position.

very successful in the past. A thorough analysis of sequences of the 16S rRNA gene, ITS1, *hsp65* and *rpoB* of the strains described in this study reveals clear relatedness with 16 other mycobacterial species which, along with the novel species represented by these strains, constitute a homogeneous group for which we suggest the name '*Mycobacterium simiae* complex'. The introduction of the *M. simiae* complex, encompassing all the species related to *M. simiae*, besides being supported by genotypic and phenotypic similarities, would represent a simplification that will certainly be welcomed by clinicians.

Description of *Mycobacterium europaeum* sp. nov.

Mycobacterium europaeum (eu.ro.pae′um. L. neut. adj. *europaeum* pertaining to Europe, as the first five known strains were isolated in four European countries).

Cells are acid-fast and Gram-stain-positive, not motile and do not produce spores. Colonies are smooth and strongly yellow pigmented and grow on egg- and agar-based solid media for mycobacteria after 2–3 weeks of incubation at 37 °C. Major biochemical features include positive catalase

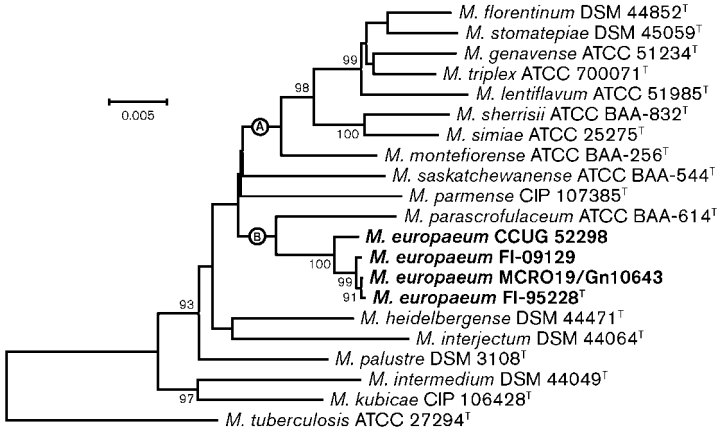

Fig. 3. Phylogenetic tree based on concatenated sequences (16S rRNA+ITS1+*hsp65*+*rpoB*) constructed using the neighbour-joining method (bootstrapped 1000 times). Bootstrap values ⩾90 % are given at nodes. *M. tuberculosis* ATCC 27294T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position. A and B represent clades described in the text.

Table 2. Comparison of variable phenotypic characters among the species belonging to the *M. simiae* complex

Species: 1, *M. europaeum* sp. nov.; 2, *M. simiae*; 3, *M. florentinum*; 4, *M. kubicae*; 5, *M. parascrofulaceum*; 6, *M. genavense*; 7, *M. lentiflavum*; 8, *M. parmense*; 9, *M. heidelbergense*; 10, *M. montefiorense*; 11, *M. interjectum*; 12, *M. saskatchewanense*; 13, *M. intermedium*; 14, *M. palustre*; 15, *M. triplex*; 16, *M. stomatepiae*; 17, *M. sherrisii*. +, Positive; −, negative; V, variable; ±, predominantly positive; ND, no data. Data for reference species were taken from Böttger *et al.* (1993), Fanti *et al.* (2004), Floyd *et al.* (1996, 2000), Haas *et al.* (1997), Karassova *et al.* (1965), Levi *et al.* (2003), Meier *et al.* (1993), Pourahmad *et al.* (2008), Selvarangan *et al.* (2004), Springer *et al.* (1993, 1996b), Torkko *et al.* (2002), Tortoli (2003, 2006), Tortoli *et al.* (1996a, b, 1997, 2002, 2005a, b), Turenne *et al.* (2004a, b) and van Ingen *et al.* (2011).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Niacin accumulation	−	V	−	ND	−	−	−	−	−	−	−	ND	−	ND	−	ND	±
Nitrate reduction	−	−	±	−	−	−	−	−	−	−	−	−	−	V	+	−	−
Tween 80 hydrolysis (10 days)	−	−	−	+	−	−	−	+	+	−	±	+	−	+	−	−	−
Tellurite reduction	+	+	+	ND	−	ND	−	−	ND	ND	+	V	+	ND	ND	+	+
Urease	−	+	+	−	+	+	−	+	+	−	+	−	+	+	+	−	+
Catalase (>45 mm of foam)	+	+	V	+	V	+	−	−	ND	−	ND	+	−	−	+	−	+
Pigmentation*	S	SP	N	S	S	N	S	S	N	N	S	S	P	SP	N	N	SPN
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	−	ND	+	+
Growth at 37 °C	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Growth at 43 °C	−	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	−
TLC pattern†	1, 4, 6	1, 2, 4	1, 3, 4	1, 3, 4	1, 4, 6	1, 2, 4	1, 2, 4	1, 4, 6	1, 2, 4	ND	1, 2, 4	ND	1, 2, 4	ND	ND	ND	ND
HPLC pattern‡	A	B	B	C	A	B	B	A	D	B	A	C	E	C	B	B	B

*S, Scotochromogenic; SP, scotochromogenic or photochromogenic; N, non-chromogenic; SPN, scotochromogenic, photochromogenic or non-chromogenic.

†1, α-Mycolates; 2, α′-mycolates; 3, methoxymycolates; 4, ketomycolates; 5, epoxymycolates; 6, wax esters; 7, omega 1-methoxymycolates.

‡A, *M. avium* complex-like; B, *M. simiae*-like; C, single late cluster; D, *M. malmoense*-like; E, unique.

tests, both semiquantitative (>45 mm) and after incubation at 68 °C, and tellurite reduction. Tests for nitrate reduction, Tween 80 hydrolysis, 3 day arylsulfatase and urease are negative; they are not useful for a certain differentiation from *M. simiae* and other related species (Table 2). The antimicrobial pattern is characterized by susceptibility to amikacin, clarithromycin, linezolid and rifamycins, and by resistance to quinolones. The mycolic acid composition is similar to that of the majority of *M. simiae*-like species but can be distinguished by the lack of α′-mycolates. The HPLC pattern can be confused with those of *M. florentinum*, *M. parascrofulaceum* and *M. parmense*. Unique genetic sequences are possessed in the 16S rRNA gene, ITS1, *hsp65* and *rpoB*.

The type strain is FI-95228^T (=DSM 45397^T =CCUG 58464^T).

Acknowledgements

We thank Mag Lafferty for linguistic revision of the manuscript.

References

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

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Böttger, E. C., Hirschel, B. & Coyle, M. B. (1993). *Mycobacterium genavense* sp. nov. *Int J Syst Bacteriol* **43**, 841–843.

CDC (1996). *Standardized method for HPLC identification of mycobacteria.* Atlanta: Centers for Disease Prevention and Control, US Department of Health and Human Services, Public Health Service.

Cingolani, A., Sanguinetti, M., Antinori, A., Larocca, L. M., Ardito, F., Posteraro, B., Federico, G., Fadda, G. & Ortona, L. (2000). Brief report: disseminated mycobacteriosis caused by drug-resistant *Mycobacterium triplex* in a human immunodeficiency virus-infected patient during highly active antiretroviral therapy. *Clin Infect Dis* **31**, 177–179.

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.

Fanti, F., Tortoli, E., Hall, L., Roberts, G. D., Kroppenstedt, R. M., Dodi, I., Conti, S., Polonelli, L. & Chezzi, C. (2004). *Mycobacterium parmense* sp. nov. *Int J Syst Evol Microbiol* **54**, 1123–1127.

Floyd, M. M., Guthertz, L. S., Silcox, V. A., Duffey, P. S., Jang, Y., Desmond, E. P., Crawford, J. T. & Butler, W. R. (1996). Characterization of an SAV organism and proposal of *Mycobacterium triplex* sp. nov. *J Clin Microbiol* **34**, 2963–2967.

Floyd, M. M., Gross, W. M., Bonato, D. A., Silcox, V. A., Smithwick, R. W., Metchock, B., Crawford, J. T. & Butler, W. R. (2000). *Mycobacterium kubicae* sp. nov., a slowly growing, scotochromogenic *Mycobacterium*. *Int J Syst Evol Microbiol* **50**, 1811–1816.

Haas, W. H., Butler, W. R., Kirschner, P., Plikaytis, B. B., Coyle, M. B., Amthor, B., Steigerwalt, A. G., Brenner, D. J., Salfinger, M. & other

authors (1997). A new agent of mycobacterial lymphadenitis in children: *Mycobacterium heidelbergense* sp. nov. *J Clin Microbiol* **35**, 3203–3209.

Karassova, V., Weissfeiler, J. & Krasznay, E. (1965). Occurrence of atypical mycobacteria in *Macacus rhesus*. *Acta Microbiol Acad Sci Hung* **12**, 275–282.

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.

Kirschner, P., Meier, A. & Böttger, E. C. (1993a). Genotypic identification and detection of mycobacteria – facing novel and uncultured pathogens. In *Diagnostic Molecular Microbiology: Principles and Applications*, pp. 173–190. Edited by D. H. Persing, T. F. Smith, F. C. Tenover & T. J. White. Washington, DC: American Society for Microbiology.

Kirschner, P., Springer, B., Vogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F. C. & Böttger, E. C. (1993b). 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.

Levi, M. H., Bartell, J., Gandolfo, L., Smole, S. C., Costa, S. F., Weiss, L. M., Johnson, L. K., Osterhout, G. & Herbst, L. H. (2003). Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic *Mycobacterium* from moray eels that is related to *Mycobacterium triplex*. *J Clin Microbiol* **41**, 2147–2152.

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.

Meier, A., Kirschner, P., Schröder, K. H., Wolters, J., Kroppenstedt, R. M. & Böttger, E. C. (1993). *Mycobacterium intermedium* sp. nov. *Int J Syst Bacteriol* **43**, 204–209.

Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. *J Gen Microbiol* **88**, 200–204.

Pourahmad, F., Cervellione, F., Thompson, K. D., Taggart, J. B., Adams, A. & Richards, R. H. (2008). *Mycobacterium stomatepiae* sp. nov., a slowly growing, non-chromogenic species isolated from fish. *Int J Syst Evol Microbiol* **58**, 2821–2827.

Rastogi, N., Goh, K. S., Guillou, N. & Labrousse, V. (1992). Spectrum of drugs against atypical mycobacteria: how valid is the current practice of drug susceptibility testing and the choice of drugs? *Zentralbl Bakteriol* **277**, 474–484.

Rogall, T., Wolters, J., Flohr, T. & Böttger, E. C. (1990). Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int J Syst Bacteriol* **40**, 323–330.

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.

Selvarangan, R., Wu, W. K., Nguyen, T. T., Carlson, L. D., Wallis, C. K., Stiglich, S. K., Chen, Y. C., Jost, K. C., Jr, Prentice, J. L. & other authors (2004). Characterization of a novel group of mycobacteria and proposal of *Mycobacterium sherrisii* sp. nov. *J Clin Microbiol* **42**, 52–59.

Springer, B., Kirschner, P., Rost-Meyer, G., Schröder, K. H., Kroppenstedt, R. M. & Böttger, E. C. (1993). *Mycobacterium interjectum*, a new species isolated from a patient with chronic lymphadenitis. *J Clin Microbiol* **31**, 3083–3089.

Springer, B., Stockman, L., Teschner, K., Roberts, G. D. & Böttger, E. C. (1996a). Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. *J Clin Microbiol* **34**, 296–303.

Springer, B., Wu, W. K., Bodmer, T., Haase, G., Pfyffer, G. E., Kroppenstedt, R. M., Schröder, K. H., Emler, S., Kilburn, J. O. & other authors (1996b). Isolation and characterization of a unique group of slowly growing mycobacteria: description of *Mycobacterium lentiflavum* sp. nov. *J Clin Microbiol* **34**, 1100–1107.

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.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Torkko, P., Suomalainen, S., Iivanainen, E., Tortoli, E., Suutari, M., Seppänen, J., Paulin, L. & Katila, M. L. (2002). *Mycobacterium palustre* sp. nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters. *Int J Syst Evol Microbiol* **52**, 1519–1525.

Tortoli, E. (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* **16**, 319–354.

Tortoli, E. (2006). The new mycobacteria: an update. *FEMS Immunol Med Microbiol* **48**, 159–178.

Tortoli, E., Bartoloni, A., Burrini, C., Colombrita, D., Mantella, A., Pinsi, G., Simonetti, M. T., Swierczynski, G. & Böttger, E. C. (1996a). Characterization of an isolate of the newly described species *Mycobacterium interjectum*. *Zentralbl Bakteriol* **283**, 286–294.

Tortoli, E., Kirschner, P., Bartoloni, A., Burrini, C., Manfrin, V., Mantella, A., Scagnelli, M., Scarparo, C., Simonetti, M. T. & Böttger, E. C. (1996b). Isolation of an unusual mycobacterium from an AIDS patient. *J Clin Microbiol* **34**, 2316–2319.

Tortoli, E., Piersimoni, C., Kirschner, P., Bartoloni, A., Burrini, C., Lacchini, C., Mantella, A., Muzzi, G., Tosi, C. P. & other authors (1997). Characterization of mycobacterial isolates phylogenetically related to, but different from *Mycobacterium simiae*. *J Clin Microbiol* **35**, 697–702.

Tortoli, E., Bartoloni, A., Erba, M. L., Levrè, E., Lombardi, N., Mantella, A. & Mecocci, L. (2002). Human infections due to *Mycobacterium lentiflavum*. *J Clin Microbiol* **40**, 728–729.

Tortoli, E., Rindi, L., Goh, K. S., Katila, M. L., Mariottini, A., Mattei, R., Mazzarelli, G., Suomalainen, S., Torkko, P. & Rastogi, N. (2005a). *Mycobacterium florentinum* sp. nov., isolated from humans. *Int J Syst Evol Microbiol* **55**, 1101–1106.

Tortoli, E., Chianura, L., Fabbro, L., Mariottini, A., Martín-Casabona, N., Mazzarelli, G., Russo, C. & Spinelli, M. (2005b). Infections due to the newly described species *Mycobacterium parascrofulaceum*. *J Clin Microbiol* **43**, 4286–4287.

Turenne, C. Y., Thibert, L., Williams, K., Burdz, T. V., Cook, V. J., Wolfe, J. N., Cockcroft, D. W. & Kabani, A. (2004a). *Mycobacterium saskatchewanense* sp. nov., a novel slowly growing scotochromogenic species from human clinical isolates related to *Mycobacterium interjectum* and Accuprobe-positive for *Mycobacterium avium* complex. *Int J Syst Evol Microbiol* **54**, 659–667.

Turenne, C. Y., Cook, V. J., Burdz, T. V., Pauls, R. J., Thibert, L., Wolfe, J. N. & Kabani, A. (2004b). *Mycobacterium parascrofulaceum* sp. nov., novel slowly growing, scotochromogenic clinical isolates related to *Mycobacterium simiae*. *Int J Syst Evol Microbiol* **54**, 1543–1551.

van Ingen, J., Tortoli, E., Selvarangan, R., Coyle, M. B., Crump, J. A., Morrissey, A. B., Dekhuijzen, P. N., Boeree, M. J. & van Soolingen, D. (2011). *Mycobacterium sherrisii* sp. nov., a slow-growing non-chromogenic species. *Int J Syst Evol Microbiol* **61**, 1293–1298.