Proposal to elevate *Mycobacterium avium* complex ITS sequevar MAC-Q to *Mycobacterium vulneris* sp. nov.

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The *Mycobacterium avium* complex (MAC) consists of four recognized species, *Mycobacterium avium*, *Mycobacterium colombiense*, *Mycobacterium intracellulare* and *Mycobacterium chimaera*, and a variety of other strains that may be members of undescribed taxa. We report on two isolates of a scotochromogenic, slowly growing, non-tuberculous *Mycobacterium* species within the *M. avium* complex from a lymph node and an infected wound after a dogbite of separate patients in The Netherlands. The extrapulmonary infections in immunocompetent patients suggested a high level of virulence. These isolates were characterized by a unique nucleotide sequence in the 16S rRNA gene, 99 % similar to *Mycobacterium colombiense*, and the MAC-Q 16S–23S internal transcribed spacer (ITS) sequence. Sequence analyses of the *hsp65* gene revealed 97 % similarity to *M. avium*. The *rpoB* gene sequence was 98 % similar to *M. colombiense*. Phenotypically, the scotochromogenicity, positive semi-quantitative catalase and heat-stable catalase tests, negative tellurite reductase and urease tests and susceptibility to hydroxylamine and oleic acid set these isolates apart from related species. High-performance liquid chromatography analysis of cell-wall mycolic acid content revealed a unique pattern, related to that of *M. avium* and *M. colombiense*. Together, these findings supported a separate species status within the *Mycobacterium avium* complex. We propose elevation of scotochromogenic *M. avium* complex strains sharing this 16S gene and MAC-Q ITS sequence to separate species status, for which the name *Mycobacterium vulneris* sp. nov. is proposed. The type strain is NLA000700772^T (=DSM 45247^T=CIP 109859^T).

Mycobacterium avium complex (MAC) bacteria are the most frequently isolated non-tuberculous mycobacteria worldwide and are capable of causing a wide spectrum of clinical disease. Pulmonary disease, mostly in patients with pre-existent pulmonary diseases, is most common, followed by lymphadenitis in immunocompetent children and disseminated disease in systemically immunocompromised patients (Griffith *et al.*, 2007).

The taxonomy of the MAC has been a subject of debate for decades. The MAC was long divided into two species, *M. avium* and *Mycobacterium intracellulare*, and a number of

unnamed bacteria not belonging to these two taxa. Frothingham and Wilson noted that sequencing of the 16S–23S internal transcribed spacer (ITS) revealed a wide range of genetic diversity among reference strains of those unnamed MAC bacteria, suggesting the presence of several as yet undefined species (Frothingham & Wilson, 1993). Two such groups have recently been elevated to species rank, *Mycobacterium chimaera* and *Mycobacterium colombiense* (Tortoli *et al.*, 2004; Murcia *et al.*, 2006). In this study, we report on a novel *M. avium* complex member, related to *M. colombiense*, which was isolated from two patients in the Netherlands.

A previously healthy, 42-year-old woman presented with a painful wound in her left lower leg 7 weeks after a dogbite. Several small white lesions with limited ulceration were noted. Surgical wound excision and oral amoxicillin and clavulanic acid had limited success. An abscess with fistula to the skin and spontaneous wound rupture prompted renewed surgical debridement, *Mycobacterium* culture, and vacuum therapy. The cultures yielded a non-tuberculous *Mycobacterium*. A third wound debridement and vacuum therapy eventually led to symptomatic improvement and wound closure. *Mycobacterium* cultures from samples of the third debridement remained negative.

A previously healthy, two-year-old girl presented at another hospital with painless swelling of a right cervical lymph node and violaceous overlying skin. No other symptoms were reported. A biopsy revealed granulomatous inflammation, but no acid–fast bacilli were visible on direct microscopy. Cultures of the biopsy material yielded a non-tuberculous *Mycobacterium* and surgery was successfully performed. No relapse has been noted since.

Both isolates were sent to the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The RIVM is the national mycobacteria reference laboratory, which provides identification, typing and drug susceptibility testing.

We investigated the biochemical and phenotypical features and performed high-performance liquid chromatography (HPLC) analysis of cell-wall mycolic acid content, using previously described procedures (Kent & Kubica, 1985; CDC, 1996). We used the HPLC mycobacterium library (available online at http://www.MycobacToscana.it) for visual comparisons.

For primary identification, we used the Inno-LiPA Mycobacteria v2 (Innogenetics, Ghent, Belgium) and Hain GenoType CM/AS (Hain Lifesciences, Nehren, Germany) reverse line-blots. To identify the isolates to the species level we sequenced the complete 16S rRNA gene, the 16S–23S ITS region and partial *hsp65* and *rpoB* genes, using previously published primers and methods (Springer *et al.*, 1996; Roth *et al.*, 1998; Telenti *et al.*, 1993; Adékambi *et al.*, 2003). The sequences obtained were compared with the GenBank/EMBL/DDBJ (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov) sequence database.

The 16S rRNA gene sequences of the *Mycobacterium* isolates of the two cases were aligned with those of reference strains in the MAC using CLUSTAL_X software (Thompson *et al.*, 1997). The resulting topology and tree, inferred by using 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). The tree was rooted with *M. tuberculosis* H37Rv[T] as an outgroup.

We tested the presence of the IS*1245* element by amplification of a 427 bp internal fragment, using P1 and P2 primers, as previously described (Guerrero *et al.*, 1995).

Drug susceptibility testing was performed using the 25-well agar dilution method (van Klingeren *et al.*, 2007). Isoniazid, rifampicin, rifabutin, ethambutol, clarithromycin, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin were included in the test panel.
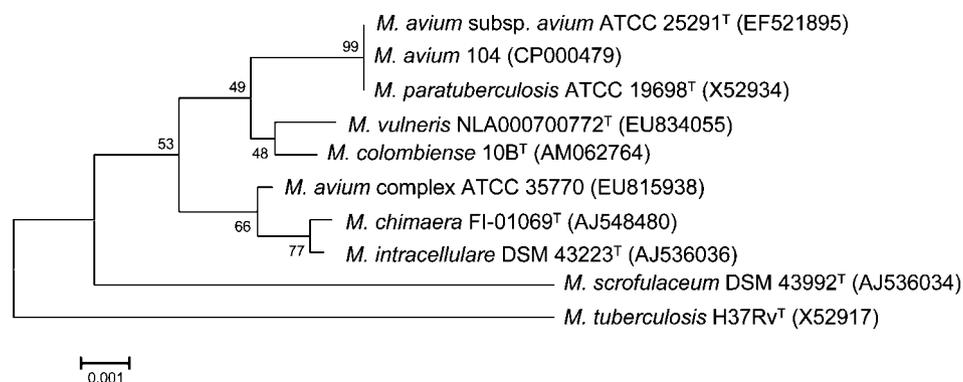

Fig. 1. Phylogenetic relationships of the type strain of *M. vulneris* sp. nov. and related species of *Mycobacterium*, based on 16S rRNA gene sequences. The neighbour-joining tree was constructed based on bootstrap analysis with 1000 resamplings and visualized with MEGA 4.0 (Tamura *et al.*, 2007). Percentage bootstrap values are given at nodes. Bar, 0.001 substitutions per nucleotide.

On Middlebrook 7H10, Ogawa and Stonebrink media (Oxoid; The Netherlands), the bacteria produce film-like growth with small, smooth, bright yellow-pigmented colonies after 3 weeks incubation at 36 °C. Growth on Middlebrook 7H10 agar was only observed at 24, 30 and 36 °C. Optimal growth occurred at 36 °C. Colony morphology on Middlebrook 7H10 agar was similar at all temperatures.

The Inno-LiPA Mycobacteria v2 assay revealed only reaction with the MAIS (*M. avium–intracellulare–scrofulaceum*) complex probe, thus identifying the isolates as *M. avium* complex members, different from *M. avium*, *M. intracellulare* and *M. scrofulaceum*. Analysis using the Hain GenoType CM assay (which uses the 23S rRNA gene as its target) incorrectly identified the strain as *M. intracellulare*. This may reflect 23S gene sequence similarity of our isolates with *M. intracellulare*. Currently available commercial identification kits for non-tuberculous mycobacteria do not have sufficient discriminative power to recognize particular subgroupings among the MAC isolates. This may suffice in the clinical setting, as the treatment of MAC disease, to date, is independent of exact speciation results (Griffith *et al.*, 2007). However, an improved recognition of clinically relevant subgroupings within the complex may improve clinical management and eventually support research on the epidemiology and pathogenesis of MAC disease.

Sequencing of the complete 16S rRNA genes of the two isolates revealed a unique sequence most closely related to MAC bacteria (Table 1). The multisequence alignment results of the 16S rRNA gene sequence clarified its taxonomical position within the MAC as being most closely related to *M. colombiense* (Fig. 1). The 16S–23S ITS sequences of both strains were identical to the previously described MAC-Q ITS sequevar (GenBank no. AF315833); it differed in 6 bp from the *M. colombiense* ITS sequevar

(Table 1). The previously published MAC-Q strain (Mijs *et al.*, 2002) was that isolated from patient two, the girl with lymphadenitis. We performed multisequence alignment of all published MAC ITS sequevars currently available in GenBank (Fig. 2). Additional sequencing of the *hsp65* and *rpoB* genes revealed unique sequences, detailed in Table 1. Again, sequences were most similar to MAC members and related to *M. avium* rather than *M. intracellulare* (Table 1). Both strains had unique *hsp65* sequences, the difference being a G→A substitution at position 140 (corresponding to codon 538 of the *M. tuberculosis* H37Rv^T *hsp65* gene). The *rpoB* sequence indicated that the isolates were related specifically to *M. colombiense*, in line with the 16S rRNA gene results (Table 1). We aligned the *rpoB* and *hsp65* sequences with those of related *Mycobacterium* species, using the same methods as for the 16S rRNA gene sequence. In addition, concatenated 16S rRNA, *hsp65* and *rpoB* gene sequences were aligned with concatenated sequences of the related *Mycobacterium* species (Stackebrandt *et al.*, 2002). The resulting topologies and trees are available as supplementary material in IJSEM Online (Supplementary Figs S1, S2 and S3).

We were able to demonstrate, by PCR, the presence of the IS*1245* element in the genomes of the two isolates (results not shown). This supported its taxonomic position within the MAC, related to *M. avium* rather than *M. intracellulare*, although a minority of the *M. intracellulare* and other MAC strains are known to harbour this element (Mijs *et al.*, 2002).

Phenotypic identification revealed a pattern generally similar to MAC strains, with negative tests for niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, 3 day arylsulfatase, urease and growth on MacConkey agar, but positive for 68 °C catalase. Our isolates were divergent in their positive semi-quantitave catalase test, susceptibility to hydroxylamine and oleic acid,

Table 1. Sequence comparison results for *M. vulneris* NLA000700772^T

| Target	Results of GenBank/EMBL/DDBJ comparison
16S rRNA (1471 bp) GenBank EU834055	99 % *M. colombiense* 10B^T (1436/1439 bp)
	99 % *M. chimaera* FI-0169^T (1432/1439 bp)
	99 % *M. avium* 104 (1463/1471 bp)
	99 % *M. paratuberculosis* ATCC 19698^T (1463/1471 bp)
16S–23S ITS (281 bp) GenBank AF315833 MAC-Q	99 % MAC-R, AF315834 (279/281 bp)
	98 % MAC-E (ATCC 35847), L07852 (278/281 bp)
	98 % MAC-F, L07853 (277/281 bp)
	97 % *M. colombiense* 10B^T (275/281 bp)
rpoB (726 bp) GenBank EU834057	98 % *M. colombiense* CIP 108962^T (690/701 bp)
	95 % *M. chimaera* DSM 44623^T (669/701 bp)
	94 % *M. avium* 104 (678/714 bp)
	94 % *M. avium* ATCC 25291^T (665/701 bp)
hsp65 (424 bp) GenBank EU834054	97 % *M. avium* ATCC 25291^T (415/424 bp)
	97 % *M. avium* 104 (415/424 bp)
	97 % *M chimaera* CIP 107892^T (414/424 bp)
	97 % *M. intracellulare* ATCC 13950^T (412/424 bp)
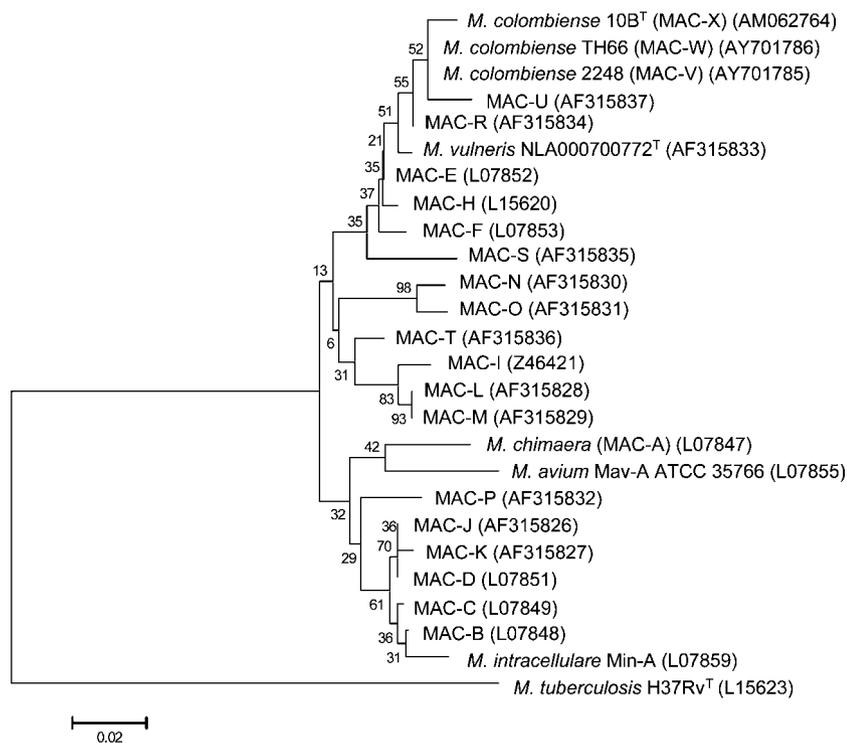
Fig. 2. Phylogenetic tree based on ITS sequences showing the relationships of *M. chimaera* and other sequevars of MAC. Each organism is indicated by the sequevar name and GenBank accession number. The neighbour-joining tree was constructed based on bootstrap analysis with 1000 resamplings and visualized with MEGA 4.0 (Tamura *et al.*, 2007). Percentage bootstrap values are given at nodes. Bar, 0.02 substitutions per nucleotide.

Table 2. Biochemical identification results

Taxa: 1, *M. vulneris* sp. nov.; 2, *M. colombiense*; 3, *M. avium*; 4, *M. intracellulare*; 5, *M. chimaera*. All species grow at 25 °C. All species do not accumulate niacin, reduce nitrate, hydrolyse Tween 80 or grow on MacConkey agar. The growth rate of all species in Middlebrook 7H10 medium at 36 °C is slow. +, Positive; −, negative; +/−, variable; ND, not determined.

Test item	1	2	3	4	5
68 °C catalase	+	+/−	+/−	+/−	+
Catalase >45 mm	+	+	−	−	−
β-Glucosidase	−	ND	−	−	−
Tellurite reduction	−	ND	+	+/−	+/−
3-day Arylsulfatase	−	+/−	−	−	−
Urease	−	+	−	−	−
Pigmentation*	SC	NC	NC	NC	NC
Colony morphology	Smooth	Rough	Smooth	Smooth	Smooth
Growth at 42 °C	−	−	+/−	+/−	−
Tolerance to:					
TCH†	+	ND	+	+	+
Thiacetazone (10 µg ml^{-1})	+	ND	+	+	+
Isoniazid (500 µg ml^{-1})‡	+	ND	+	+	+
p-Nitrobenzoic acid	+	ND	+	+	+/−
Hydroxylamine (1 µg ml^{-1})‡	−	ND	+/−	+/−	+/−
Oleic acid	−	ND	+	+	+/−

*NC, Non-chromogenic; SC, scotochromogenic.
†Thiophen-2-carboxylic acid hydrazide.
‡On Middlebrook 7H10 agar.

as well as their scotochromogenicity. Positive semi-quantitative catalase tests were also noted for *M. colombiense*, which differs from our isolates in its negative urease test and colony morphology and pigmentation (Table 2).

The HPLC pattern of the isolate comprises 3 clusters of peaks; the first cluster was the main one and included four major peaks. The second and third emerged later and were close to each other, presenting four and three peaks, respectively (Fig. 3). This pattern was consistent among most strains included in the MAC, although the *M. colombiense* pattern is characterized by increasing peak heights within the first cluster and the second cluster is absent in *M. chimaera* (Tortoli *et al.*, 2004; Murcia *et al.*, 2006). The relative heights of the peaks in the second and third cluster varied within the MAC. *M. avium* mostly presents lower peaks in the third cluster, as for the *M. vulneris* isolates. *M. colombiense* usually presents lower peaks in the second cluster (Fig. 3).

Applying the 25-well agar dilution method for drug susceptibility testing (van Klingeren *et al.*, 2007), we recorded resistance to isoniazid (MIC >20 µg ml^{-1}),

ethambutol (MIC 20 µg ml^{-1}), streptomycin (MIC >20 µg ml^{-1}), amikacin (MIC 20 µg ml^{-1}) and ciprofloxacin (MIC >16 µg ml^{-1}), with susceptibility to rifampicin (MIC 1 µg ml^{-1}), rifabutin (MIC 1 µg ml^{-1}), clarithromycin (MIC 4 µg ml^{-1}), clofazimine (MIC= <0.5 µg ml^{-1}), cycloserine (MIC 20 µg ml^{-1}) and prothionamide (MIC 2 µg ml^{-1}).

Previous authors have suggested the presence of multiple species within MAC, based on various phenotypic and genetic traits (Frothingham & Wilson, 1993; Wayne *et al.*, 1996; Wayne & Sramek, 1992). Based on the phenotypic and genotypic features reported above, we believe that our isolates represent one such species. The extrapulmonary infections in immunocompetent patients suggest a high level of virulence.

Description of *Mycobacterium vulneris* sp. nov.

Mycobacterium vulneris [vul′ne.ris; L. gen. n. *vulneris* of a wound (*vulnus*), from which the type strain was isolated].

Stains acid–alcohol-fast. Cells are short rods, with occasional coccoid forms. No cording, spores or filaments are present. On Middlebrook 7H10, Ogawa and Stonebrink media, mature growth develops after 28 days incubation at 36 °C; growth is slower at 25 and 30 °C and no growth occurs at 42 °C. Colonies are small, scotochromogenic and bright yellow in appearance. Negative for niacin accumulation, nitrate reduction, *β*-glucosidase, Tween 80 hydrolysis, tellurite reduction, 3 day arylsulfatase, urease and growth on MacConkey agar, but positive for 68 °C catalase and semi-quantitative catalase. Tolerant to isoniazid, thiophen-2-carboxylic acid hydrazide, *p*-nitrobenzoic acid and thiacetazone, but not to oleic acid or hydroxylamine. Readily identifiable by its unique HPLC pattern and 16S rRNA, *hsp65* and *rpoB* gene sequences. The 16S–23S ITS region was previously described as the MAC-Q ITS sequevar.

The type strain, NLA000700772[T] (=DSM 45247[T]=CIP 109859[T]), was isolated from a wound debridement specimen.
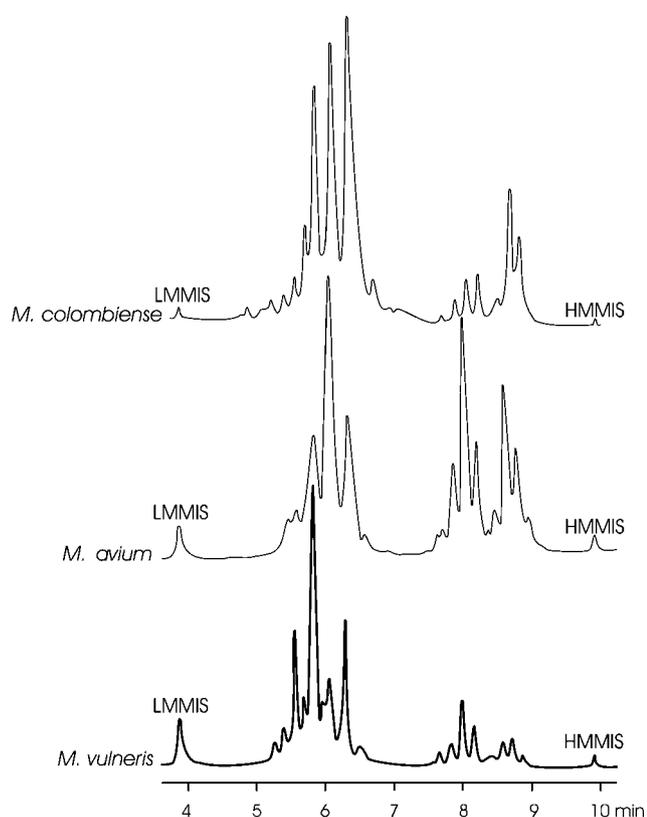

Fig. 3. Mycolic acid patterns of *M. vulneris*, *M. avium* and *M. colombiense* obtained by HPLC analysis. The *M. vulneris* pattern is similar to that of other *M. avium* complex members, but does not share the increasing peak heights within the first cluster, typical for *M. colombiense*. HMMIS, high molecular mass internal standard; LMMIS, low molecular mass internal standard.

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.

CDC (1996). *Standardized Method for HPLC Identification of Mycobacteria.* Atlanta, USA: Department of Health and Human Services, Centers for Disease Control and Prevention.

Frothingham, R. & Wilson, K. H. (1993). Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *J Bacteriol* **175**, 2818–2825.

Griffith, D. E., Aksamit, T., Brown-Elliot, B. A., Catanzaro, A., Daley, C., Gordin, F., Holland, S. M., Horsburgh, R., Huitt, G. & other authors (2007). An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med* **175**, 367–416.

Guerrero, C., Bernasconi, C., Burki, D., Bodmer, T. & Telenti, A. (1995). A novel insertion element from *Mycobacterium avium*, IS*1245*, is a specific target for analysis of strain relatedness. *J Clin Microbiol* **33**, 304–307.

Kent, P. T. & Kubica, G. P. (1985). *Public Health Mycobacteriology. A Guide For The Level III Laboratory*. Atlanta, USA: Department of Health and Human Services.

Mijs, W., de Haas, P., Rossau, R., van der Laan, T., Rigouts, L., Portaels, F. & van Soolingen, D. (2002). Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and '*M. avium* subsp. *hominissuis*' for the human/porcine type of *M. avium*. *Int J Syst Evol Microbiol* **52**, 1505–1518.

Murcia, M. I., Tortoli, E., Menendez, M. C., Palenque, E. & Garcia, M. J. (2006). *Mycobacterium colombiense* sp. nov., a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant. *Int J Syst Evol Microbiol* **56**, 2049–2054.

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.

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

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C., 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.

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.

Tortoli, E., Rindi, L., Garcia, M. J., Chiaradonna, P., Dei, R., Garzelli, C., Kroppenstedt, R. M., Lari, N., Mattei, R. & other authors (2004). Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. *Int J Syst Evol Microbiol* **54**, 1277–1285.

van Klingeren, B., Dessens-Kroon, M., van der Laan, T., Kremer, K. & van Soolingen, D. (2007). Drug susceptibility testing of *Mycobacterium tuberculosis* complex using a high throughput, reproducible, absolute concentration method. *J Clin Microbiol* **45**, 2662–2668.

Wayne, L. G. & Sramek, H. A. (1992). Agents of newly recognized or infrequently encountered mycobacterial diseases. *Clin Microbiol Rev* **5**, 1–25.

Wayne, L. G., Good, R. C., Böttger, E. C., Butler, R., Dorsch, M., Ezaki, T., Gross, W., Jonas, V., Kilburn, J. & other authors (1996). Semantide- and chemotaxonomy-based analyses of some problematic phenotypic clusters of slowly growing mycobacteria, a cooperative study of the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* **46**, 280–297.