Northumbria Research Link

Northumbria
University
NEWCASTLE

Mycolicibacterium stellerae sp. nov., a rapidly growing scotochromogenic strain isolated from *Stellera chamaejasme*

Imen Nouioui[1*], Vartul Sangal[2], Carlos Cortés[1], Marlen Jando[3], José Mariano Igual[4], Hans-Peter Klenk[1], Yu-Qin Zhang[5], Michael Goodfellow[1]

[1]School of Natural and Environmental Sciences, Ridley Building 2, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom

[2]Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, UK

[3]Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany

[4]Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), c/Cordel de Merinas 40-52, 37008 Salamanca, Spain

[5]Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, No. 1 of Tiantanxili Road, Beijing, Beijing 100050, China

Corresponding author: **Imen Nouioui** imen.nouioui@newcastle.ac.uk

Section: Actinobacteria

Keywords: Actinobacteria, polyphasic taxonomy, draft-whole genome sequence

Abbreviations: ANI, Average Nucleotide Identity; A2pm, diaminopimelic acid, BLAST, Basic Local Alignment Search Tool; CDP-x, CDP-alcohol phosphatidyltransferase family protein; dDDH, Digital DNA:DNA Hybridization; GGDC, Genome-to-Genome Distance Calculator; GTR, General Time-Reversible; GYM, Glucose-Yeast extract-Malt extract; LJ, Löwenstein Jensen; Mb, Megabases (millions of base pairs); MB7H10, Middlebrook 7H10; MIDI, Microbial IDentification Inc.; ML, Maximum-Likelihood; MP, Maximum-Parsimony; MUSCLE, MUltiple Sequence Comparison by Log-Expectation; PAUP, Phylogenetic Analysis Using Parsimony; PMG, Proteose peptone-Meat extract-Glycerol; RAxML, Randomized Axelerated Maximum Likelihood; MRE, Maximal-Relative-Error; TSA, Tryptic

Soy Agar; TNT, Tree analysis New Technology; PE, PhosphatidylEthanolamine; RAST, Rapid Annotation using Subsystem Technology.

The GenBank accession numbers of 16S rRNA gene and genome sequences for strain CECT 8783^{T} are MH935827 and RARC00000000, respectively.

Abstract

A polyphasic study was undertaken to establish the taxonomic provenance of a rapidly growing *Mycolicibacterium* strain CECT 8783^T, recovered from the plant *Stellera chamaejasme* L. in Yunnan Province, China. Phylogenetic analyses based upon 16S rRNA and whole-genome sequences showed that the strain formed a distinct branch within the evolutionary radiation of the genus *Mycolicibacterium*. The strain was most closely related to *Mycolicibacterium moriokaense* DSM 44221^T with 98.4% 16S rRNA gene sequence similarity, but was distinguished readily from this taxon by a combination of chemotaxonomic and phenotypic features and by low average nucleotide identity and digital DNA:DNA hybridization values of 79.5% and 21.1%, respectively. Consequently, the strain is considered, to represent a novel species of *Mycolicibacterium* for which the name *Mycolicibacterium stellerae* sp. nov is proposed; the type strain is I10A-01893^T (= CECT 8783^T = KCTC 19843^T = DSM 45590^T).

Mycobacterium [1], the type genus of the family *Mycobacteriaceae* [2], can be distinguished from other genera classified in the order *Corynebacteriales* by using a combination of genotypic and phenotypic criteria [3]. The genus includes pathogenic and non-tuberculous mycobacteria that are common in the environment and can cause opportunistic infections in immunocompetent and immunosuppressed patients [4]. Mycobacteria can be divided into two groups based on their growth rates on solid media; slowly growing strains need seven or more days of incubation at optimal temperatures to form visible colonies from highly diluted inocula whereas colonies of rapidly growing strains are seen within seven days under comparable conditions [5]. Species can be assigned to these two groups using polyphasic procedures, as exemplified by the circumscription of strains previously lumped together within the *Mycobacterium abscessus* and *Mycobacterium avium* complexes [6, 7]. However, genomic based methods provide much better resolution between mycobacterial species compared to 16S rRNA generated phylogenies and also provide insights into their evolution [8-10]. The availability of whole genome sequences and associated bioinformatic tools has led to the unambigious delineation of new mycobacterial species [11] and the recognition of heterotypic synonyms of *Mycobacterium tuberculosis* [10].

The genus *Mycobacterium* encompasses nearly 200 validly named species [12] representatives of which formed a monophyletic taxon in a genome-based classification of the phylum *Actinobacteria* [11]. Most of these species have been classified into five distinct monophyletic groups based on extensive phylogenomic and comparative genome analyses [13]. Gupta and his colleagues [13] recognised an emended genus *Mycobacterium*, which included all of the

major known pathogens as "Tuberculosis-Simiae" clade, and the novel genera *Mycolicibacillus*, *Mycolicibacter*, *Mycolicibacterium* and *Mycobacteroides* which contain species assigned to clades designated as "Terrae", "Triviale", "Fortuitum-Vaccae", and "Abscessus-Chelonae", respectively [13]. The genus *Mycolicibacterium* encompasses 88 rapidly growing species, including *Mycolicibacterium fortuitum* the type species; members of this taxon have genomes that range in size from 3.95 – 8.0 Mbp and in G+C content 65.4 - 70.3 mol% [13].

In a continuation of our studies on mycobacterial diversity, strain CECT 8783^T recovered from *Stellera chamaejasme* L. in Yunnan Province, China was the subject of a polyphasic study. The resultant datasets show that the strain represents a novel *Mycolicibacterium* species for which the name *Mycolicibacterium stellerae* sp. nov. is proposed.

A culture of CECT 8783^T representing strain I10A-01893^T was obtained from the Spanish Type Culture Collection (CECT), but was originally deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) and given the accession number DSM 45590^T. The strain was maintained together with its closest phylogenetic neighbour, *Mycolicibacterium moriokaense* CIP105393^T [13, 14], on proteose peptone-meat extract-glycerol agar (PMG; DSMZ 250 medium) and as suspensions of cells in 35%, v/v glycerol at -80°C.

Cultural and morphological features of CECT 8783^T were carried out under light and dark conditions on glucose-yeast extract-malt extract agar (GYM; DSMZ medium 65), Löwenstein-Jensen medium (LJ; [15]), Middlebrook 7H10 agar (MB7H10; [16]), nutrient agar (NA; [17]) and peptone-meat extract-glucose agar (PMG; DSMZ medium 250) and tryptic soy agar (TSA; [18]) after incubation for 14 days at 37°C. In addition the strain was examined for its ability to grow at 4°C, 10°C, 15°C, 25°C, 28°C, 37°C and 45°C as well as for its ability to grow anaerobically on PMG agar, using an anaerobic bag system (Sigma-Aldrich 68061). Strain CECT 8783^T was examined for acid-alcohol-fastness [19]. The strain was acid-alcohol-fast, grew optimally on GYM, NA, PMG and TSA agar after 5 days at 37°C, but not under anaerobic conditions. Yellow pigmented colonies were produced on all of these media under both light and dark conditions.

Genomic DNA was extracted from a culture of CECT 8783^T after Amaro *et al.* [20]. The genome of the strain was sequenced using an Illumina MiSeq instrument as described by Sangal *et al*. [21] and assembled into contigs using SPAdes 3.9.0 with a kmer length of 127 [22]. A complete 16S rRNA gene sequence was extracted from the draft genome (accession number MH935827). Corresponding 16S rRNA gene sequences of the type strains of closely related *Mycolicibacterium* species were retrieved from the EzBioCloud server [23]. Maximum-

likelihood (ML) and maximum-parsimony (MP) phylogenetic trees derived from the 16S rRNA were inferred using the genome-to-genome distance calculator (GGDC) web server [24] adapted to single gene inferences; the latter was also used to calculate pairwise sequence similarities [25, 26]. Multiple sequence alignments were generated using MUSCLE software [27] and a ML tree inferred from the alignment with RAxML [28] using rapid bootstrapping and the auto MRE criterion [29]. In turn, an MP tree was inferred from alignements with the Tree analysis New Technology (TNT) program [30] using 1000 bootstraps together with tree bisection and reconnection branch swapping and ten random sequence replicates. The X^2 test implemented in PAUP* [31] was used to check for compositional bias in the sequences.

Blast analysis of the complete 16S rRNA gene sequence of strain CECT 8783^T (1533 nucleotides) showed that it was most closely related to *M. moriokaense* CIP105393^T and *Mycolicibacterium goodii* ATCC 700504^T [13, 32], showing 16S rRNA gene sequence similarities with them of 98.4% and 98.6%, respectively; the corresponding pairwise 16S rRNA gene sequence similarities based on the GGDC platform were 98.3% and 98.4%. The strain formed a distinct branch in the 16S rRNA gene tree (Fig. 1), between *Mycolicibacterium madagascarense* ATCC 49865^T branch [13, 33] and a poorly supported subclade that contained *Mycolicibacterium celeriflavum* AFPC-000207^T [13, 34], *M. moriokaense* CIP105393^T, *Mycolicibacterium phlei* DSM 43239^T [13, 35], *Mycolicibacterium pulveris* DSM 44222^T [13, 36]; strain CECT 8783^T shared 16S rRNA gene sequence similarities with these organismes within the range 97.8%-98.4%. An adjacent well supported clade contained *M. goodii* ATCC 700504^T and *Mycolicibacterium smegmatis* NCTC 8159^T [13, 35, 37].

Due to poor bootstrap support for several nodes in the 16S rRNA gene tree, ML trees were constructed from core proteins and 400 universal protein sequences using PhyloPhlAn [38]. In brief, genome sequences were annotated using Prokka 1.11 [39] and the protein sequences were compared using BPGA 1.3 pipeline [40] to calculate the core genome. Poorly aligned regions and sites with the missing data was removed from the sequence alignment of the core proteins using Gblocks [41]. A ML phylogenetic tree was generated from the resulting alignment of 23,359 amino acids using IQ-Tree [42] with LG+F+I+G4 substitution model and 100,000 ultrafast bootstrap iterations and SH-like approximate likelihood ratio tests. Another ML tree was generated from the protein sequences using PhyloPhlAn which extracts subset of amino acids from 400 universal sequences and calculates ML phylogeny [38].

It can be seen from Figure 2 that strain CECT 8783^T formed a well supported branch in the phylogenomic tree together with, *M. celeriflavum* DSM 46765^T, *Mycolicibacterium flavescens* M6^T [13, 43], *M. moriokaense* CIP 105393^T and *M. phlei* CCUG21000^T that was clearly separated from a corresponding clade contained *M. goodii* X7B^T and *M. smegmatis* NCTC8159^T (Fig 2a). The same topology was observed on the concatenated 400 universal amino acid sequences based tree (Fig. 2b).

The genome size of the strain was found to be ~6.9 Mb with 37.7X coverage, 88 contigs, 52 RNAs and 6836 coding sequences and an *in silico* G+C content of 65.8 mol%. Similarly, the type strain of *M. moriokaense* has a genome size of 6.2 Mb, 51 RNAs, 6114 coding sequences and an *in silico* G+C content of 66.0 mol%. The average nucleotide identity (ANI), estimated using blastANI algorithm [44], between the genomes of the two strains was 79.5%, a value well below the threshold of 95-96% used to delineate prokaryotic species [44-46]. Similarly, the digital DNA:DNA hybridization (dDDH) value between the two strains, calculated using GGDC based on formula 2 of Meier-Kolthoff *et al.* [25] (http://ggdc.dsmz.de/), was 21.1% [18.9 - 23.5%], this is well below the 70% cut-off recommended for assigning strains to the same species [47]. Using Artemis software [48], the molecular signatures specific to the genus *Mycolicibacterium* (LacI family transcriptional regulator (WP_036341761); CDP-x (WP_036344961); and CDP-diacylglycerol–serine (WP_066811333)), identified by Gupta *et al.* [13], were found in the genome of the strain CECT 8783^T.

Biomass for most of the chemotaxonomic analyses on strain CECT 8783^T was prepared in shake flasks (200 resolutions per minute) of PMG broth following incubation at 37°C for 5 days. Cells were washed three time in sodium chloride solution (0.9%, w/v), freeze dried and stored at room temperature. Standard chromatographic procedures were used to detect isomers of diaminopimelic acid (A_2pm) [49]; mycolic acids [50], polar lipids [51] and cell wall sugars [52]. Strain CECT 8783^T and *M. moriokaense* DSM 44221^T produced whole organism hydrolysates rich in *meso*-A_2pm, arabinose, galactose, glucose, mannose and ribose and had polar lipid profiles containing diphosphatidylglycerol (DPG), glycophospholipid (GPL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), unidentified glycolipids (GL), unidentified lipids and phospholipid. In addition strain CECT 8783^T contained an aminoglycolipid and *M. moriokaense* DSM 44221^T an aminolipid (Fig. S1).

Wet biomass for the fatty acid was prepared in Middelbrook 7H10 broth following incubation for 5 days at 37°C. Cellular fatty acids were extracted and fatty acids methyl esters (FAMES) prepared after saponification and methylation using the procedure introduced by Miller

[53] .The FAMES were analysed by gas chromatography (Agilent 6890N) instrument and identified based on myco6 database [54] and the standard Microbial Identification (MIDI) system, version 4.5.

Quantitative differences in the mixtures of saturated, unsaturated and 10-methyloctadecanoic (tubercolostearic) fatty acids were found between the two strains (Table S1). The major fatty acid of strain CECT 8783^T (>25%) were $C_{18:1}$ ω9c and $C_{16:0}$ and that of *M. moriokaense* DSM 44221^T was $C_{16:0}$ though the latter unlike the former, contained $C_{17:1}$ ω 7c (Table S1). The mycolic acid profile of the strain CECT 8783^T consisted of α, α'-mycolates, *keto*-mycolates, ω-carboxymycolate while *M. moriokanese* DSM 44221^T lacked α'-mycolates (Fig. S2).

The two strains were examined, in duplicate, for a broad range of standard biochemical tests, namely: arylsulfatase after 3 and 20 days [55], reduction of potassium tellurite [56, 57], degradation of Tween 80 [58] and urea hydrolysis [59]. In addition, the strains were examined for their ability to metabolise a broad range of sole carbon and nitrogen sources, to grow on the presence of several concentrations of sodium chloride, at a range of pH values and in the presence of inhibitory compounds, using GENIII microplates and an Omnilog device (Biolog Inc., Hayward, USA). These tests were carried out in duplicate using freshly prepared inocula harvested from the mid-logarithmic growth phase of PMG agar plates incubated at 37℃ for 7 days, as described by Nouioui *et al.* [60]. Opm package version 1.3.36 [61-62] was used to analyse the resultant data. Identical results were obtained for all of the duplicated tests.

Phenotypic features summarised in Table 1 clearly distinguish strain CECT 8783^T from *M. moriokaense* DSM 44221^T. Both strains found to be able to reduce potassium tellurite, but were unable to degrade Tween 80 or hydrolyse urea. However, only strain DSM 44221^T produced arylsulfatase after 3 and 14 days.

It can be concluded from the chemotaxonomic, genomic and phenotypic data that strain CECT 8783^T clearly forms a new centre of taxonomic variation within the genus *Mycolicibacterium.* Consequently, it is proposed that the strain be recognised to represent a new species, namely as *Mycolicibacterium stellerae* sp. nov.

Description of *Mycolicibacterium stellerae* sp. nov.

Mycolicibacterium stellerae (*stel'le.rae* N.L. gen. n. *stellerae* of *Stellera*, named referring to the host plant, *Stellera chamaejasme,* from which the strain was isolated).

Strictly aerobic Gram-stain positive, acid-alcohol fast, fast growing organism which produces orange coloured colonies on Middelbrook 7H10, nutrient agar, proteose peptone-meat extract-glycerol, glucose-yeast extract-malt extract and tryptic soy agar plates within 5 days at 37°C under dark and light conditions. Grows between 25°C and 37°C, optimally ~ 37°C, and at pH7. Arylsulfatase negative after 3 and 14 days at 37°C. Strain CECT 8783^T reduced potassium tellurite, but was unable to degrade Tween 80 or hydrolyse urea. It was able to utilise L-arginine, L-aspartic acid and D-serine (amino acids); L-glutamic acid, L-lactic acid, D-malic acid, α-*hydroxy*-butyric acid, p-*hydroxy*-phenylacetic acid and L-pyroglutamic acid (organic acids); D-cellobiose, dextrin, glycerol, D-maltose, methyl pyruvate, sucrose, D-trehalose and turanose (sugars) and degraded pectin. Whole cell hydrolysates contained *meso*-diaminopimelic acid and arabinose, galactose, glucose, mannose and ribose; the polar lipid consists of diphosphatidylglycerol, a glycophospholipid, phosphatidyethanolamine, phosphatidylinositol, an aminoglycolipid, a phospholipid, three unidentified glycolipids and two unidentified lipids. The major fatty acids are $C_{18:1}$ ω9c and $C_{16:0}$ with α, α'-mycolates, *keto*-mycolates, ω-carboxymycolate as mycolic acids. The genome size is 6.9 Mb with an *in silico* DNA G+C content of 65.8%.

The type strain I10A-01893^T (= CECT 8783^T = KCTC 19843^T =DSM 45590^T) was isolated from *Stellera chamaejasme* in Yunnan Province, China

Acknowledgements

This project was supported by the School of Natural and Environmental Sciences at Newcastle University. IN is grateful to Newcastle University for a postdoctoral fellowship.

Conflicts of interest

The authors declare that they have no conflicts of interest.

References:

1. **Lehmann KB, Neumann R.** Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik. München 1896.
2. **Chester FD**. Report of mycologist: bacteriological work. *Del Agric Exp Sta Bull*. 1897;9:38-145.

3. **Goodfellow M, Jones AL.** Order V. *Corynebacteriales* ord. nov. In: Goodfellow M, Kämpfer P, Busse HJ, Trujillo ME, Suzuki K, Ludwig W, et al. (editors). Bergey's Manual of Systematic Bacteriology. 2nd edn, Vol. 5, The *Actinobacteria*, Part A. New York: Springer; 2012. p. 232-43.
4. **Magee JG, Ward AC.** Genus I. *Mycobacterium*. In: Goodfellow M, Busse HJ, Trujillo ME, Suzuki KI, Ludwig W, Whitman WB (editors). Bergey's Manual of Systematic Bacteriology. 2nd edn, Vol. 5, The *Actinobacteria* Part A. New York: Springer; 2012. p. 312-75.
5. **Wayne LG, Kubica GP.** The mycobacteria. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (editors). Bergey's Manual of Systematic Bacteriology. vol. 2. Baltimore: Williams & Wilkins; 1986. p. 1435-57.
6. **Ben Salah I, Cayrou C, Raoult D, Drancourt M**. *Mycobacterium marseillense* sp. nov., *Mycobacterium timonense* sp. nov. and *Mycobacterium bouchedurhonense* sp. nov., members of the *Mycobacterium avium* complex. *Int J Syst Evol Microbiol* 2009;59:2803-2808.
7. **Tortoli E, Kohl TA, Brown-Elliott BA, Trovato A, Leao SC, Garcia MJ, et al.** Emended description of *Mycobacterium abscessus, Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* and designation of *Mycobacterium abscessus* subsp. *massiliense* comb. nov. *Int J Syst Evol Microbiol* 2016;66:4471-4479.
8. **Prasanna AN, Mehra S**. Comparative phylogenomics of pathogenic and non-pathogenic mycobacterium. *PLoS One* 2013;8:e71248.
9. **Wee WY, Dutta A, Choo SW**. Comparative genome analyses of mycobacteria give better insights into their evolution. *PLoS One* 2017;12:e0172831.
10. **Wang J, McIntosh F, Radomski N, Dewar K, Simeone R, Enninga J, et al.** Insights on the emergence of *Mycobacterium tuberculosis* from the analysis of *Mycobacterium kansasii*. *Genome Biol Evol* 2015;7:856-870.
11. **Nouioui I, Carro L, Garcia-Lopez M, Meier-Kolthoff JP, Woyke T, Kyrpides NC, et al.** Genome-based taxonomic classification of the phylum *Actinobacteria*. *Front Microbiol* 2018;9:2007.
12. **Parte AC**. LPSN - List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Microbiol* 2018;68:1825-1829.
13. **Gupta RS, Lo B, Son J**. phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. *Front Microbiol* 2018;9:67.
14. **Tsukamura M, Yano I, Imaeda T.** *Mycobacterium moriokaense* sp. nov., a rapidly growing, nonphotochromogenic *Mycobacterium*. *Int J Syst Bacteriol* 1986;36: 333-338.
15. **Jensen KA**. Reinzuch und Typen Bestimmung von Tuberkelbazillenstämen. *Zentralbl Bakteriol* 1932;125:222-239
16. **Lorian V**. Differentiation of *Mycobacterium tuberculosis* and Runyon Group 3 "V" strains on direct cord-reading agar. *Am Rev Respir Dis* 1968;97:1133-1135.
17. **American Public Health Association ACS, Association of Official Agricultural Chemists.** Standard Methods for the Examination of Water and Sewage: American Public Health Association; 1920. p 95.
18. **MacFaddin JF.** Media for Isolation–Cultivation–Identification–Maintenance of Medical Bacteria. Baltimore: Williams & Wilkins; 1985.
19. **Runyon EH, Karlson AG, Kubica Gp, Wayne LG.** *Mycobacteriurn*. Washington, D.C: American Society for Microbiology; 1980.
20. **Amaro A, Duarte E, Amado A, Ferronha H, Botelho A**. Comparison of three DNA extraction methods for *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *avium*. *Lett Appl Microbiol* 2008;47:8-11.
21. **Sangal V, Jones AL, Goodfellow M, Hoskisson PA, Kämpfer P, Sutcliffe IC**. Genomic analyses confirm close relatedness between *Rhodococcus defluvii* and *Rhodococcus equi* (*Rhodococcus hoagii*). *Arch Microbiol* 2015;197:113-116.

22. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al.** SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;19:455-77.
23. **Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, et al.** Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613-1617.
24. **Meier-Kolthoff JP, Hahnke RL, Petersen J, Scheuner C, Michael V, Fiebig A, et al.** Complete genome sequence of DSM 30083(T), the type strain (U5/41(T)) of *Escherichia coli*, and a proposal for delineating subspecies in microbial taxonomy. *Stand Genomic Sci* 2014;9:2.
25. **Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M**. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics*. 2013;14:60.
26. **Meier-Kolthoff JP, Göker M, Spröer C, Klenk HP**. When should a DDH experiments be mandatory in microbial taxonomy? *Arch Microbiol* 2013;195:413-418.
27. **Edgar RC**. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792-1797.
28. **Stamatakis A**. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;30:1312-1313.
29. **Pattengale ND, Alipour M, Bininda-Emonds OR, Moret BM, Stamatakis A**. How many bootstrap replicates are necessary? *J Comput Biol* 2010;17:337-354.
30. **Goloboff PA, Farris JS, Nixon KC**. TNT, a free program for phylogenetic analysis. *Cladistics* 2008;24:774-786.
31. **Swofford DL.** PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0. Sunderland: Sinauer Associates; 2002.
32. **Brown BA, Springer B, Steingrube VA, Wilson RW, Pfyffer GE, Garcia MJ, et al.** *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* 1999;49 Pt 4:1493-1511.
33. **Kazda J, Muller HJ, Stackebrandt E, Daffe M, Muller K, Pitulle C**. *Mycobacterium madagascariense* sp. *Int J Syst Bacteriol* 1992; 42:524-528.
34. **Shahraki AH, Cavusoglu C, Borroni E, Heidarieh P, Koksalan OK, Cabibbe AM, et al.** *Mycobacterium celeriflavum* sp. nov., a rapidly growing scotochromogenic bacterium isolated from clinical specimens. *Int J Syst Evol Microbiol* 2015;65:510-515.
35. **Lehmann KB, Neumann R**. Lehmann's Medizin, Handatlanter X. Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik. 2 Aulf. *JF Lehmann, München*. 1899: pp. 1-497.
36. **Tsukamura M, Mizunol S, Toyama H**. *Mycobacterium pulveris* sp. nov., a nonphotochromogenic mycobacterium with an intermediate growth rate. *Int J Syst Bacteriol* 1983;33:811-815.
37. **Trevisan V**. Generi e le Specie delle Batteriacee, Zanaboni and Gabuzzi, Milano 1889.
38. **Segata N, Bornigen D, Morgan XC, Huttenhower C**. PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes. *Nat Commun* 2013;4:2304.
39. **Seemann T**. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068-9.
40. **Chaudhari NM, Gupta VK, Dutta C**. BPGA- an ultra-fast pan-genome analysis pipeline. *Sci Rep* 2016;6:24373.
41. **Castresana J**. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;17:540-552.
42. **Minh BQ, Nguyen MA, von Haeseler A**. Ultrafast approximation for phylogenetic bootstrap. *Mol Biol Evol* 2013;30:1188-1195.
43. **Bojalil LF, Cerbon J, Trujillo A**. Adansonian classification of mycobacteria. *J Gen Microbiol* 1962;28:333-346.

44. **Richter M, Rosselló-Móra R**. Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* 2009;106:19126-19131.
45. **Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM**. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81-91.
46. **Chun J, Rainey FA**. Integrating genomics into the taxonomy and systematics of the *Bacteria* and *Archaea*. *Int J Syst Evol Microbiol* 2014;64:316-324.
47. **Wayne LG BD, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, et al**. Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. *Int J Syst bacteriol* 1987; 37:463–464.
48. **Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA**. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics 2012;28:464–469.
49. **Staneck JL, Roberts GD**. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* 1974;28:226-31.
50. **Minnikin DE, Goodfellow M.** Lipid composition in the classification and identification of nocardiae and related taxa. In: Goodfellow M, Brownell GH, Serrano JA, editors. The Biology of the Nocardiae. London: Academic Press; 1976. p. 160-219.
51. **Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal A, et al.** An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Meth* 1984;2:233-41.
52. **Lechavalier MP, Lechevalier HA.** Composition of whole-cell hydrolysates as a criterion in the classification of aerobic actinomycetes. In: Prauser H, editor. The *Actinomycetales*. Jena: Gustav Fischer Verlag; 1970. p. 311-6.
53. **Miller LT**. Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* 1982;16:584-6.
54. **Sasser MJ.** Identification of bacteria by gas chromatography of cellular fatty acids. Technical Note 101, Microbial ID. USA: Inc, Newark, Del, ; 1990.
55. **Tomioka H, Saito H, Sato K, Dawson DJ**. Arylsulfatase activity for differentiating *Mycobacterium avium* and *Mycobacterium intracellulare*. *J Clin Microbiol* 1990;28:2104-2106.
56. **Kilburn JO, Silcox VA, Kubica GP**. Differential identification of mycobacteria. V. The tellurite reduction test. *Am Rev Respir Dis* 1969;99:94-100.
57. **Kent PT**, **Kubica GPW**. Public Health Mycobacteriology A Guide for the Level III Laboratory. Atlanta GA; 1985.
58. **Ribón W.** Chemical isolation and identification of mycobacteria. In: Jimenez-Lopez JC, editor. Biochemical testing: InTech; 2012.
59. **Palomino JC**, **Leão SC**, **Ritacco V**. Tuberculosis 2007: From basic science to patient care. Amedeo Challenge; 2007.
60. **Nouioui I, Carro L, Teramoto K, Igual JM, Jando M, Montero-Calasanz MDC, et al.** *Mycobacterium eburneum* sp. nov., a non-chromogenic, fast-growing strain isolated from sputum. *Int J Syst Evo Microbial* 2017;67:3174-3181.
61. **Vaas LA, Sikorski J, Hofner B, Fiebig A, Buddruhs N, Klenk HP, et al.** opm: an R package for analysing OmniLog® phenotype microarray data. *Bioinformatics* 2013;29:1823-1824.
62. **Vaas LA, Sikorski J, Michael V, Göker M, Klenk HP**. Visualization and curve-parameter estimation strategies for efficient exploration of phenotype microarray kinetics. *PLoS One* 2012;7:e34846.

383 **Table 1**. Phenotypic features that distinguish strain CECT 8783^T from *M. moriokaense* DSM
384 44221^T

	CECT 8783^T	***M. moriokaense* DSM 44221^T**
Utilisation of amino acids		
L-Arginine, D-serine #2	+	-
L-histidine, L-serine	-	+
Utilisation of organic acids		
D-Gluconic acid, glucuronamide	-	+
L-aspartic acid, L-glutamic acid, L-lactic acid, D-malic acid, α-*hydroxy*-butyric acid, p-*hydroxy*-phenylacetic acid, L-pyroglutamic acid	+	-
Utilisation of sugars :		
D-Cellobiose, dextrin, glycerol, D-maltose, methyl pyruvate, sucrose, D-trehalose turanose	+	-
D-arabitol, fructose, D-mannitol, D-sorbitol	-	+
Resistance to		
Fusidic acid, lincomycin, minocycline, rifamycin sv, 1% sodium lactate, tetrazolium blue, tetrazolium violet, vancomycin	-	+
Degradation tests		
Tween 40	-	+
Pectin	+	-

385 + Positive reaction; - negative reaction.

All of the strains metabolised acetic acid, acetoacetic acid, bromo-succinic acid, butyric acid, β-*hydroxy*-butyric acid, L-malic acid andpropionic acid (organic acids), glycine-proline (amino acid); gelatin; grew in presence of aztreonam and nalidixic acid (antibiotics) and lithium acid, potassium tellurite, sodium bromate and sodium formate (salts). In contrast, none of the strains oxidised alanine, D-aspartic acid, γ -*amino*-n butyric acid, n-*acetyl*-D-galactosamine, n-*acetyl*-D-glucosamine, n-*acetyl*-β-d-mannosamine, n-*acetyl*-neuraminic acid, citric acid, D-fructose-6-phosphate, D-fucose, L-galactonic acid-γ-lactone, D-galacturonic acid, D-glucose-6-phosphtae D-glucuronic acid, guanidine hydrochloride, α-*keto*-butyric acid, α-keto-glutaric acid, mucic acid, D-galactose, β-gentiobiose, inosine, D-lactic acid, α-D-lactose, D-lactic acid methyl ester, mannose, 3-o-methyl-D-glucose, β-methyl-D-glucoside, *myo*-inositol, melibiose, niaproof, quinic acid, D- raffinose, L-rhamnose, D-saccharic acid, D-salicin, D-serine, stachyose and troleandomycin.

Figures

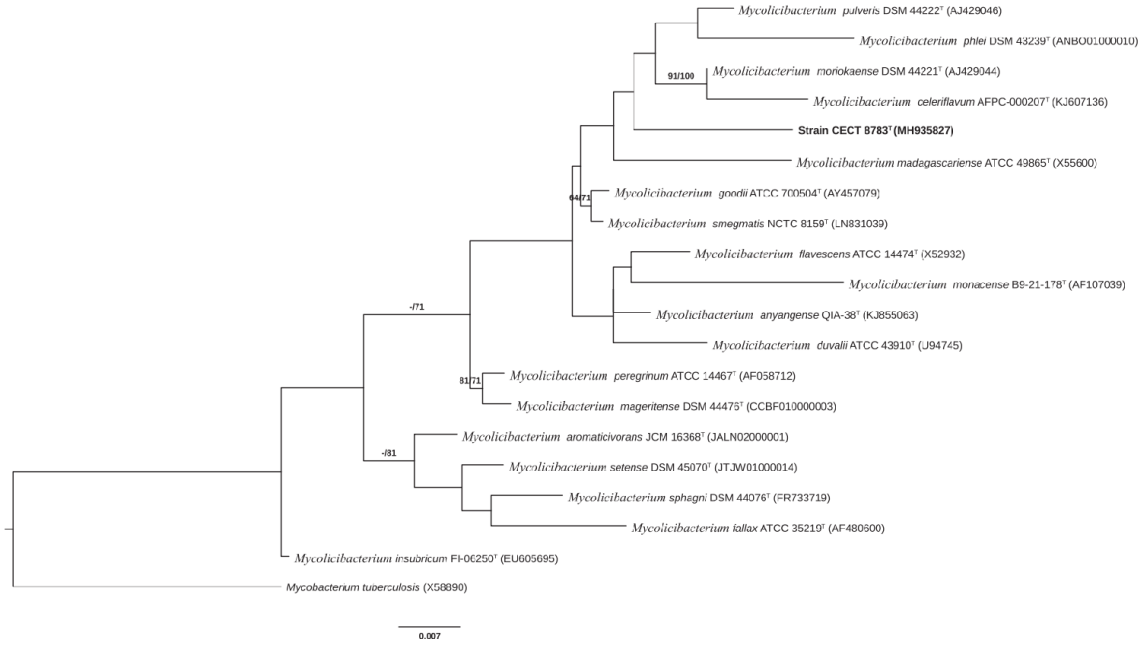

Fig. 1. Maximum-likelihood phylogenetic tree based on almost complete16S rRNA gene sequences inferred using the GTR+GAMMA model showing relationships between strain CECT 8783^T and its closest phylogenetic neighbours. The numbers above the branches are bootstrap support values greater than 60% for ML (left) and MP (right). The scale bar is 0.007.

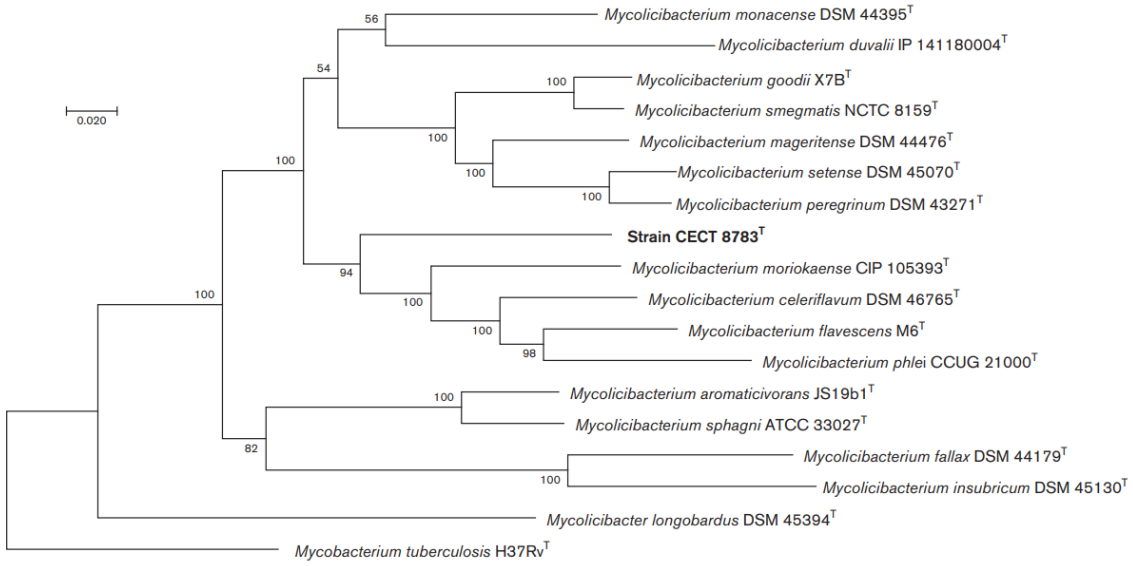

Fig. 2. Maximum-likelihood phylogenomic trees based on core genome sequences (a) and on concatenated amino acid sequences from 400 universal proteins (b) showing relationships between strain CECT 8783^T and its nearest neighbours.