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1 **Formal description of *Mycobacterium neglectum* sp. nov. and *Mycobacterium palauense***  
2 **sp. nov., rapidly growing actinobacteria**

3

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18

## 19 **Abstract**

20 The taxonomic positions of two fast growing mycobacteria (CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup>)  
21 were established using a polyphasic approach. The strains were shown to have  
22 chemotaxonomic, cultural and morphological properties consistent with their classification in  
23 the genus *Mycobacterium*. Multi-locus sequence analyses (MLSA) show that strain CECT  
24 8778<sup>T</sup> forms a well-supported clade together with the type strains of *Mycobacterium aurum*,  
25 *Mycobacterium austroafricanum* and *Mycobacterium vanbaalenii* while strain CECT 8779<sup>T</sup>  
26 presents as a distinct branch that is well separated from its nearest phylogenetic neighbours;  
27 it is also apparent from the MLSA genetic distances that these strains are most closely related  
28 to the type strains of *Mycobacterium mageritense* and *M. vanbaalenii*, respectively. Digital  
29 DNA:DNA hybridization and average nucleotide identity values between each of the strains  
30 and its close phylogenetic neighbour are below the 70 and 96% threshold values for definition  
31 of prokaryotic species; these results are underpinned by corresponding phenotypic data. Based  
32 upon the consensus of the phenotypic and phylogenetic analyses, it can be concluded that the  
33 two strains represent novel species within the genus *Mycobacterium* for which the following  
34 names are proposed: *Mycobacterium neglectum* sp. nov., with the type strain CECT 8778<sup>T</sup> (BN  
35 3150<sup>T</sup> = DSM 44756<sup>T</sup>) and *Mycobacterium palauense* sp. nov., with the type strain CECT  
36 8779<sup>T</sup> (= DSM 44914<sup>T</sup>).

37

38 **Keywords:** Actinobacteria, phenotyping, phylogeny, polyphasic taxonomy

39

## 40 **Introduction**

41 The genus *Mycobacterium* (Lehmann and Neumann 1896), the sole representative of the  
42 family *Mycobacteriaceae* (Chester 1897) can be distinguished from all of the other genera  
43 classified in the order *Corynebacteriales* by using a selection of genotypic and phenotypic  
44 methods (Goodfellow and Jones 2012). The genus encompasses pathogenic and non-  
45 tuberculous mycobacteria (Magee and Ward 2012; Forbes 2017; Gcebe et al. 2017) which can  
46 be assigned to two groups based on growth rates. Slowly growing strains require 7 or more  
47 days of incubation at optimal temperature to produce visible colonies from highly diluted  
48 inocula whereas those of rapidly growing strains are evident in fewer than 7 days under  
49 comparable conditions (Wayne and Kubica 1986). Polyphasic taxonomic procedures are now  
50 used to detect novel mycobacterial species, as exemplified by the delineation of species  
51 previously aggregated within the *Mycobacterium abscessus* and *Mycobacterium avium*

52 complexes (Ben Salah et al. 2009; Tortoli et al. 2016). Developments such as these are needed  
53 to detect the causal agents of mycobacterial infections and to establish the primary reservoirs  
54 of individual mycobacterial species within natural habitats (Tran and Dahl 2016; Shahraki et  
55 al. 2017).

56

57 Environmental mycobacteria are common in aquatic and terrestrial ecosystems  
58 (Nishiuchi et al. 2017; Roguet et al. 2016), including biofilms of water distribution systems  
59 (September et al. 2004; Feazel et al. 2009; Gomez-Smith et al. 2015). This study was  
60 undertaken to establish the taxonomic status of two rapidly growing mycobacteria: strain  
61 CECT 8778<sup>T</sup> (DSM 44756<sup>T</sup>) was isolated from a biofilm of a water distribution system and  
62 strain CECT 8779<sup>T</sup> from marine sediment. A 16S rRNA gene sequence of strain DSM 44756  
63 (then coded BN 3150) was deposited in GenBank (accession number AJ580802) under the  
64 name “*Mycobacterium neglectum*”. This code and species epithet have been used quite  
65 extensively in the literature (Thomas et al. 2008; Hussein et al. 2009; Jenkins et al. 2009; Salah  
66 et al. 2009; Loret and Creub 2010; Pontiroli et al. 2013; Nishiuchi et al. 2017). However, at no  
67 stage has a formal description been given for “*M. neglectum*” hence this name has no standing  
68 in nomenclature (Rule 29 of International Code of Nomenclature of Prokaryotes [2008  
69 revision]; Parker et al. 2015). In the present polyphasic study, we provide the first formal  
70 description of *Mycobacterium neglectum* sp. nov., with the type strain CECT 8778<sup>T</sup>, while a  
71 second novel species represented by strain CECT 8779<sup>T</sup> is named *Mycobacterium palauense*  
72 sp. nov.

73

## 74 **Materials and methods**

75

76 Source, maintenance and cultivation of strains

77

78 Strain CECT 8778<sup>T</sup>, isolated from a biofilm of an underground drinking water system in  
79 Duisburg, Germany in 1999, and strain CECT 8779<sup>T</sup>, isolated from a marine sediment collected  
80 from the Republic of Palau in 2004, were obtained from the Spanish Type Culture Collection.  
81 The strains, together with *Mycobacterium aurum* DSM 43999<sup>T</sup> (Tsukamura 1966),  
82 *Mycobacterium austroafricanum* DSM 44191<sup>T</sup> (Tsukamura et al. 1983), *Mycobacterium*  
83 *mageritense* DSM 44476<sup>T</sup> (Domenech et al. 1997) and *Mycobacterium vanbaalenii* DSM 7152<sup>T</sup>  
84 (Khan et al. 2002) were maintained as suspensions in 35% (v/v) glycerol at -80°C. Biomass for

85 the chemotaxonomic and molecular systematic studies on the isolates was cultured in shake  
86 flasks (200 revolutions per minute) of proteose peptone-meat extract-glycerol agar medium  
87 (PMG; DSMZ medium 250); after incubation at 28°C for 5 days, cells were harvested and  
88 washed three times in sodium chloride solution (0.9%, w/v). Cells for the chemotaxonomic  
89 analyses were freeze dried and stored at room temperature; wet biomass for the fatty acid  
90 analyses was prepared under the same conditions.

91

## 92 Phylogeny

93 Genomic DNA was extracted from strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> using the procedure  
94 described by Amaro et al. (2008). The genomes of the strains were sequenced using an MiSeq  
95 instrument (Illumina), as described by Sangal et al. (2015) and assembled into contigs using  
96 SPAdes 3.9.0 with a kmer length of 127 (Bankevich et al. 2012). Annotation of the genomes  
97 was achieved using the RAST pipeline available on the RAST server (Aziz et al. 2008, 2012).  
98 Complete 16S rRNA gene sequences of strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> were extracted  
99 from the draft genomes (accession numbers NVQE000000000 and NVQF000000000,  
100 respectively) and deposited in GenBank under accession numbers MF769621 and MF769712.  
101 Corresponding 16S rRNA gene sequences of the type strains of closely related *Mycobacterium*  
102 spp. were retrieved from the EzBioCloud server (Yoon et al. 2017) and pairwise sequence  
103 similarities calculated using the Genome-to-Genome Distance Calculator (GGDC) web server  
104 (Meier-Kolthoff et al. 2013a, b). Phylogenies derived from the 16S rRNA gene sequences were  
105 inferred using the GGDC web server adapted to single genes (Meier-Kolthoff et al. 2014).  
106 Multiple sequence alignments were generated using MUSCLE software (Edgar 2004) and a  
107 maximum-likelihood (ML) tree inferred from the alignment with RAxML (Stamatakis 2014)  
108 using rapid bootstrapping together with the auto Maximal-Relative-Error (MRE) criterion  
109 (Pattengale et al. 2010). Similarly, a maximum-parsimony (MP) tree was inferred from the  
110 alignments with the ‘Tree analysis New Technology’ (TNT) program (Goloboff et al. 2008)  
111 using 1000 bootstraps together with tree bisection and reconnection branch swapping and ten  
112 random sequence replicates. The sequences were checked for computational bias using the X<sup>2</sup>  
113 test implemented in PAUP\* (Phylogenetic Analysis Using Parsimony) (Swofford 2002).

114 Partial sequences of three housekeeping genes, *hsp65* (heat shock protein), *rpoB* (RNA  
115 polymerase beta subunit) and *recA* (recombination protein A) (McNabb et al. 2004;  
116 Ramaprasad et al. 2016), were drawn from the draft genomes of strains CECT 8778<sup>T</sup> and CECT  
117 8779<sup>T</sup> and deposited in GenBank under the accession numbers MF774022, MF774023,

118 MF774024 , MF77402 , MF774026, MF774027, respectively. A multilocus sequence  
119 analysis (MLSA) tree was generated from 3203 nucleotides (nt) of concatenated sequences of  
120 the three housekeeping genes and corresponding 16S rRNA gene sequences and ML and MP  
121 trees inferred as described above. In addition, a neighbour-joining (NJ) tree (Saitou and Nei  
122 1987) was generated from the MEGA 7 software package (Kumar et al. 2015). The alignment  
123 of the concatenated sequences and the corresponding evolutionary distances were carried out  
124 using CLUSTAL W software (Thompson et al. 1997) and the Kimura two parameter model  
125 (Kimura 1980), respectively.

126 The average nucleotide identity (ANI) between strain CECT 8778<sup>T</sup> and *M. aurum* DSM  
127 43999<sup>T</sup> (genome accession number is NZ\_CVQQ000000000), *M. austroafricanum* DSM  
128 44191<sup>T</sup> genome accession number is (NZ\_HG964469) and *M. vanbaalenii* PYR1<sup>T</sup> (genome  
129 accession number is CP000511) and between strain CECT 8779<sup>T</sup> and *M. mageritense* DSM  
130 44476<sup>T</sup> (genome accession number is NZ\_CCBF000000000), their respective near  
131 phylogenetic neighbours, were calculated according to Rodriguez and Konstantinidis (2014).  
132 Similarly, digital DNA-DNA hybridization (dDDH) similarities were determined between the  
133 two strains and their close phylogenetic neighbours using the GGDC server (Meier-Kolthoff et  
134 al. 2013a).

135

#### 136 Chemotaxonomy

137 The chemotaxonomic profiles of strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and their respective  
138 close phylogenetic neighbours were determined using standard thin-layer chromatographic  
139 procedures. To this end, the strains were examined for diaminopimelic acid isomers (A<sub>2</sub>pm)  
140 (Staneck and Roberts 1974), predominant menaquinones (Collins 1985), mycolic acids  
141 (Mininkin et al. 1980), diagnostic sugars (Lechevalier and Lechevalier 1970) and polar lipids  
142 (Mininkin et al. 1984). In addition, cellular fatty acids were extracted from freeze dried biomass  
143 of the strains and fatty acid methyl esters (FAMES) prepared following saponification and  
144 methylation using the procedure introduced by Miller (1982), as modified by Kuykendall et al.  
145 (1988). The FAMES were analysed by gas chromatography (Agilent 6890 instrument) and the  
146 resultant peaks automatically integrated. The identities of the fatty acids were determined using  
147 the standard Microbial Identification (MIDI) System, version 4.5, and the Myco 6 database  
148 (Sasser 1990).

149

#### 150 Growth and cultural properties

151 Strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> were examined for their ability to grow and form  
152 colonies and pigments on glucose-yeast extract-malt extract agar (GYM, DSMZ medium 65),  
153 Löwenstein-Jensen (LJ) medium (Jensen 1932), Middlebrook 7H10 agar supplemented with  
154 oleic acid, albumin dextrose and catalase (MB7H10, Lorian 1968), proteose peptone-meat  
155 extract-glycerol agar (PMG; DSMZ medium 250) and tryptic soy agar (TSA, MacFaddin 1985)  
156 for 14 days at 4, 10, 20, 25, 28 and 42°C under light and dark conditions. The strains were also  
157 examined for acid-alcohol-fastness using the Ziehl-Neelsen method (Runyon et al. 1980) and  
158 for their ability to grow on PMG agar under anaerobic conditions at 28°C using an anaerobic  
159 bag system (Sigma-Aldrich 68061).

#### 160 Phenotypic tests

161 The two strains and their close phylogenetic neighbours were examined for phenotypic tests  
162 found to be useful in mycobacterial systematics (Magee and Ward 2012, Nouioui et al. 2017).  
163 The strains were tested for their ability to use sole carbon and sole nitrogen sources, to grow in  
164 the presence of several concentrations of sodium chloride, at a range of pH values and in the  
165 presence of antibiotics using GENIII microplates and an Omnilog device (BIOLOG, Hayward,  
166 CA). The tests were carried out in duplicate using freshly prepared inocula (OD<sub>600</sub>-0.3-0.6)  
167 harvested from the mid-logarithmic growth phase of PMG agar plates incubated at 28°C for 7  
168 days. The resultant data were exported and analysed using the opm package version 1.3.36  
169 (Vaas et al. 2012, 2013). The strains were also examined for their ability to produce  
170 arylsulfatase after 3 and 14 days (Tomioka et al. 1990), catalase (Palomino et al. 2007) and  
171 heat stable catalase (Sequeira de Latini and Barrera 2008) and for niacin accumulation (Kent  
172 and Kubica 1985), resistance to potassium tellurite (Kent and Kubica 1985; Kilburn et al. 1969),  
173 degradation of Tween 80 (Ribón 2012) and urea hydrolysis (Palomino et al. 2007) using the  
174 media and incubation conditions described in these references. All of these tests were carried  
175 out in duplicate using the standard inoculum.

176

#### 177 **Results and discussion**

178 The chemotaxonomic, growth and staining properties of strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup>  
179 were shown to be consistent with their classification in the genus *Mycobacterium* (Magee and  
180 Ward 2012). The organisms were found to be strictly aerobic, Gram-positive, acid-alcohol fast,  
181 rapid growing, rod-shaped bacteria which contain *meso*-diaminopimelic acid, arabinose,  
182 galactose, glucose, rhamnose and ribose in whole organism hydrolysates (wall chemotype IV  
183 *sensu* Lechevalier and Lechevalier 1970); mixtures of saturated, unsaturated and 10-methyl



184 octadecanoic (tuberculostearic) fatty acids; mycolic acids; dihydrogenated menaquinones with  
185 nine isoprene units (MK9(H<sub>2</sub>)) as the predominant isoprenologue; and a polar lipid profile that  
186 includes diphosphatidylglycerol, phosphatidylethanolamine (diagnostic phospholipid),  
187 phosphatidylinositol, as well as a glycopospholipid and glycolipids (phospholipid type II;  
188 Lechevalier et al. 1977). Both strains were found to produce unpigmented colonies under both  
189 light and dark conditions on LJ, MB7H10, PMG and TSA plates after 5 days at 28°C; moderate  
190 growth was observed at 20°C, 25°C and 37°C; optimal growth was detected at 28°C on GYM,  
191 MB7H10 and PMG agar after 5 days. The strains were unable to grow on any of these media  
192 at 4°C, 15°C, 42°C, or 45°C or under anaerobic conditions at 28°C on PMG agar. Strain CECT  
193 8778<sup>T</sup> and its nearest phylogenetic neighbours, *M. aurum* DSM 43999<sup>T</sup>, *M. austroafricanum*  
194 DSM 44191<sup>T</sup> and *M. vanbaalenii* DSM 7152<sup>T</sup>, share several features; they are all acid acid-  
195 alcohol fast, rapid growing bacteria that grew on MB7H10 and PMG media at 28°C though  
196 only the test strain formed non-pigmented colonies. Strain CECT 8779<sup>T</sup> and *M. mageritense*  
197 DSM 44476<sup>T</sup> were found to have very similar cultural and morphological traits though only  
198 the latter grew at 22, 30, 37 and 45°C on LJ media (Domenech, et al. 1997).

199 The pairwise 16S rRNA gene similarities between strain CECT 8778<sup>T</sup> and *M. aurum*  
200 NCTC 10437<sup>T</sup>, *M. austroafricanum* DSM 44191<sup>T</sup>, *Mycobacterium pyrenivorans* DSM 44605<sup>T</sup>  
201 (Derz et al. 2004), *Mycobacterium vaccae* ATCC 25954<sup>T</sup> (Bönicke and Juhasz 1964) and *M.*  
202 *vanbaalenii* PYR-1 were found to be 99.2%, 99.3%, 98.6%, 98.9%, 99.2%, respectively. It can  
203 be seen from Figure 1 that the two strains were found to be well separated from the type strains  
204 of the remaining fast growing *Mycobacterium* species. Strain CECT 8778<sup>T</sup> was shown to form  
205 a distinct branch at the periphery of a well-supported subclade that included all of the organisms  
206 cited above. In turn, the pairwise 16S rRNA gene similarities between strain CECT 8779<sup>T</sup> and  
207 the type strains of *M. mageritense*, *Mycobacterium peregrinum* ATCC 14467<sup>T</sup> (Kusunoki and  
208 Ezaki 1992) and *Mycobacterium wollinskyi* ATCC 700010<sup>T</sup> (Brown et al. 1999) were found to  
209 be 98.8%, 98.8% and 98.9%, respectively; strain CECT 8779<sup>T</sup> formed a well defined branch in  
210 a subclade that also contained these organisms, albeit one that was supported by a high  
211 bootstrap value only in the ML analysis. It can also be seen from Figure 1 that all of the rapidly  
212 growing strains were sharply separated from the type strain of *Mycobacterium Tuberculosis*  
213 (Zopf 1883; Lehmann and Neumann 1896), the type species of the genus.

214 The MLSA trees based on the sequences of the three housekeeping genes and the  
215 corresponding 16S rRNA sequences are shown in Figure 2; this tree was inferred from 3257  
216 nt, 604 of which were variable and 324 of which were parsimony-informative. The average  
217 bootstrap supports for the ML and MP trees were found to be 90.0% and 92.3%, respectively.



218 It is evident from Figure 2 that strain CECT 8778<sup>T</sup> forms a well supported subclade together  
219 with the type strains of *M. aurum*, *M. austroafricanum* and *M. vanbaalenii* while strain CECT  
220 8779<sup>T</sup> forms a branch distinct from all of the other mycobacteria, including the type strains of  
221 *M. mageritense*, *M. peregrinum* and *M. wollinskyi*. Moreover, it is clear from Table 1 that  
222 strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> share close genetic distances, namely 0.02 and 0.03, with  
223 the type strains of *M. vanbaalenii* and *M. mageritense*, respectively. However, in the  
224 corresponding NJ tree, strain CECT 8778<sup>T</sup> was shown to be more closely related to the *M.*  
225 *austroafricanum* and *M. vanbaalenii* type strains than to the *M. aurum* strain though these  
226 relationships were not supported by high bootstrap values (Fig. S1). The phylogenetic trees  
227 based on the sequences of the individual housekeeping genes are shown in Figures S2-S4; the  
228 relationships between strain CECT 8778<sup>T</sup> and the type strains of *M. aurum*, *M. austroafricanum*  
229 and *M. vanbaalenii* are evident in all of the trees though bootstrap values are low. In contrast,  
230 strain CECT 8779<sup>T</sup> was found to form a distinct branch in all of these trees.

231 The genome sizes of strains CECT 8778<sup>T</sup> (NVQE000000000) and CECT 8779<sup>T</sup>  
232 (NVQF000000000) were both found to be ~6.2Mb with average *in silico* G+C contents of  
233 65.3mol% and 69.4mol%, respectively. The draft genome of strain CECT 8778<sup>T</sup> was generated  
234 from 123 contigs with N lengths of 116368 and was found to contain 6159 predicted protein  
235 coding sequences and 51 tRNA genes. Similarly, the draft genome of strain CECT 8779<sup>T</sup> was  
236 compiled from 210 contigs with N lengths of 61684 and was shown to have 5802 predicted  
237 protein coding sequences and 71 tRNA genes. The coverage for strains CECT 8778<sup>T</sup> and CECT  
238 8779<sup>T</sup> were 79X and 67X, respectively. Strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> contain, very  
239 similar subsystem gene functions as exemplified in Table S1.

240 Strain CECT 8778<sup>T</sup> and *M. aurum* NCTC 10437<sup>T</sup>, *M. austroafricanum* DSM 44191<sup>T</sup>  
241 and *M. vanbaalenii* PYR-1<sup>T</sup>, currently its closest phylogenetic neighbours, were found to share  
242 dDDH similarities of 20.1%, 21.1% and 21.1%, respectively, values well below the 70% cut  
243 off point recommended for the delineation of prokaryotic species (Wayne et al. 1987). The  
244 corresponding ANI similarities between strain CECT 8778<sup>T</sup> and the three strains mentioned  
245 above were found to be 78.4%, 78.7% and 79.6%, values well below the 95-96% threshold  
246 used to distinguish between closely related species of prokaryotes (Goris et al. 2007; Richter  
247 and Rosselló-Móra 2009; Chun and Rainey 2014). Similarly, the dDDH and ANI values  
248 between strain CECT 8779<sup>T</sup> and *M. mageritense* DSM 44476<sup>T</sup>, its current closest phylogenetic  
249 neighbour, were 20.9% and 79.1%, respectively, values well below the species thresholds cited  
250 above.

251 Identical results were recorded for all of the phenotypic tests that were carried out in  
252 duplicate. It can be seen from Table 2 that strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> can be  
253 distinguished from one another and from their respective reference strains using a combination  
254 of phenotypic properties though it is apparent that all of these organisms share a common set  
255 of features. Strain CECT 8778<sup>T</sup>, unlike the type strains of *M. aurum*, *M. austroafricanum* and  
256 *M. vanbaalenii*, was shown to grow in the presence of tetrazolium blue and tetrazolium violet  
257 and also differed from these strains by its inability to utilise L-alanine, butyric acid,  $\alpha$ -hydroxy-  
258 butyric acid,  $\alpha$ -keto-butyric acid, galactose, glycerol, L-lactic acid, pectin and sucrose or to  
259 grow in the presence of lithium chloride, nalidixic acid and sodium chloride (up to 8%, w/v).  
260 Similarly, strain CECT 8779<sup>T</sup>, unlike *M. mageritense* DSM 44476<sup>T</sup>, was shown to use D-  
261 arabitol, D-glucose-phosphate, D-maltose, D-mannose, methyl pyruvate, pectin, D-sorbitol,  
262 sucrose, D-trehalose and D-turanose as sole carbon sources and to grow in the presence of  
263 lithium chloride and sodium chloride (8%, w/v). In turn, strain CECT 8778<sup>T</sup>, unlike strain  
264 CECT 8779<sup>T</sup>, was seen to metabolise dextrin, *myo*-inositol, D- and L-malic acid, quinic acid,  
265 D- saccharic acid, D- salicin, L-serine and bromo-succinic acid. Conversely, strain CECT  
266 8779<sup>T</sup> was found to use L-alanine, L-aspartic acid, butyric acid, citric acid, D-glucose-6-  
267 phosphate, glycerol,  $\alpha$ -keto-glutaric acid, L-histidine, L-lactic acid, D-maltose, N-acetyl- $\beta$ -D-  
268 mannosamine, methyl pyruvate, pectin, L-rhamnose and sucrose and to grow in the presence  
269 of lithium chloride, nalidixic acid and sodium chloride.

270 The kind of mycolic acids synthesised by representatives of *Mycobacterium* species fall  
271 into several well established patterns of taxonomic value (Mininkin et al. 1985; Magee and  
272 Ward 2012). In the present study, the test strains were found to have different mycolic acid  
273 profiles: strain CECT 8778<sup>T</sup> was shown to contain  $\alpha$ - and *decarboxy*- mycolates and strain  
274 CECT 8779<sup>T</sup>  $\alpha$ -, *keto*- and *methoxy*- mycolates. This latter pattern serves to distinguish strain  
275 CECT 8779<sup>T</sup> from *M. mageritense* DSM 44476<sup>T</sup> which is characterised by the presence of  $\alpha$ -,  
276  $\alpha'$ - and *epoxy*-mycolates (Domenech et al. 1997). Similarly, the mycolic acid profile  
277 distinguishes strain CECT 8778<sup>T</sup> from *M. aurum* DSM 43999<sup>T</sup> and *M. austroafricanum* DSM  
278 44191<sup>T</sup> as these strains have  $\alpha$ - and *keto*-mycolates and wax esters (Mininkin et al. 1985; Magee  
279 and Ward 2012). Similarly, complex polar lipid pattern of strain CECT 8778<sup>T</sup> serves to  
280 distinguish it from both strain CECT 8779<sup>T</sup> and from the type strains of *M. aurum*, *M.*  
281 *austroafricanum* and *M. vanbaalenii*; all of these strains were found to contain  
282 diposphatidylglycerol, phosphatidylethanolamine (diagnostic lipid), phosphatidylinositol and

283 glycophospholipids. Strain CECT 8779<sup>T</sup>, unlike its near phylogenetic neighbour, *M.*  
284 *mageritense* DSM 44476<sup>T</sup>, was shown to have a lipid pattern that lacked phosphatidylglycerol.

285 All of the strains produced complex mixtures of straight-chain saturated, unsaturated  
286 and 10-methyl-octadecanoic (tuberculostearic) fatty acids, a profile typical of members of the  
287 genus *Mycobacterium* (Magee and Ward 2012). With few exceptions, strains CECT 8778<sup>T</sup>, *M.*  
288 *aurum* DSM 43999<sup>T</sup>, *M. austroafricanum* DSM 44191<sup>T</sup> and *M. vanbaalenii* DSM 7152<sup>T</sup> were  
289 found to have major proportions (>10% of total fatty acid) of C<sub>16:0</sub> (13.3-72.6%), C<sub>18:1 ω9c</sub>  
290 (7.6-19.7.0%) and summed features 2 (12.6-43.2%) and 3 (12.3-16.8%) though the  
291 predominant component varied (Table 3). The fatty acid profiles of CECT 8778<sup>T</sup> and the type  
292 strain of *M. vanbaalenii* were distinct; the test strain, for instance, produced higher proportions  
293 of summed feature 2 (43.2 against 27.6%). Even greater differences were found between the  
294 fatty acid profiles of CECT 8778<sup>T</sup> and *M. aurum* DSM 43999<sup>T</sup>; the latter, for instance, was  
295 especially rich in C<sub>16:0</sub> (25.4% against 13.3.0%). Marked differences were found between the  
296 fatty acid profiles of strain CECT 8779<sup>T</sup> and *M. mageritense* DSM 44476<sup>T</sup> as the former  
297 contained moderate proportions of C<sub>16:1 ω9c</sub> and summed features 2 and a lower amount of  
298 C<sub>16:0</sub> (25.1 against 40.0%) (Table 3).

299 In summary, strain CECT 8778<sup>T</sup> can be distinguished readily from *M. aurum* DSM  
300 43999<sup>T</sup>, *M. austroafricanum* DSM 44191<sup>T</sup> and *M. vanbaalenii* DSM 7251<sup>T</sup>, its close  
301 phylogenetic neighbours, in the 16S rRNA and MLSA gene trees, by low ANI and dDDH  
302 scores and by a range of chemotaxonomic and phenotypic markers. A similar wealth of  
303 taxonomic data separate strain CECT 8779<sup>T</sup> from the type strain of *M. mageritense*, its close  
304 phylogenetic neighbour. These datasets clearly show that strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup>  
305 represent new centres of taxonomic variation within the genus *Mycobacterium*; the names  
306 chosen for these species are *Mycobacterium neglectum* sp. nov. and *Mycobacterium palauense*  
307 sp. nov., respectively. The Digital Protologue database TaxoNumbers for strains CECT 8778<sup>T</sup>  
308 and CECT 8779<sup>T</sup> are TA00318 and TA000312, respectively.

### 309 **Description of *Mycobacterium neglectum* sp. nov.**

310 *Mycobacterium neglectum* (neg.lec'tum. L. adj. *neglectum*, neglected reflecting the history of  
311 the strain)

312 Strict aerobic, Gram-stain positive, acid-alcohol fast, rapid growing organism which  
313 forms unpigmented colonies after growth on Middelbrook 7H10, proteose peptone-meat  
314 extract-glycerol and LJ media after incubation under the light and dark conditions after 5 days

315 at 28°C. Grows between 25°C and 28°C, optimally at 28°C and at pH 7. Produces arylsulfatase  
316 after 3 and 14 days, catalase, nitrate reductase, urease and grows in the presence of potassium  
317 tellurite. Additional phenotypic data are given in the text and in Table 1. Whole cell  
318 hydrolysates are rich in *meso*-diaminopimelic acid, arabinose, galactose, glucose, ribose and  
319 rhamnose; the polar lipid profile contains diphosphatidylglycerol, glycopospholipids (GPL1-  
320 2), phosphatidylethanolamine, phosphatidylinositol and a glycolipid; MK9 (H<sub>2</sub>) is the  
321 predominant menaquinone and the major fatty acid is C<sub>17:1</sub> ω<sub>7c</sub>/18 alcohol. Contains α- and  
322 *decarboxy*-mycolic acids. The DNA G+C content determined from the draft genome of the  
323 type strain is 65.3 mol%.

324 The type strain, CECT 8778<sup>T</sup> (= BN3150<sup>T</sup> = DSM 44756<sup>T</sup>) was isolated from a biofilm  
325 of an underground drinking water system in Germany. The Genbank accession number of the  
326 draft genome sequence of strain CECT 8778<sup>T</sup> is NVQE000000000.

### 327 **Description of *Mycobacterium palauense* sp. nov.**

328 *Mycobacterium palauense* (pa.lau.en'se N.L. neut. adj. *palauense* referring to the Republic  
329 Palau, the source of the strain)

330 Strict aerobic, Gram-stain positive, acid-alcohol fast, rapid growing organism which  
331 forms unpigmented colonies after growth on Middelbrook 7H10, proteose peptone-meat  
332 extract-glycerol and LJ media after incubation under the light and dark conditions for 5 days  
333 at 28°C. Grows between 25°C and 28°C, optimally at 28°C and at pH 7 and in the presence of  
334 up to 8% w/v NaCl. Produces arylsulfatase after 3 and 14 days, catalase, accumulates niacin,  
335 degrades Tween 80 and grows in presence of potassium tellurite. Additional phenotypic data  
336 are given in the text and in Table 1. Whole cell hydrolysates are rich in *meso*-diaminopimelic  
337 acid, arabinose, galactose, glucose, ribose and rhamnose; the polar lipid profile contains  
338 diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, as well as  
339 glycopospholipids (GPL<sub>1-2</sub>) and a glycolipid; MK9 (H<sub>2</sub>) is the predominant menaquinone and  
340 the major fatty acids are C<sub>16:0</sub> and C<sub>18:1</sub> ω<sub>9c</sub>. Contains α, *keto-methoxy* mycolic acids. The DNA  
341 G+C content determined from the draft genome of the type strain is 69.4 mol %.

342 The type strain, CECT 8779<sup>T</sup> (= DSM 44914<sup>T</sup>) was isolated from marine sediment from  
343 the Republic of Palau. The Genbank accession number of the draft genome sequence of strain  
344 CECT 8779<sup>T</sup> is NVQF000000000.

345

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349 **Compliance with ethical standards**

350 **Conflict of interest** The authors declare that they have no conflicts of interest.

351 **Ethical statement** This article does not contain any studies inoculating human participants or  
352 animals.

353

354 **References**

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566

567

568 **Table 1.** Genetic distances between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours

		MLSA (Kimura 2-parameter) distance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Strain CECT 8779 <sup>T</sup>														
2	Strain CECT 8778 <sup>T</sup>	0,051													
3	<i>Mycobacterium peregrinum</i>	0,031	0,039												
4	<i>Mycobacterium mageritense</i>	0,030	0,039	0,009											
5	<i>Mycobacterium wolinskyi</i>	0,032	0,036	0,012	0,012										
6	<i>Mycobacterium chlorophenicum</i>	0,036	0,034	0,031	0,034	0,034									
7	<i>Mycobacterium chubuense</i>	0,037	0,036	0,033	0,036	0,037	0,006								
8	<i>Mycobacterium psychrotolerans</i>	0,037	0,039	0,035	0,036	0,037	0,015	0,016							
9	<i>Mycobacterium austroafricanum</i>	0,048	0,028	0,046	0,048	0,049	0,029	0,032	0,036						
10	<i>Mycobacterium vanbaalenii</i>	0,047	0,026	0,044	0,046	0,046	0,026	0,029	0,035	0,003					
11	<i>Mycobacterium aurum</i>	0,050	0,029	0,038	0,041	0,041	0,032	0,030	0,037	0,028	0,026				
12	<i>Mycobacterium rufum</i>	0,040	0,036	0,034	0,037	0,036	0,009	0,012	0,018	0,035	0,034	0,036			
13	<i>Mycobacterium arcueilense</i>	0,054	0,044	0,030	0,035	0,036	0,049	0,051	0,046	0,054	0,052	0,046	0,051		
14	<i>Mycobacterium alvei</i>	0,056	0,043	0,032	0,036	0,039	0,049	0,052	0,047	0,059	0,057	0,046	0,052	0,008	
15	<i>Tsukamurella paurometabola</i>	0,071	0,077	0,074	0,076	0,073	0,073	0,073	0,073	0,083	0,081	0,087	0,077	0,084	0,086

569

570

571 **Table 2.** Phenotypic features that distinguish strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> from one another and from their near phylogenetic  
572 neighbours. All data are from the present study.  
573

	strain CECT 8778 <sup>T</sup>	<i>M. aurum</i> DSM 43999 <sup>T</sup>	<i>M. austroafricanum</i> DSM 44191 <sup>T</sup>	<i>M. vanbaalenii</i> DSM 7152 <sup>T</sup>	strain CECT 8779 <sup>T</sup>	<i>M. mageritense</i> DSM 44476 <sup>T</sup>
<b>Biochemical tests:</b>						
Arylsulfatase 3 days	+	-	+	+	+	+
Heat stable catalase 68°C	+	-	-	-	-	-
Niacin, Tween 80	+	+	-	-	+	+
Urea hydrolysis	+	-	-	+	-	-
<b>GEN III Biolog microplate tests</b>						
<b>Utilisation of sugars:</b>						
D-Arabitol,	+	-	+	-	+	-
Dextrin	+	-	-	+	-	+
D-Galactose	-	+	+	-	-	-
<i>N</i> -acetyl-D-Glucosamine	+	-	+	-	+	+
3- <i>o</i> -methyl-D-Glucose	-	-	+	-	-	-
D-Glucose-6-phosphate	-	-	+	-	+	-
Glycerol	-	+	+	+	+	+
<i>myo</i> -Inositol	+	+	+	+	-	-
D-Maltose	-	-	+	-	+	-
<i>N</i> -acetyl-β-D-Mannosamine	-	-	+	-	+	+
D-Mannose, D-sorbitol, D-trehalose	+	+	+	+	+	-
L-Rhamnose	-	-	+	-	+	+
D-Salicin	+	-	+	-	-	-

Sucrose	-	+	+	+	+	-
D-Turanose	+	+	-	+	+	-
<b>Utilisation of organic acids:</b>						
Butyric acid	-	+	+	+	+	+
$\beta$ -amino- <i>n</i> -Butyric acid	+	+	-	-	+	+
$\alpha$ -hydroxy-Butyric acid, $\alpha$ -keto-Butyric acid	-	+	+	+	-	+
Citric acid	-	-	-	-	+	+
D-Galacturonic acid	-	+	-	-	-	-
$\alpha$ -keto-Glutamic acid	-	+	-	+	+	+
L-Lactic acid	-	+	+	+	+	+
D-and L-Malic acid	+	+	+	+	-	+
Methyl pyruvate	-	+	+	+	+	-
Quinic acid	+	-	+	-	-	-
D-Saccharic acid	+	+	+	+	-	-
Bromo-Succinic acid	+	+	+	+	-	+
<b>Utilisation of amino acids:</b>						
L-Alanine	-	+	+	-	+	+
L-Aspartic acid	-	-	+	-	+	+
Glycyl- L-proline	+	-	+	+	+	+
L-Histidine	-	-	-	-	+	+
D-Serine #2, L- Pyroglutamic acid	-	+	-	-	-	+
L-Serine	+	-	+	-	-	+
<b>Resistance to:</b>						
Lincomycin	-	-	-	-	-	+
Lithium chloride	-	+	+	+	+	-
Nalidixic acid, sodium chloride (1% w/v)	-	+	+	+	+	+

Rifamycin SV, sodium bromate	+	+	-	+	+	+
Sodium chloride (4% w/v)	-	+	-	-	+	+
Sodium chloride (8% w/v)	-	+	-	-	+	-
Sodium formate	+	+	+	+	-	+
Tetrazolium violet	+	-	-	-	+	+
Tetrazolium blue	+	-	-	-	-	-
Troleandomycin	-	+	-	-	-	-
Vancomycin	-	+	-	+	-	-
<b>Growth in presence of:</b>						
Gelatin	-	+	-	-	-	+
Pectin	-	+	+	+	+	-
Tween 40	+	-	+	+	+	+
<b>Chemotaxonomic traits</b>						
Polar lipids	DPG, PE, PI, GL GPL1-2	DPG, PE, PI, GL GPL1-2	DPG, PE, PI, GL GPL1-2	DPG, PE, PI, GL GPL1-2	DPG, PE, PI, GL GPL1-2	DPG, PE, PI, PG, GL GPL1-2
Fatty acids (>20 %)	Summed feature 2	C <sub>16:0</sub>	C <sub>16:0</sub>	Summed feature 2	C <sub>16:0</sub> , C <sub>18:1</sub> ω9c	C <sub>16:0</sub> , C <sub>18:1</sub> ω9c
Mycolic acids	<i>α</i> - and <i>decarboxy</i> -mycolates	<i>α</i> - <i>keto</i> -mycolates and wax esters*	<i>α</i> - <i>keto</i> -mycolates and wax esters*	<i>α</i> - <i>keto</i> -mycolates and wax esters*	<i>α</i> , <i>keto</i> - <i>methoxy</i> mycolic acids	<i>α</i> -, <i>α</i> '- and <i>epoxy</i> -mycolates**
<b>DNA G+C content (%)</b>	65.3	67.5	67.4	67.8	69.4	66.6

574 + Positive reaction; - negative reaction.

575 Positive results recorded for all of the strains: arylsulfatase (14 days) and catalase (biochemical tests); utilisation of acetic acid, acetoacetic acid, L-arginine, β-*hydroxy*-butyric acid, D-glucose, D-fructose, D-gluconic acid, L-glutamic acid, D-mannitol and propionic acid; growth at pH 6 and in presence of aztreonam, potassium tellurite and 1% sodium lactate. Negative results detected for all of the strains: utilisation of D-aspartic acid, D-

578 cellobiose, D-fructose-6-phosphate, D-and L-fucose, L-galactonic acid- $\gamma$ -lactone, *N*-acetyl-D-galactosamine,  $\beta$ -gentiobiose,  $\beta$ -methyl-D-glucoside,  
579 glucuronamide, D-glucuronic acid, D-lactic acid methyl ester,  $\alpha$ -D-lactose, D-melibiose, mucic acid, *N*-acetyl-neuraminic acid, *p*-hydroxy-  
580 phenylacetic acid, D-raffinose and stachyose; growth at pH 5 and in the presence of fusidic acid, guanidine hydrochloride, inosine, minocycline  
581 and niaproof. Abbreviation: DPG: diphosphatidylglycerol; GPL1-2: glycophospholipids; GL: glycolipid; PE: phosphatidylethanolamine; PI:  
582 phosphatidylinositol; PG: glycophospholipid; summed features 2, C<sub>17:1</sub>  $\omega$ 7c /18 alcohol; \*data taken from Mininkin et al. 1985, Magee and Ward 2012;  
583 \*\* data taken from Domenech et al. 1997.

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594 **Table 3.** Fatty acid profiles (%) of strain CECT 8778<sup>T</sup> (1), *M. aurum* DSM 43999<sup>T</sup> (2), *M.*  
 595 *austroafricanum* DSM 44191<sup>T</sup> (3), *M. vanbaalenii* DSM 7152<sup>T</sup> (4), strain CECT 8779<sup>T</sup> (5) and  
 596 *M. mageritense* DSM 44476<sup>T</sup> (6). All data are from the present study.

	1	2	3	4	5	6
C <sub>14:0</sub>	3.6	3.4	2.6	2.4	5.2	7.3
C <sub>16:1</sub> ω9c	1.0	0.8	0.9	1.5	3.6	-
C <sub>16:1</sub> ω6c	4.2	6.9	10.7	6.8	6.5	10.3
C <sub>16:1</sub> ω7c	0.5	-	-	-	1.6	4.1
C <sub>16:0</sub>	13.3	25.4	27.6	17.0	25.4	40.0
Summed feature 2	43.2	12.6	15.7	27.6	7.6	-
C <sub>18:1</sub> ω9c	7.6	19.7	8.9	13.8	26.2	28.3
C <sub>18:0</sub>	-	1.6	2.7	1.6	-	-
10 Me-C <sub>18:0</sub>	11.0	12.6	14.2	8.6	7.8	8.7
Summed feature 3	12.3	15.2	15.0	16.8	-	-
C <sub>20:0</sub>	-	-	0.3	0.3	-	-

597 Summed features 2, C<sub>17:1</sub> ω7c /18 alcohol and summed feature 3, 20:0 ALC 18.838/ECL20:0  
 598 ALC.

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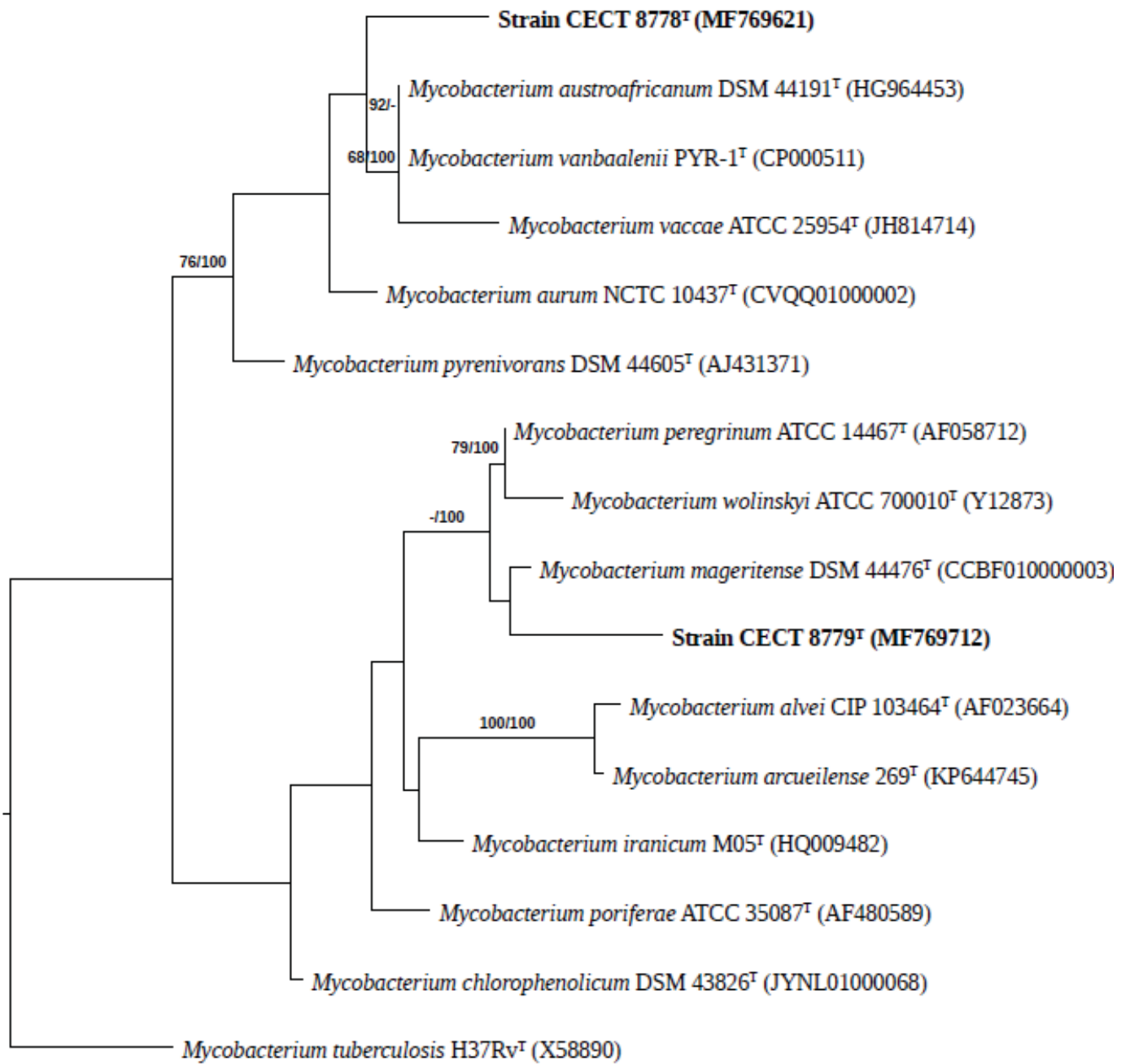
603 **Figure legends**

604 Fig. 1. Maximum-likelihood phylogenetic tree based on almost complete 16S rRNA gene  
605 sequences generated using the GTR+GAMMA model and midpoint-rooting showing  
606 relationships between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close  
607 phylogenetic neighbours. The numbers above the branches are bootstrap support values greater  
608 than 60% for ML (left) and MP (right). The scale bar indicates 0.007 substitutions per site.

609 Fig. 2. Maximum-likelihood phylogenetic tree based on concatenated sequences of 16S rRNA,  
610 *hsp65*, *rpoB* and *recA* gene sequences (3257 nt) showing relationships between strains CECT  
611 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours. The tree  
612 was inferred using the GTR+GAMMA model. The branches are scaled in terms of the expected  
613 number of substitutions per site. The numbers above the branches are bootstrap support values  
614 when larger than 60% from ML (left) and MP (right). The scale bar indicates 0.02 substitutions  
615 per site. The accession numbers of the MLSA gene sequences are listed in Table S2.

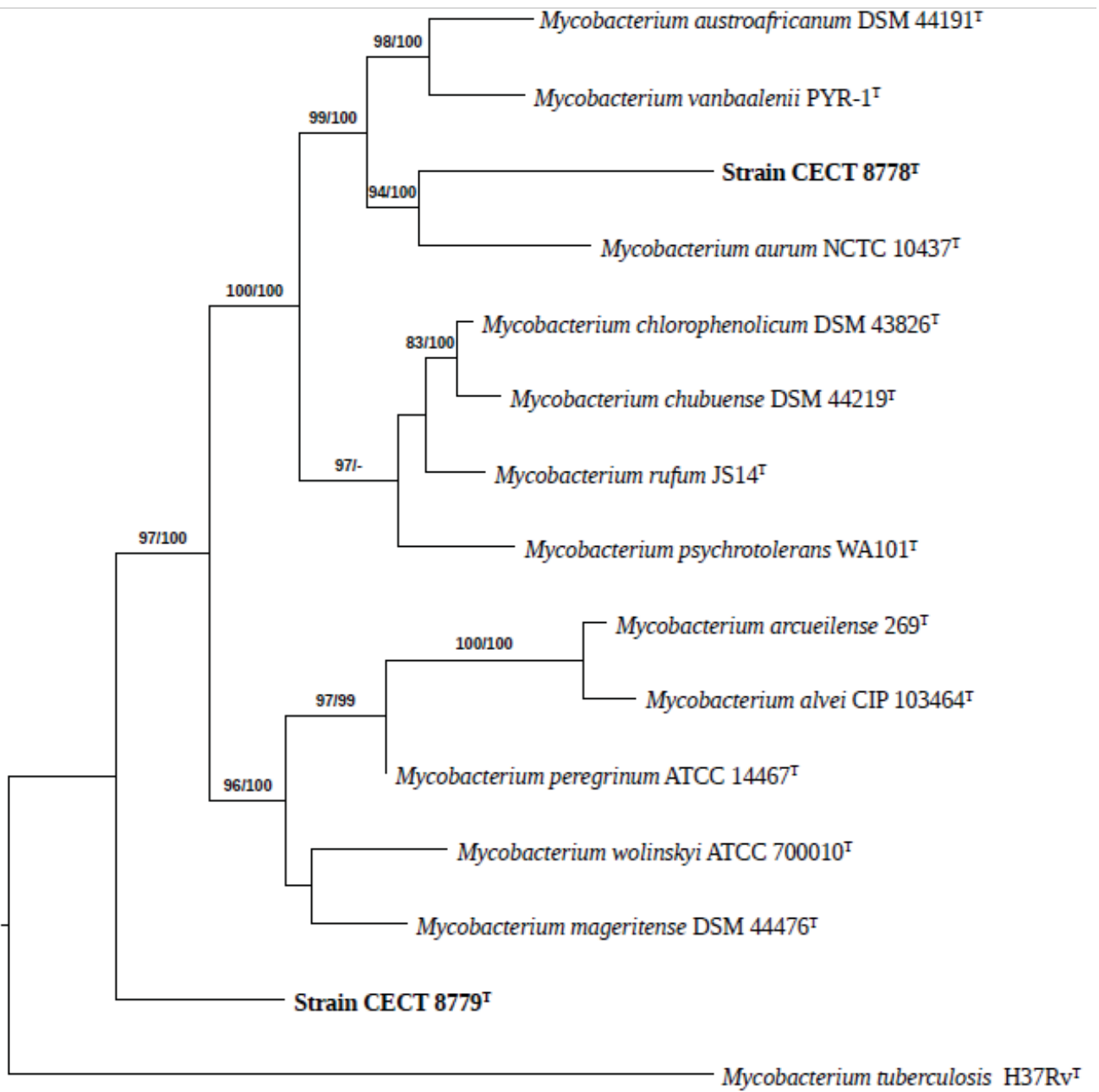
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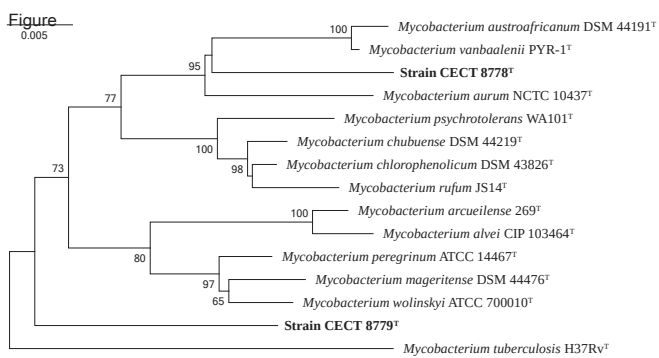
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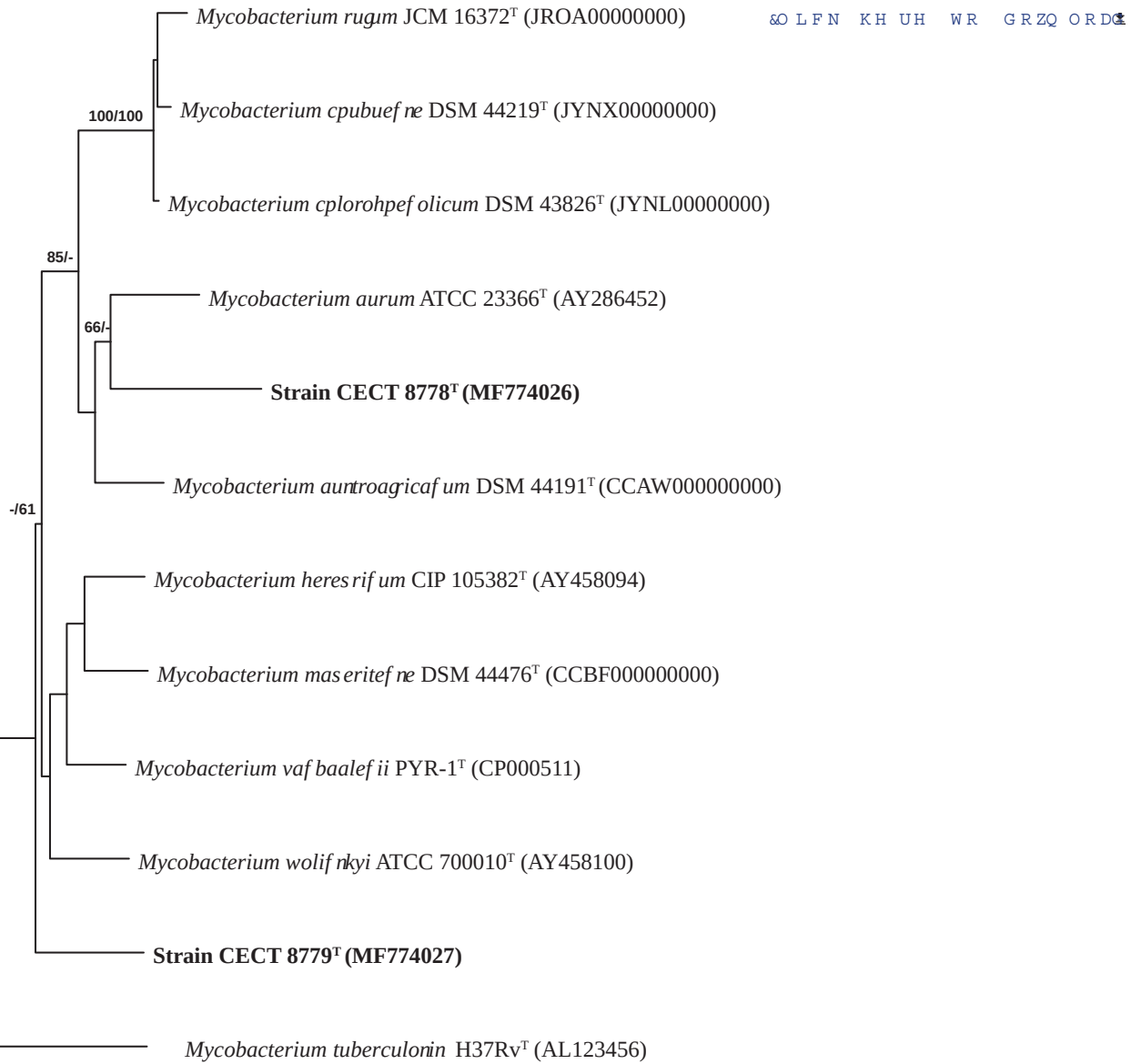
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**Fig. S1.** Neighbour-joining MLSA phylogenetic tree based on concatenated sequences of 16S rRNA, *hsp65*, *rpoB* and *recA* genes showing relationships between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours. The numbers above the branches are bootstrap support values greater than 60%. The scale bar indicates 0.005 substitutions per site.

Figure  
0.06



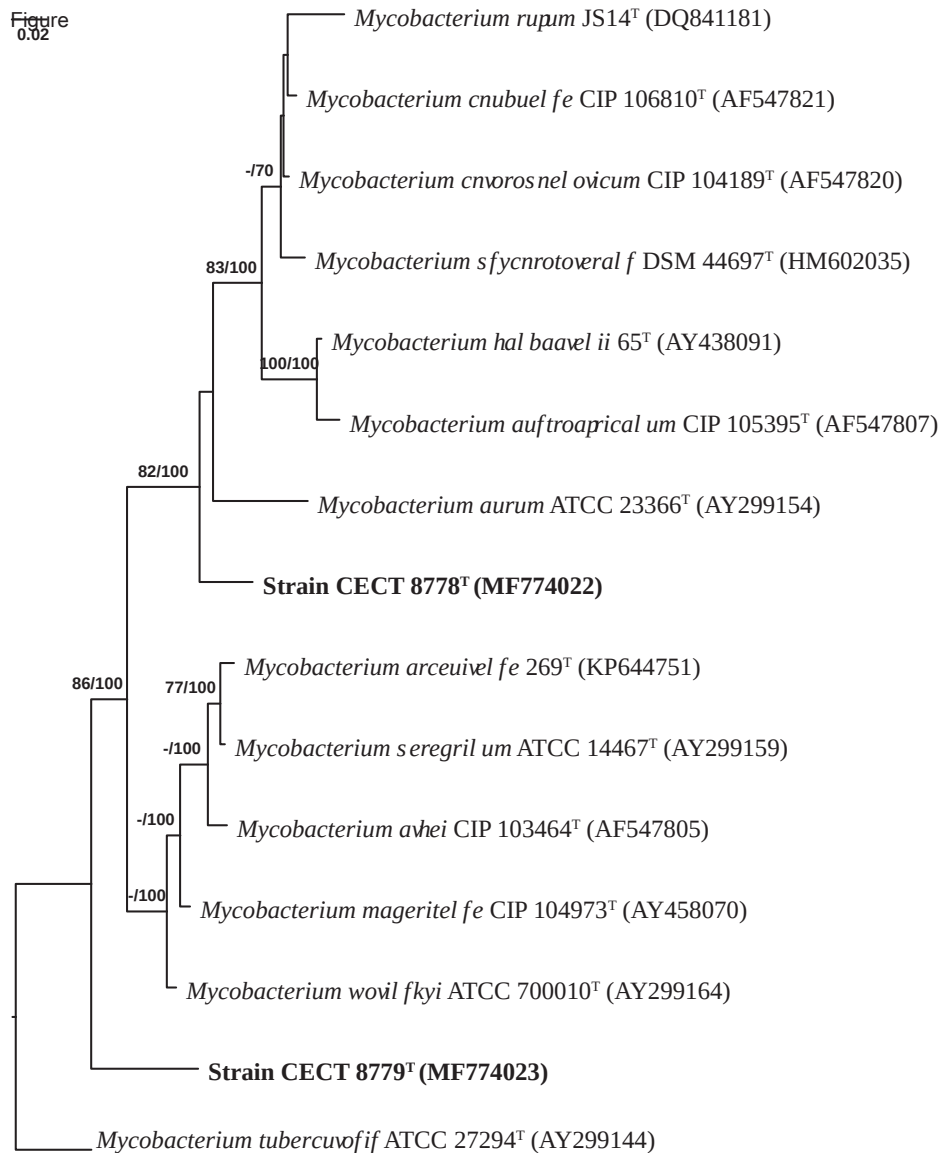
**Fig. S2.** Maximum-likelihood phylogenetic tree based on *recA* partial gene sequences generated using the GTR+GAMMA model and midpoint-rooting showing relationships between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours. The numbers above the branches are bootstrap support values greater than 60% for ML (left) and MP (right). The scale bar indicates 0.06 substitutions per site.



**Fig. S3.** Maximum-likelihood phylogenetic tree based on *rpoB* partial gene sequences generated using the GTR+GAMMA model and midpoint-rooting showing relationships between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours. The numbers above the branches are bootstrap support values greater than 60% for ML (left) and MP (right). The scale bar indicates 0.02 substitutions per site.

Figure  
0.02

⊗ LFN KH UH WR GRZQ ORDC



**Fig. S4.** Maximum-likelihood phylogenetic tree based on *hsp65* partial gene sequences generated using the GTR+GAMMA model and midpoint-rooting showing relationships between strains CECT 8778<sup>T</sup> and CECT 8779<sup>T</sup> and between them and their close phylogenetic neighbours. The numbers above the branches are bootstrap support values greater than 60% for ML (left) and MP (right). The scale bar indicates 0.02 substitutions per site.