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1 ***Mycobacterium eburneum* sp. nov., a non-chromogenic, slowly growing species isolated**
2 **from sputum**

3
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21
22 **Keywords:** Polyphasic taxonomy, phylogeny, phenotyping, mycobacteria

23
24
25 **Abstract**

26 A non-pigmented slowly-growing *Mycobacterium* strain isolated from sputum was
27 characterised using a polyphasic approach. The isolate, strain X82^T, has chemotaxonomic
28 properties consistent with its classification in the genus *Mycobacterium*. The organism formed
29 a distinct subclade in the mycobacterial 16S rRNA gene tree together with *Mycobacterium*
30 *paraense* DSM 46749^T. The two strains were distinguished based on their fatty acid, mycolic
31 acid and polar lipid profiles and by a combination of phenotypic features. These data support
32 the conclusion that the isolate represents a novel mycobacterial species for which the name of
33 *Mycobacterium eburneum* sp. nov. is proposed. The type strain is X82^T (DSM 44358^T = CECT
34 8775^T).

35 *Mycobacterium* (Lehmann & Neumann, 1896) is the type genus of the family
36 *Mycobacteriaceae* (Magee & Ward, 2012) in the order *Corynebacteriales* (Goodfellow &
37 Jones, 2012). The genus can be distinguished from other genera in the order *Corynebacteriales*
38 using a combination of chemotaxonomic and morphological properties. At the time of writing
39 the genus contains 175 species with validly published names and 13 subspecies
40 (<http://www.bacterio.net>) which can be divided into two groups based on growth rate. Slowly-
41 growing mycobacteria require seven or more days of incubation at optimal temperature to form
42 visible colonies from highly diluted inocula, whereas colonies of rapidly growing strains are
43 seen in less than seven days under compatible conditions (Wayne & Kubica, 1986). Species
44 assigned to the two groups can be distinguished using a combination of genotypic and
45 phenotypic criteria (Magee & Ward, 2012), including the characterisation of mycolic acids
46 (Teramoto *et al.*, 2015). The genus encompasses obligate pathogens, saprophytes, and non-
47 tuberculosis mycobacteria which are common in the environment and can cause opportunistic
48 infections in humans (Tortoli, 2009).

49 The aim of the present study was to establish the taxonomic position of a slowly-growing
50 mycobacterial strain that was isolated many years ago from sputum collected in Switzerland in
51 1998 and was deposited in a public culture collection without thorough taxonomic
52 characterisation. During a systematic screening of mycobacterial cultures for distinct 16S rRNA
53 gene sequences strain X82^T was identified as a potential type strain of new species and
54 subjected to polyphasic taxonomic analyses which showed that the strain represents a novel
55 species for which we propose the name *Mycobacterium eburneum*.

56

57 *Mycobacterium eburneum* strain X82^T and *Mycobacterium paraense* DSM 46749^T (Costa *et*
58 *al.*, 2015) were obtained from the Spanish Type Culture Collection (CECT) and Leibniz
59 Institute DSMZ – German Collection of Microorganisms and Cell Cultures, respectively. Both
60 strains were maintained in 50% v/v glycerol at -80°C. DNA extraction and PCR amplification
61 on strain X82^T were achieved using the method of Amaro *et al.* (2008). An almost complete
62 16S rRNA gene sequence (1394 nucleotides [nt]) has been deposited in the GenBank NCBI
63 database under the accession number KX879093.

64 A BLAST search of the 16S rRNA gene sequence of strain X82^T in EzTaxon database (Kim *et*
65 *al.*, 2012) was performed and the pairwise sequence similarities were calculated using the

66 method of Meier-Kolthoff *et al.* (2013a). Maximum likelihood (ML) and maximum parsimony
67 (MP) trees were constructed based on a comparison of the sequences of strain X82^T and its
68 closest relatives using the DSMZ phylogenomics pipeline (Meier-Kolthoff *et al.*, 2014) adapted
69 to single genes with RAxML (Stamatakis, 2014) and TNT (Goloboff *et al.*, 2008), respectively.
70 This pipeline is accessible through the GGDC web server (Meier-Kolthoff *et al.*, 2013b)
71 available at <http://ggdc.dsmz.de/>. A multiple sequence alignment was performed with
72 MUSCLE (Edgar, 2004). Rapid bootstrapping replicates in conjunction with the auto
73 MREbootstopping criterion (Pattengale *et al.*, 2010) and tree-bisection-and-reconnection
74 branch swapping were used for the ML and MP analyses, respectively. The X² test, as
75 implemented in PAUP* (Swofford, 2002), was used to check the sequences for compositional
76 bias. A neighbour joining (NJ) tree (Saitou & Nei, 1987) was constructed using MEGA software
77 version 7.0 (Kumar *et al.*, 2015) and the evaluation of its robustness was performed in a
78 bootstrap analyses based on 1000 replicates. The alignment of the sequences were achieved
79 using ClustalW (Thompson *et al.*, 1997).

80 In the BLAST analyses of the 16S rRNA gene sequence of strain X82^T, in the EzTaxon
81 database, the closest similarity, 97.9 %, was with *Mycobacterium conceptionense* DSM 45102^T.
82 However using the method recommended by Meier-Kolthoff *et al.* (2013a), *M. conceptionense*
83 DSM 45102^T kept the same 16S rRNA gene similarity value to strain X82^T and the highest 16S
84 rRNA gene similarity was 98.0% between strain X82^T and *M. paraense* DSM 46749^T which
85 was found in the 14th position in EzTaxon. These results are in line with the close phylogenetic
86 position of strain X82^T to *M. paraense* DSM 4674^T highlighting the distinct phyletic position
87 of *M. conceptionense* DSM 45102^T (Adékambi *et al.*, 2006) from the present studied strain
88 X82^T (Fig. 1). The pairwise 16S rRNA gene similarities between strain X82^T and its closest
89 relatives, namely *M. paraense* DSM 46749^T (Costa *et al.*, 2015), *M. kumamotonense* DSM
90 45093^T (Masaki *et al.*, 2007), *M. longobardum* DSM 45394^T (Tortoli *et al.*, 2013), *M. poriferae*
91 DSM 44585^T (Padgitt & Moshier, 1987), *M. rufum* DSM 45406^T (Hennessee *et al.*, 2009) and
92 *M. iranicum* DSM 44162^T (Shojaei *et al.*, 2013) were 98.0%, 98.0%, 97.8%, 97.6%, 97.4%,
93 97.9% respectively. All of these strains were recovered in the same subclade. *M. paraense* DSM
94 46749^T and *M. kumamotonense* DSM 45093^T showed the highest 16S rRNA gene similarities,
95 98.0%, with strain X82^T but the *M. paraense* strain was most closely related to strain X82^T with
96 33 nt differences in the 16S rRNA gene sequences. The phyletic relatedness of strain X82^T to
97 *M. paraense* DSM 46749^T was underpinned in the ML, MP (Fig. 1a) and NJ (Fig. 1b) analyses.

98 According to Meier-Kolthoff *et al.* (2013a), up to half of the currently conducted DDH
99 experiments could safely be omitted without a significant risk for wrongly classifying species.
100 For *Actinobacteria* they recommended a 16S rRNA sequence identity threshold of 99.0% for a
101 maximal 1% probability of error in case of omitted DDH experiments. The 98.0% 16S rRNA
102 sequence similarity determined for strain X82^T and *M. paraense*'s type strain DSM 46749^T
103 indicate a 0.05% [meaning 1 in 2000] chance for missing a positive DDH value (above 70%)
104 in omitting wet lab DDH experiments. All other type strains in the close vicinity of X82^T (see
105 Fig. 1a) indicate even lower error probabilities of 0.01-0.025% [meaning 1 in 4000 to 10000].
106 High costs for DDH experiments and low risk for missed DDH values above the established
107 threshold for species discrimination (Wayne *et al.*, 1987) prompted us to relinquish wet lab
108 DDH experiments.

109 Biomass preparation of the strains for the chemotaxonomic and phenotypic tests were harvested
110 from flasks of Middelbrook 7H10 broth supplemented with oleic acid, albumin, dextrose and
111 catalase (MB; Lorian, 1968) that was incubated at 37°C for 7 days. Bacterial cells for the
112 chemotaxonomic analyses were washed three times in 0.9%, v/v sodium chloride solution and
113 freeze dried. Phenotypic tests relevant to mycobacterial systematics, as recommended by Kent
114 and Kubica (1985), were performed from fresh cultures prepared at mid logarithmic phase
115 (OD₆₀₀ nm 0.30 - 0.60). Strain X82^T and its closest phylogenetic neighbour were examined for
116 chemotaxonomic properties known to be of value in mycobacterial systematics (Magee &
117 Ward, 2012). Cellular fatty acids were extracted from fresh biomass of the strains and fatty acid
118 methyl esters (FAMES) prepared following saponification, methylation and extraction using
119 the protocol introduced by Miller (1982), as modified by Kuykendall *et al.* (1998). The FAMES
120 were analyzed using a gas chromatography instrument (Agilent 6890 N) and the resultant peaks
121 automatically integrated fatty acids names and properties using the standard Microbial
122 Identification (MIDI) system version 4.5 and the Myco 6 database (Sasser, 1990). Mycolic
123 acid methyl esters prepared according to Minnikin and Goodfellow (1976) were analysed by
124 matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-
125 FMS) using Spiral Ion Trajectory (MALDI spiral-TOFMS), according to Teramoto *et al.*
126 (2015). Standard chromatographic procedures were used to establish additional
127 chemotaxonomic properties. Isomers of diaminopimelic acids were determined following the
128 procedure of Schleifer and Kandler (1972). Whole cell sugars extract (Lechevalier &
129 Lechevalier, 1970) were examined according to Stanek and Roberts (1974). Isoprenoid
130 quinones were extracted according to Collins (1985) and identified by high pressure liquid

131 chromatography as designed by Kroppenstedt (1982). Polar lipids were extracted and identified
132 following the protocol established by Minnikin *et al.* (1984), as modified by Kroppenstedt and
133 Goodfellow (2006). Functional groups were revealed using spraying reagent namely α -
134 naphthol-sulfuric acid (40.5 ml ethanol, 4 ml distilled water, 6.5 ml concentrated sulfuric acid
135 and 10.5 ml α -naphthol (15g/l ethanol)) to detect sugar containing lipids, Dragendorff's solution
136 (Merck) to identify choline-containing lipids (Tindall, 1990), ninhydrin reagent (0.2 %
137 ninhydrin in acetone) to reveal amino-groups (Skipski *et al.*, 1964), molybdenum blue (Sigma
138 119KG123) to detect lipids with phospho-groups and molybdatophosphoric acid (Sigma
139 P1518) to identify total lipid content.

140 Isolate X82^T and *M. paraense* DSM 46747^T were found to have chemotaxonomic properties in
141 accordance with their classification in the genus *Mycobacterium* (Magee & Ward, 2012). They
142 produced whole-organism hydrolysates rich in *meso*-2,6-diaminopimelic acid, arabinose,
143 galactose, glucose and ribose (wall chemotype IV; Lechevalier & Lechevalier, 1970), straight
144 chain saturated, unsaturated and 10 methyl-octadecanoic (tuberculostearic) fatty acids (Table
145 S1), mycolic acids, dihydrogenated menaquinones with nine isoprene units as the predominant
146 menaquinone (>50%) and polar lipid patterns that included diphosphatidylglycerol (DPG),
147 phosphatidylethanolamine (PE), phosphatidylinositol (PI), aminolipids (AL) and unidentified
148 lipids (L₁₋₃) and glycolipids (GL₁₋₅) (Fig. S1; phospholipids pattern 2 according to Lechevalier
149 *et al.* (1977)). However, the two strains can be distinguished using several chemotaxonomic
150 features. The fatty acid profile of strain X82^T showed major amounts of C_{16:0} (38.2%) and
151 C_{10Me}-C_{18:0} (25.5%) with summed feature 3 (16.8%) containing 20:0 ALC 18.838 ECL /20
152 alcohol / 19:0 Cycloprop ω 10c / 19:0 Cycloprop ω 8c (Table S1) while *M. paraense* strain DSM
153 46747^T exhibited C_{18:0}, higher amounts of C_{18:1} ω 9c (39.4%) and C_{16:0} (32.1%), lower amount
154 of C_{10Me}-C_{18:0} (8.1 %) and lacked summed feature 3. In addition, the polar lipid profile of strain
155 X82^T contains phosphatidyl glycerol (PG) and an unknown phospholipid while *M. paraense*
156 DSM 46747^T, has two aminolipids (AL₁₋₂) and lacked PG. Finally, strain X82^T was
157 characterised by the presence of dicarboxy-mycolic acids with 64 carbons while the *M.*
158 *paraense* strain has α - *keto* -mycolic acids with 87 carbons (Fig. 2).

159 Strain X82^T was examined for its ability to grow on GYM (glucose-yeast-malt extract)
160 *Streptomyces* agar media ([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium_65)
161 [65](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium_65)), peptone-meat extract-glycerol (PMG;
162 http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium_250), Lowenstein-Jensen

163 (LJ; Jensen, 1932), MB (Lorian, 1968) and on tryptic soy agar (TSA; MacFaddin, 1985) after
164 7 days at 25°C, 28°C, 37°C and 45°C.

165 Rough, non-pigmented colonies of isolate X82^T grew on almost all of the media (GYM, 7H10,
166 LJ, MB, PMG,) at 37 °C after 14 days irrespective of whether plates were incubated in the light
167 or dark. Optimal growth was observed on the MB and PMG agar plates after 7 days. Growth
168 was not observed on plates incubated at either 25°C or 45°C.

169 Strains X82^T and *Mycobacterium paraense* DSM 46749^T were examined for a broad range of
170 phenotypic properties. To this end, they were tested for their ability to use different carbon
171 compounds, to grow at a range of pH values and in presence of sodium chloride and antibiotics
172 using GENIII, PM 11 and PM 12 microplates and an Omnilog device (BIOLOG Inc. Hayward,
173 CA, USA). All of the microplates were inoculated with a mycobacterial suspension in IFC
174 solution (Inoculating Fluid provided by the manufacturer) and in MB 7H10 broth supplemented
175 with Dye mix G, following the protocols of Khatri *et al.* (2013). The inoculation solution
176 showed 71 % transmittance. The tests were carried out in triplicate using freshly prepared
177 inocula harvested at mid-logarithmic phase (OD₆₀₀ nm 0.30 - 0.60) (Khatri *et al.*, 2013). The
178 plates were incubated at 37 °C for 7 days and the resultant data were exported and analysed
179 using the opm package version 1.3.36 (Vaas *et al.*, 2012; 2013). Strain X82^T can be
180 distinguished from *M. paraense* DSM 44749^T using a combination of phenotypic properties
181 through it is evident that these strains have many properties in common (Table 1).

182 The strains were also tested for their ability to produce catalase (Palomino *et al.*, 2007), heat
183 stable catalase (Organización Panamericana de la Salud, 2008), arylsulfatase after 3 and 20 days
184 (Tomioka *et al.*, 1990), niacin accumulation (Kent & Kubica, 1985), nitrate reductase
185 (Bernardelli, 2007), growth in presence of potassium tellurite (Kilburn *et al.*, 1969; Kent &
186 Kubica, 1985), degradation of Tween 80 (Ribón, 2012) and urease hydrolysis (Palomino *et al.*,
187 2007). Enzymatic features were determined using API ZYM kits following the manufacturer's
188 Instructions (BioMérieux, France). Strain X82^T, unlike *M. paraense* strain, produced
189 arylsulfatase after 3 and 20 days, acid phosphatase, β glucosidase, valine arylamidase, degraded
190 Tween 80 and metabolised esterase (C4) while only the *M. paraense* DSM 46747^T was positive
191 for niacin accumulation, nitrate reduction and α-chymotrypsin. Additional phenotypic features
192 for each of these strains are displayed in Table 1.

193 In conclusion, strain DSM 44358^T can be distinguish from *M. paraense* DSM 44749^T, its
194 nearest phylogenetic neighbour, using a combination of chemotaxonomic, genotypic and
195 phenotypic data. Consequently, it is proposed that this strain be recognised as a new species in
196 the genus *Mycobacterium*, *Mycobacterium eburneum* sp. nov.

197 **Description of *Mycobacterium eburneum* sp. nov.**

198 *Mycobacterium eburneum* (*e. bur'ne.um*. N. L. masc. adj. referring to the cream coloured
199 colonies produced by the organism).

200 Gram-stain-positive, acid-alcohol fast, non-motile, non-spore forming organism which
201 produces cream coloured colonies on Middelbrook 7H10 and peptone- meat extract-glycerol
202 agar media after 7 days at 37 °C in both the dark and light. Grows from 28 °C to 37 °C, optimally
203 at 37 °C, from pH 7 to 8; optimal pH is 7.0. Produces arylsulfatase after 3 and 20 days, acid
204 phosphatase, alkaline phosphatase, β glucosidase, catalase, catalase (68 °C), esterase lipase
205 (C8), esterase (C4), lipase (C14), leucine arylamidase, naphthol-AS-BI-phosphohydrolase,
206 urease, valine arylamidase and grows in presence of potassium tellurite and degrades Tween
207 80. The major cellular fatty acids (>20%) are C_{16:0}, and C_{10Me} C_{18:0}, contains dicarboxy mycolic
208 acids with 64 carbons chain length. The predominant menaquinone (>50%) is MK-9 (H₂). The
209 phospholipid pattern is composed of phosphatidylinositol, diphosphatidylglycerol,
210 phosphatidylglycerol and phosphatidylethanolamine.

211 The type strain of *M. eburneum* is X82^T (= DSM 44358^T = CECT 8775^T). The GenBank
212 accession number of the 16S rRNA gene sequence is KX879093.

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214

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371 **Figure legends**

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373 **Fig. 1.** Maximum likelihood (ML) (a) and neighbour joining (b) phylogenetic trees based on
374 16S rRNA gene sequences. The branches are scaled in terms of the expected number of
375 substitutions per site. The numbers above the branches are bootstrap support values greater than
376 60%.

377 **Fig. 2.** Mycolic acid profiles of *M. eburneum* strain X82^T (a) and *M. paraense* DSM 46749^T (b)
378 using high through put MALDI TOF according to Teramoto *et al.* (2015).

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385 **Table 1.** Phenotypic features that distinguish strain X82^T from *M. paraense* DSM 46749^T

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	<i>M. eburneum</i> X82 ^T	<i>M. paraense</i> DSM 46749 ^T
Biochemical tests		
Arylsulfatase 3 and 20 days, esterase (C4), Tween 80	+	-
Niacin accumulation, nitrate reduction, α -chymotrypsin	-	+
Biolog GENIII microplates		
Utilisation of sugars[#]		
D-cellobiose, D-fructose, D-fucose, D-galactose, D-glucose-6-phosphate, D-maltose, L-fucose, β -methyl-d-glucoside, <i>myo</i> -inositol, <i>N</i> -acetyl-D-glucosamine, stachyose, sucrose, turanose	+	-
Dextrin, D-raffinose, D-sorbitol, glucuronamide	-	+
Utilisation of amino acids[#] :		
D-serine	-	+
L-alanine, L-glutamic acid	+	-
Utilisation of organic acids :		
Acetoacetic acid, citric acid, D-malic acid, L-malic acid, mucic acid	-	+
D-gluconic acid, D-glucuronic acid, D-saccharic acid, α - <i>keto</i> -butyric acid	+	-
Inhibition tests[#] :		
Sodium bromate, sodium chloride 1% ,4%, 8% ,	-	+
Sodium formate	+	-
Growth in presence of[#] :		
Fusidic acid, guanidine hydrochloride, lincomycin lithium chloride, nalidixic acid, niaproof tetrazolium blue, troleandomycin, vancomycin	-	+
Biolog PM 11 microplates		

Growth in presence of

Amikacin, cephalothin, chlortetracycline	+	-
Amoxicillin, cefazolin, ceftriaxone, demeclocycline, enoxacin, gentamicin, lomefloxacin, minocycline, neomycin	-	+

Biolog PM 12 microplates**Growth in presence of[#]**

Carbenicillin, spiramycin, tetracycline, tobramycin,	+	-
2,4-diamino-6,7-diisopropylpteridine, d, l-serine hydroxamate, 5-fluoroorotic acid, l-aspartic- β -hydroxamate, polymyxin b, spectinomycin, sulfamethazine, sulfamethoxazole, sulfathiazole	-	+

Polar lipids profile	PI, DPG, PG, PE, GPL, GL ₁₋₅ , L ₁₋₃ , PL, AL	PI, DPG, PE, GPL, GL ₁₋₅ , L ₁₋₃ , AL ₁₋₂
Mycolic acids (carbon chain length)	dicarboxy-mycolic acids (C64)	α -keto-mycolic acids (C87)

387388 + Positive reaction; - negative reaction; [#] concentrations of antibiotics as specified for Biolog.

389 Isolate X82^T and *M. paraense* DSM 46747^T were positives for catalase, catalase (68 °C), potassium tellurite, urease, produced alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase and naphthol-AS-BI-phosphohydrolase (API ZYM tests), metabolised acetic acid, butyric acid, D- glucose, D-fructose-6-phosphate, dodecyltrimethyl ammonium bromide, D-trehalose, glycerol, propionate, Tween 40, α -keto-glutaric acid and β -hydroxy-butyric acid. Able to grow in presence of methyl pyruvate, tetrazolium violet and 1% sodium lactate. In contrast, neither strains metabolised aspartic acid, D -arabitol, D-aspartic acid, D-lactic acid methyl ester, D-mannitol, D-mannose, D-melibiose, gelatin, glycine-proline, inosine, L-arginine, L-galactonic acid- γ -lactone, L-histidine, L-lactic acid, L-pyroglutamic acid, L-serine, *N*-acetyl-d-galactosamine, *N*-acetylneuraminic acid, *N*-acetyl- β -D-mannosamine, pectin, *p*-hydroxy-phenylacetic acid, L-rhamnose, α -D-lactose, β -gentiobiose and 3-*O*-methyl-D-glucose. Both strains were resistant to aztreonam, capreomycin, carbenicillin, cephalothin, chloramphenicol, cloxacillin, colistin, demeclocycline, d-salicin (anti-inflammatory), nafcillin, ofloxacin, oxacillin, paromomycin, penicillin G and sisomicin but sensitive to amikacin, benzethonium chloride,

401 bleomycin, chlortetracycline, erythromycin, kanamycin, lincomycin, minocycline, novobiocin, penimepicycline,
402 rifampicin, spiramycin, sulfadiazine and vancomycin.

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Figure 1a

60 LFN KH UH WR GRZQ RDG JL

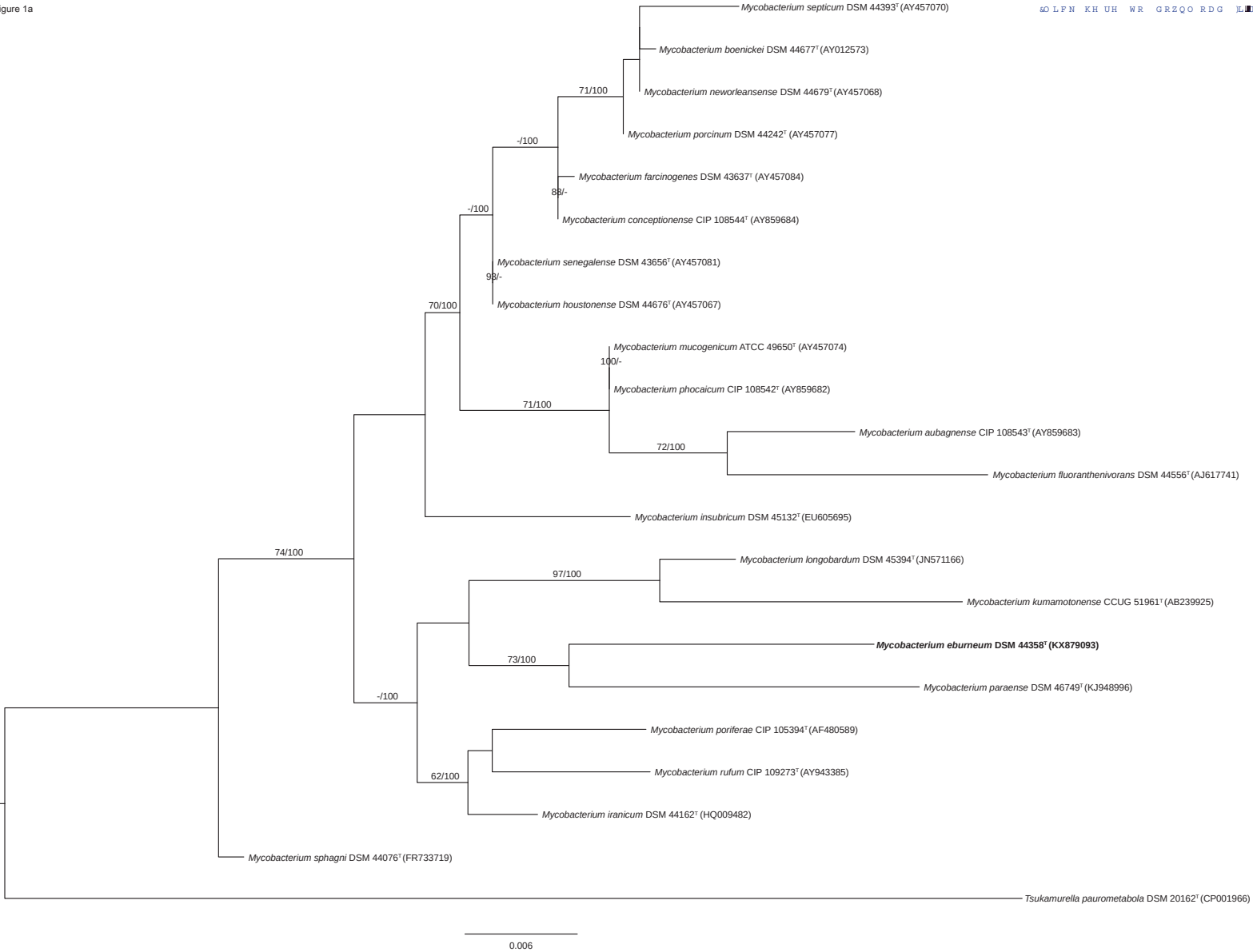


Figure 1b

[Click here to download Figure Fig 1b NJ Tree.pdf](#)

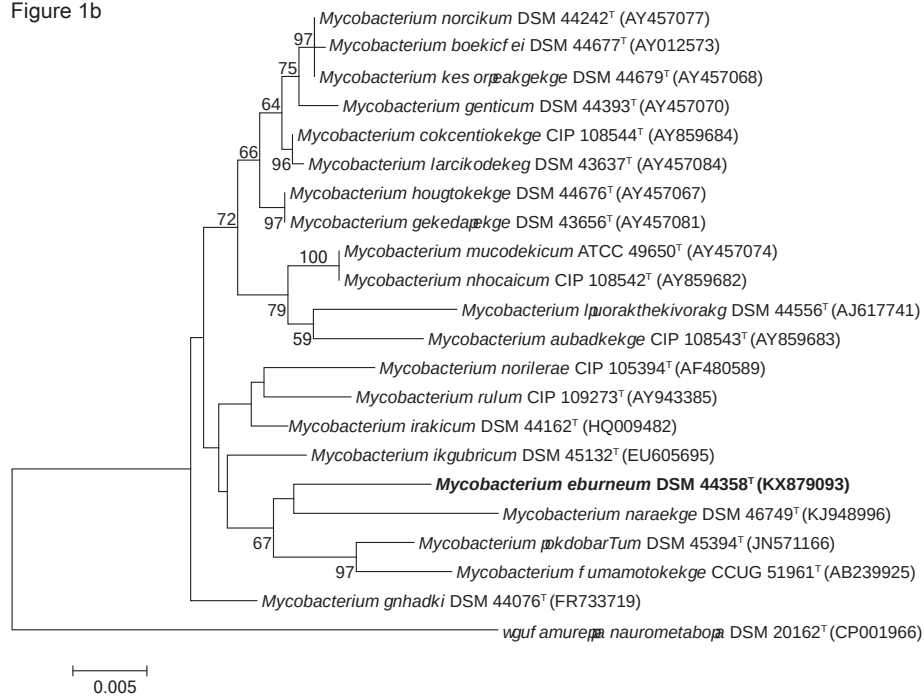
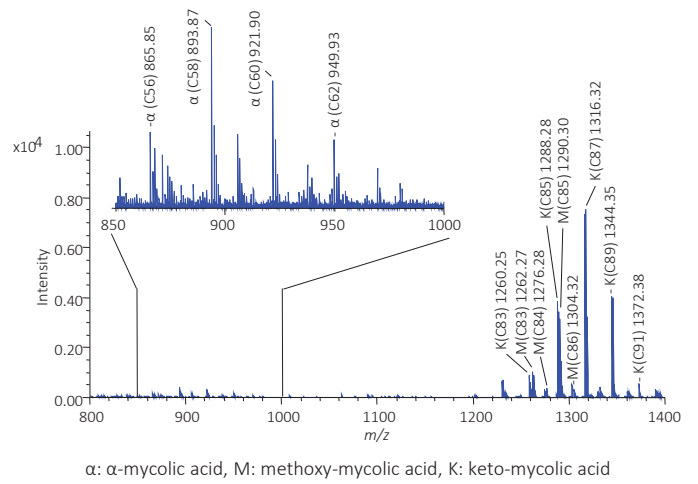
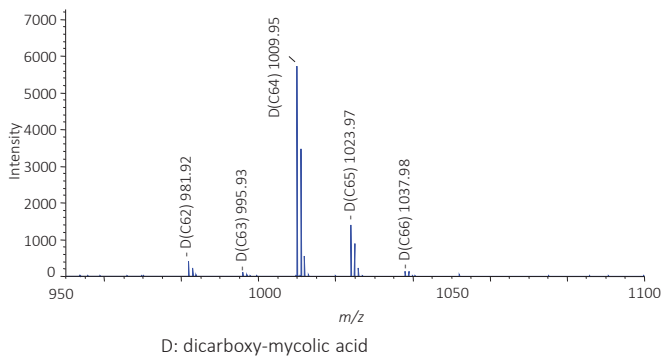
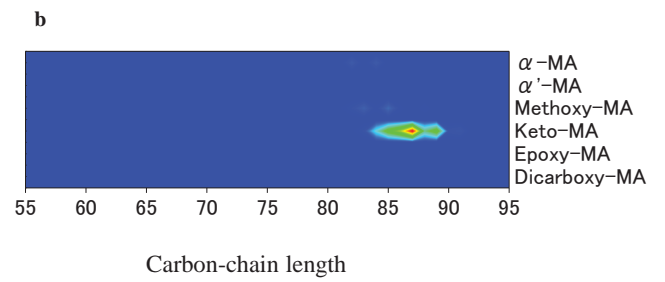
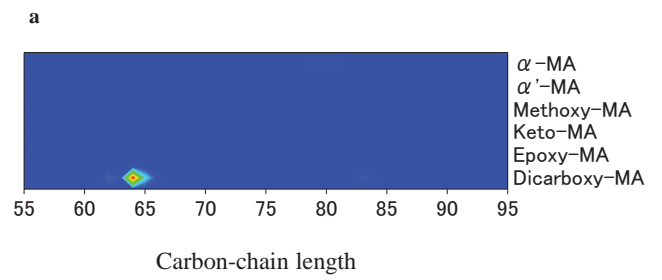


Figure 2

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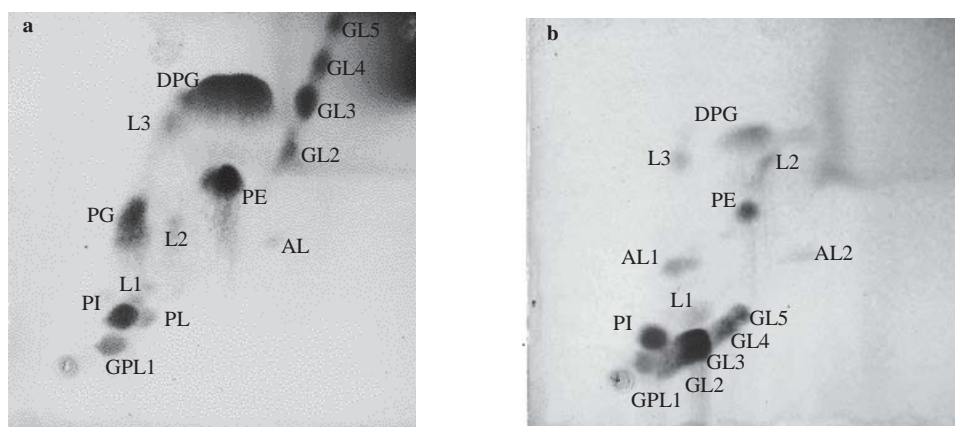


Fig. S1. Two-dimensional TLC plates of polar lipids extracted from *M. eburneum* X82^T (a) and *M. paraense* DSM 46749^T (b) using molybdotophosphoric acid (Sigma P1518) to identify total lipid content.

Table S1. Fatty acids patterns of *M. eburneum* X82^T and its closest relatives *M. paraense* DSM 46749^T.

	<i>M. eburneum</i> X82 ^T	<i>M. paraense</i> DSM 46749 ^T
C _{16:0}	38.2	32.1
C _{18:1 w9c}	19.4	39.4
C _{18:2 w6,9c}	-	-
C _{18:0}	-	9.1
C _{10Me} -C _{18:0}	25.5	8.1
C _{14:0}	-	-
C _{16:1 w9c}	-	-
C _{16:1 w6c}	-	-
Summed	-	-
feature 2*		
Summed	16.8	-
feature 3**		

* 2 17:1 W7c / C_{17:1 w6c}; ** 20:0 ALC 18.838 ECL /20 alcohol / 19:0 Cycloprop w10c / 19:0 Cycloprop w8c