Nakamurella Leprariae Sp. Nov., Isolated From a Lichen Sample

De-Feng An
Yunnan Institute of Microbiology
Shao-Juan Yang
Yunnan Institute of Microbiology
Long-Qian Jiang
Yunnan Institute of Microbiology
Xin-Yu Wang
Kunming Institute of Botany Chinese Academy of Sciences
Xiao-Yu Huang
Yunnan Institute of Microbiology
Lei Lang
Yunnan Institute of Microbiology
Xue-Mei Chen
Yunnan Institute of Microbiology
Ming-Qun Fan
Yunnan Institute of Microbiology
Gui-Ding Li
Yunnan Institute of Microbiology
Ming-Guo Jiang
Guangxi University for Nationalities
Li-Song Wang
Kunming Institute of Botany Chinese Academy of Sciences
Cheng-Lin Jiang
Yunnan Institute of Microbiology
Yi Jiang (jiangyi@ynu.edu.cn)
Yunnan Institute of Microbiology, Yunnan University https://orcid.org/0000-0002-1847-252X

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Abstract

A novel actinobacterium, YIM 132084^T, was isolated from *Lepraria* sp. lichen collected from Yunnan province, south-west PR China and identified by a polyphasic taxonomic approach. The strain was Gram-stain-positive, aerobic, catalase-positive, oxidase-negative, non-motile and coccus-shaped. Colonies were round, convex, smooth and light orange yellow in colour. It grew at 10–40 °C (optimum 28 °C), at pH 6.0–11.0 (optimum pH 7.0) and in the presence of 0–4 % NaCl (optimum 0 %). Strain YIM 132084^T comprised diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol as the major polar lipids, MK-8(H_4) as the predominant menaquinone, and *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, *iso*-$C_{15:0}$ and *iso*-$C_{16:0}$ as major fatty acids. Strain YIM 132084^T had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, and mannose, ribose, glucose and rhamnose as whole-cell sugars. The 16S rRNA gene sequence showed high level of similarity with *Nakamurella flavida* KCTC 19127^T (97. 7%) and *Nakamurella flava* CGMCC 4.7524^T (97.7%). The G + C content of the genomic DNA was 72.4 mol%. Strain YIM 132084^T showed an average nucleotide identity value of 76.1 % and 74.9 %, a digital DNA-DNA hybridizations value of 20.9 % and 20.6 % with the reference strains *Nakamurella flavida* and *Nakamurella flava* based on draft genome sequences, respectively. The results of the phenotypic, chemotaxonomic and phylogenetic analyses, showed that strain YIM 132084^T represents a novel species of the genus *Nakamurella*, for which the name *Nakamurella leprariae* sp. nov. is proposed. The type strain is YIM 132084^T (= CGMCC 4.7667^T = NBRC 114280^T = KCTC 49367^T).

Introduction

The genus *Nakamurella* was proposed by Tao in 2004, as a substitute for the illegitimate genus *Microsphaera* by Yoshimi et al., at the same time, the family of *Nakamurellaceae* replaced *Microsphaeraceae* (Tao et al. 2004 and Yoshimi et al. 1996). *Nakamurella* species are distributed in different natural ecosystems, including activated sludge (Yoshimi et al. 1996), rock (Lee et al. 2008), soil (Yoon et al. 2007), faeces (Kim et al. 2017), lichen (Jiang et al. 2020), automobile air conditioning system (Chaudhary et al. 2021), plant (Yan et al. 2020) and bark (Tuo et al. 2016). At the time of writing, the genus *Nakamurella* is composed of 10 species with validly published names and two species with not validly published nomenclature (https://www.bacterio.net/genus/nakamurella), and *Nakamurella multipartita* is the type species of this genus. During an investigate the diversity of cultivable actinobacteria from lichen samples collected in Yunnan province, south-west PR China, a new actinobacterium strain YIM 132084^T was isolated from *Lepraria* sp..The strain was identified by polyphasic approach, which indicated that it represented a new species of the genus *Nakamurella*.

Material And Methods

Isolation and Culture of Strains

Lichens provide an extremely rich reservoir for the isolation of novel species. Strain YIM 132084^T was isolated from the lichen of genus *Lepraria*, The lichen *Lepraria* sp. sample was collected from Yunnan province (99°39'E, 22°23'N), south-west PR China. The lichen sample was transferred into a sterile paper bag and air-dried at 28 °C for 1 week, then washed three times with sterile water and homogenized with 18 ml of sterile 0.1% $Na_4P_2O_7$ using a sterile glass homogenizer. Strain YIM 132084^T was isolated using a standard dilution plate method on humic acid-vitamin (HV) agar (Hayakawa et al. 1987). The isolated colony was selected and further purified on YIM 38 medium (Li et al. 2016). Strain YIM 132084^T was stored in tubes of aqueous glycerine (20 %, v/v) and then in a -80 ℃ refrigerator. The reference strain, *Nakamurella flavida* KCTC 19127^T was gained from Korean Collection for Type Cultures (KCTC), Japan. *Nakamurella flava* CGMCC 4.7524^T was gained from China General Microbiological Culture Collection Centre (CGMCC).

Phenotypic and Biochemical Tests

Cultural characteristics of strain YIM 132084^T were observed after 3 days of incubation under aerobic conditions at 28 ℃ on YIM 38 medium. Morphological characteristics were observed by transmission electron microscopy (JEM-2100; JEOL). Growth in different culture media was performed using YIM 38 medium, tryptic soy agar (TSA, BD Difco), R2A agar (MB cell, Republic of Korea), Luria-Bertani (LB) agar, International Streptomyces Project Medium 2 (ISP 2, BD Difco), ISP 4 (BD Difco) at 28 °C for 3 days. Growth at different temperatures (4, 10, 15, 20, 25, 28, 30, 35, 37, 40 and 45℃) was tested on YIM 38 medium. The pH range for growth (pH 4.0-13.0, at intervals of 1.0 pH unit) was tested on YIM 38 at 28 °C. NaCl tolerance test for growth was performed using YIM 38 medium supplemented with different concentrations of NaCl (0-10 %, w/v, in increments of 1.0 %) at 28 °C. Anaerobic growth was tested after incubation on YIM 38 agar at 28 ℃ for 14 days using a GasPak EZ Anaerobe Pouch System (Becton Dickinson). Cell motility was determined in semisolid medium (Tittsler et al. 1936). Oxidase activity was determined by using 1 % (w/v) tetramethyl-p-phenylenediamine reagent and catalase activity was determined as the production of bubbles after the addition of 3 % (v/v) H_2O_2 (Jiang et al. 2019). The Gram reaction of strain YIM 132084^T was examined using a standard Gram reaction and was confirmed by the 3 % KOH lysis test (Cerny 1978 and Buck 1982). Hydrolysis of starch, cellulose, tyrosine and casein, Tweens (20, 40, 60 and 80), gelatin liquefaction, H_2S production, coagulation and peptonizationn of milk were tested using the methods described by Smibert et al. (1994). Susceptibility to antibiotics was tested on YIM 38 medium plate using filter paper containing the following antibiotics: ofloxacin (5 μg) , vancomycin (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), polymyxin B (300 IU), gentamicin (10 μg), ampicillin (10 μg), chloramphenicol (30 μg), ceftriaxone (30 μg), penicillin G (10 IU), neomycin (30 μg), kanamycin (30 μg), streptomycin (50 μg), novobiocin (5 μg), lincomycin (15 μg), tetracycline (30 μg). Sole carbon and nitrogen source utilization were determined using Biolog GEN III MicroPlate, other biochemical properties and enzyme activities were tested using API 20NE, API 50CH and API ZYM kits (bioMérieux) according to the manufacturer's instructions.

Phylogenetic Analysis and 16S rRNA Gene Sequencing

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Li et al. (2007). The purified product was cloned by using the pEASY-T1 sample cloning kit to obtain the almost-complete 16S rRNA gene sequence. The sequence obtained was compared with available 16S rRNA gene sequences of validly named species using the EzBioCloud server databases (https://www.ezbiocloud.net/) (Yoon et al. 2017). Phylogenetic trees were constructed with neighbour-joining (Saitou et al. 1987), maximum-likelihood algorithms (Tamura et al. 2011) and maximum parsimony (Fitch 1971) methods using the software package MEGA version 7.0 (Kumar et al. 2016). Kimura's two-parameter model was used to calculate evolutionary distance matrices (Kimura 1980). Bootstrap values were calculated based on 1000 replications (Felsenstein 1985).

Genomic Analysis

The draft genome sequence of strain YIM 132084^T and *Nakamurella flavida* KCTC 19127^T were determined using the Illumina NovaSeq PE150 sequencing platform. The processed reads data were assembled using SOAPdenovo version 2.04 short sequence group assembly software (Li et al. 2008). The average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values were determined based on the genome sequences of YIM 132084^T and closely related species of *Nakamurella* using the EzBioCloud server databases and formula 2 Genome-to-Genome Distance Calculator website (http://ggdc.dsmz.de/ggdc.php) (Meier-Kolthoff et al. 2013), respectively. Gene annotations were conducted through the NCBI prokaryotic genome annotation pipeline.

Chemotaxonomic Analysis

The strain YIM 132084^T and the reference strains were cultured on YIM 38 agar at 28 °C for 3 days to obtain the amount needed for chemotaxonomic characterization. Polar lipids were extracted and analyzed by the method of Minnikin et al. (1984). Menaquinones were extracted by the method of Collins et al. (1977) and detected by HPLC (Tamaoka et al. 1983). The composition of cellular fatty acids were extracted and analyzed according to the standard protocol of the Microbial Identifcation System (MIDI) (Sasser 1990 and Kämpfer et al. 1996). Cell wall amino acids and whole cell sugars were extracted, detected and analyzed according to procedures described by Schleifer and Kandler (1972) and Tang et al. (2009).

Results And Discussion

Phenotypic and Biochemical Tests

Cells of strain YIM 132084^T were Gram-stain-positive, aerobic, non-spore forming, non-motile, coccus-shaped and 0.7-0.9 μm in a diameter (Fig. S1). Colonies were round, convex, smooth and light orange yellow on YIM 38 agar at 28 °C for 3 days. The strain was found to growth on ISP 2, R2A, TSA, LB and YIM 38 agar, No growth occurs on ISP 4 agar. The growth range of strain YIM 132084^T at 10-40 ℃ (optimum 28 ℃), at pH 6.0-11.0 (optimum pH 7.0) and at 0-4 % NaCl (optimum 0 %). Hydrolysis of

starch, cellulose, tyrosine and casein, Tweens (40, 60 and 80), gelatin liquefaction, H_2S production, coagulation and peptonisation of milk were negative, except for hydrolysis of Tween 20. Susceptibility to ofloxacin, vancomycin, ciprofloxacin, norfloxacin, polymyxin B, gentamicin, chloramphenicol, neomycin, kanamycin were positive, susceptibility to ampicillin, ceftriaxone, penicillin G, streptomycin, novobiocin, lincomycin and tetracycline were negative. In the API ZYM tests, alkaline phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *α*-glucosidase activities were positive, but lipase (C_{14}), *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase and α-fucosidase activities were negative. In the API 20NE strips, hydrolysis of L-arginine, urease, esculine and PNPG were positive, but other tests were negative. In the API 50CH strips, acid was produced from D-glucose, D-fructose, D-mannose, esculin citrate, D-maltose, D-sucrose, D-mycose, D-turanose and D-lyxose. The detailed physiological and biochemical characteristics of strain YIM 132084^T are shown in the species description and Table 1.

Phylogenetic Analysis and 16S rRNA Gene Sequencing

The almost-complete 16S rRNA gene sequence of strain YIM 132084^T was 1480bp (GenBank accession number MZ050064). Phylogenetic analyses based on the 16S rRNA gene sequence of strain YIM 132084^T indicated that it should be recognized as a member of the genus *Nakamurella*. Strain YIM 132084^T showed a high level of similarity with *Nakamurella flavida* KCTC 19127^T (97. 7 %) and *Nakamurella flava* CGMCC 4.7524^T (97.7 %). Phylogenetic trees were constructed by the neighbour-joining, maximum-likelihood algorithms and maximum parsimony based on the 16S rRNA gene sequence (Fig. 1, Fig. S2 and Fig. S3). The results of three tree-making algorithms showed that strain YIM 132084^T groups within the genus *Nakamurella*.

Genomic Analysis

Based on the draft genome sequencing, strain YIM 132084^T contained 39 contigs, with a total length of 4,472,446 bp and an N50 length of 232,774 bp (GenBank accession number JAERWK000000000). Based on the genomic annotation, the genome of strain YIM 132084^T contains 4,101 genes, included 4,009 protein-coding genes, 3 rRNA genes, 46 tRNA genes, 3 ncRNA genes and 40 pseudogenes. The DNA G+C content of strain YIM 132084^T was determined to be 72.4 mol% based on the draft genome. The ANI value between strain YIM 132084^T and the type strains of *Nakamurella flavida* KCTC 19127^T and *Nakamurella flava* CGMCC 4.7524^T were 76.1 and 74.9 %, respectively. The ANI value was lower than the 95.0% cut-off for species demarcation (Richter et al. 2009). The dDDH value between strain YIM 132084^T and the type strain: *Nakamurella flavida* KCTC 19127^T and *Nakamurella flava* CGMCC 4.7524^T were 20.9 and 20.6 %, respectively, which were much lower than the threshold value (70 %) recommended for distinguishing novel prokaryotic species.

Chemotaxonomic Analysis

The polar lipids profile of strain YIM 132084^T contained the predominant compounds diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), an unidentified aminophospholipid (APL), an unidentified glycolipid (GL) and two unidentified phosphoglycolipids (PGL1-2) (Fig. S4). The predominant menaquinone was MK-8(H_4) in agreement with the genus *Nakamurella* (Chaudhary et al. 2021), in addition, MK-8(H_2) and MK-7(H_4) were detected in strain YIM 132084^T. The major cellular fatty acids consist of *anteiso*-$C_{15:0}$ (27.9 %), *anteiso*-$C_{17:0}$ (20.7 %), *iso*-$C_{15:0}$ (12.5 %) and *iso*-$C_{16:0}$ (16.0 %), which were similar to other members of the genus *Nakamurella*. The fatty acids composition and content comparison between strain YIM 132084^T and other closely related species of the genus *Nakamurella* are shown in Table 2. Strain YIM 132084^T had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, which concurs with the members of the genus *Nakamurella*. The whole-cell sugars detected in strain YIM 132084^T were mannose, ribose, glucose and rhamnose.

In conclusion, based on phenotypic, chemotaxonomic and phylogenetic analyses, strain YIM 132084^T is considered to represent a novel species of genus *Nakamurella*, for which the name *Nakamurella leprariae* sp. nov. is proposed.

Description of *Nakamurella leprariae* sp. nov.

Nakamurella leprariae (le.pra'ri.ae. N.L. gen. n. *leprariae* referring to the isolation of the organism from the lichen genus *Lepraria*).

Cells are Gram-stain-positive, catalase-positive, oxidase-negative, aerobic, non-motile, non-spore-forming and coccus-shaped (0.7-0.9 μm in diameter). Colonies on YIM 38 medium are round, smooth and convex, light orange yellow in colour. Growth occurs at 10-40 ℃ (optimum 28 ℃), at pH 6.0-11.0 (optimum pH 7.0) and at 0-4 % NaCl (optimum 0 %). Hydrolysis of starch, cellulose, tyrosine and casein, Tweens (40, 60 and 80), gelatin liquefaction, H_2S production, coagulation and peptonisation of milk are negative, except for hydrolysis of Tween 20. In the Biolog GEN III system, the following substrates are used as a source of energy: *β*-methyl-D-glucoside, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, D-mannose, D-fructose, D-galactose, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose-6-phosphate, D-fructose-6-phosphate, D-aspartic acid, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-glucuronic acid, D-saccharic acid, L-lactic acid, citric acid, *α*-keto-glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The predominant menaquinone is MK-8(H_4). The major fatty acids are *anteiso*-$C_{15:0}$, *anteiso*-$C_{17:0}$, *iso*-$C_{15:0}$ and *iso*-$C_{16:0}$. Strain YIM 132084^T contain *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, and mannose, ribose, glucose and rhamnose as whole-cell sugars. The G+C content of the genomic DNA is 72.4 mol%. The type strain, YIM 132084^T (=CGMCC 4.7667^T = NBRC 114280^T=KCTC 49367^T) was isolated from *Lepraria* sp. lichen collected from Yunnan province, south-west PR China. The GenBank accession

number for the 16S rRNA gene sequence and draft genome sequence of strain YIM 132084^T are MZ050064 and JAERWK000000000, respectively.

Abbreviations

ANI, Average Nucleotide Identity; dDDH, digital DNA-DNA hybridization; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; APL, aminophospholipid; PGL, phosphoglycolipids; GL, glycolipid.

Declarations

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Author contributions

De-Feng An and Shao-Juan Yang performed the experiments and wrote the manuscript; Long-Qian Jiang collected the lichen samples; Xiao-Yu Huang, Xue-Mei Chen, Ming-Qun Fan, Gui-Ding Li, and Lei Lang analyzed the data; Xin-Yun Wang identified the lichen samples; Yi Jiang and Ming-Guo Jiang guided the experiments and revised the manuscript; Cheng-Lin Jiang and Li-Song Wang designed the study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Tables

Table 1

Differential characteristics between strain YIM 132084^T and closely related species of the genus *Nakamurella*

Characteristic	**1**	**2**	**3**
Isolation source	Lichen	Soil	*Mentha haplocalyx* Briq.
Colony colour	Light orange yellow	Light-yellow	Brilliant orange yellow
Cell size (μm)	0.7–0.9	0.6–1.2[a]	1.0–1.8[b]
Growth at (°C)	10–40	4–35	4–40
pH	6–11	5–9	6–10
NaCl concentration (%, w/v)	0–4	0–3	0–5
Acidification of D-glucose	-	-	+
Hydrolysis of:			
Tween 20	+	-	+
Tween 40	-	-	+
Starch	-	+	+
Casein	-	-	+
Gelatin	-	+	+
Assimilation of:			
D-Glucose	-	-	+
L-Arabinose	-	-	+
D-Mannose	-	-	+
D-Mannitol	-	-	+
N-acetyl-D-glucosamine	-	-	+
Potassium gluconate	-	-	+
Enzyme activity:			
Cystine arylamidase	+	+	-
Trypsin	-	+	-
Chymotrypsin	-	+	-
β-galactosidase	-	+	+
β-glucosidase	-	+	+

Characteristic	1	2	3
Acid produced from:			
Glycol	-	-	+
Erythritol	-	-	+
L-arabinose	-	-	+
D-ribose	-	+	-
L-xylose	-	+	+
L-sorbose	-	+	-
N-acetylglucosamine	-	+	+
D-cellobiose	-	-	+
D-maltose	+	-	+
D-melibiose	-	-	+
D-trehalose	+	-	+
Inulin	-	+	-
D-melezitose	-	-	+
D-raffinose	-	+	+
D-gentiobiose	-	-	+
D-turanose	+	-	+
Susceptibility to Antibiotics:			
Norfloxacin	+	+	-
Polymyxin B	+	+	-
Neomycin	+	+	-
Kanamycin	+	+	-
Streptomycin	-	+	-
Novobiocin	-	+	-
Lincomycin	-	+	-
Tetracycline	-	+	-
DNA G + C content (mol%)	72.4	72.4	71.6[b]

Strains: 1, YIM 132084^T; 2, *Nakamurella flavida* KCTC 19127^T; 3, *Nakamurella*

flava CGMCC 4.7524^T. +, Positive; −, negative. All data were obtained from this study except where indicated.

Milk coagulation and peptonization, H_2S production, hydrolysis of cellulose, tyrosine , Tween 60 and Tween 80 were negative in both strains. In API 20NE tests, all strains were positive for hydrolysis of L-arginine, urease, esculine and PNPG. In the API ZYM kits, all strains were positive for alkaline phosphatase, esterase (C_4), esterase lipase (C_8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-glucosidase. In the API 50CH kits, all strains were positive for acid production from D-glucose, D-fructose, D-mannose, esculin citrate, D-sucrose and D-lyxose.

[a]Data from Yoon et al. (2007)

[b]Data from Yan et al. (2020)

Table 2. Cellular fatty acid compositions of strain YIM 132084^T and other closely related species of the genus *Nakamurella*

Fatty acid	1	2	3
Straight-chain			
$C_{16:0}$	5.8	**14.5**	6.9
$C_{17:0}$	1.2	**10.5**	1.7
$C_{18:0}$	3.0	3.4	4.3
Branched			
anteiso-$C_{15:0}$	**27.9**	**37.2**	**21.7**
anteiso-$C_{16:0}$	0.4	2.1	1.4
anteiso-$C_{17:0}$	**20.7**	**10.5**	**13.8**
iso-$C_{14:0}$	0.7	0.5	2.3
iso-$C_{15:0}$	**12.5**	**13.0**	**12.4**
iso-$C_{16:0}$	**16.0**	3.8	7.1
iso-$C_{17:0}$	8.2	1.8	7.2
Summed Feature 3*	1.5	0.9	5.4

Strains: 1, YIM 132084^T; 2, *Nakamurella flavida* KCTC 19127^T; 3, *Nakamurella*

flava CGMCC 4.7524^T

Values are percentages of total fatty acids. The major fatty acids (greater than 10.0 %) are shown bold. The data of YIM 132084^T, *Nakamurella flavida* KCTC 19127^T and *Nakamurella flava* CGMCC 4.7524^T were obtained from this study.

*Summed features represent groups of two fatty acids that could not be separated by HPLC with the Microbial Identification System (MIDI, Inc.). Summed feature 3 consisted of $C_{16:1}$ $\omega 6c$ and/or $C_{16:1}$ $\omega 7c$.

Figures

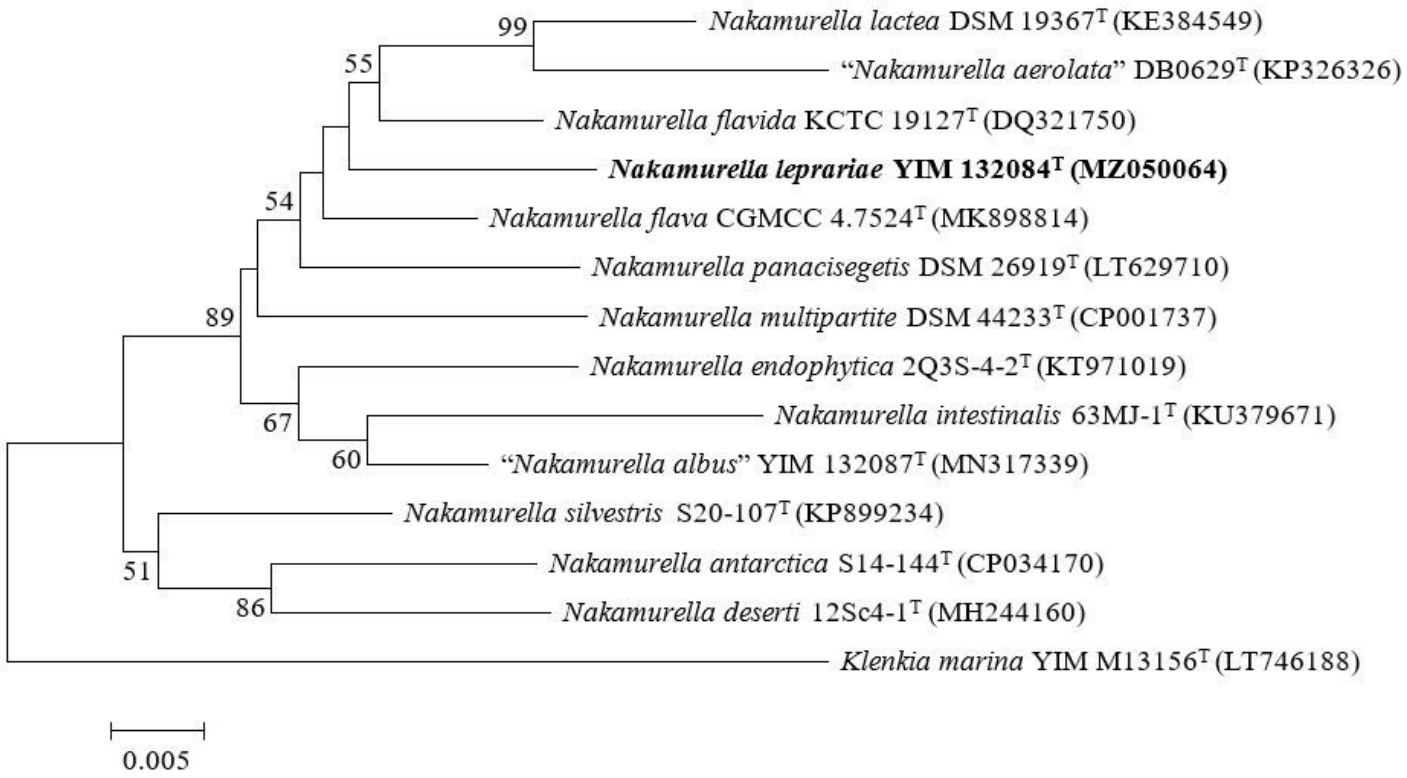

Figure 1

Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain YIM 132084T in relation to its nearest phylogenetical neighbours. Numbers at nodes indicate the level of bootstrap support (> 50 %) based on 1000 resamplings. Klenkia marina YIM M13156T (LT746188) was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 132084supplementarymaterials.docx