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Agromyces mangrovi sp. nov., a Novel Actinobacterium Isolated from Mangrove Soil

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Abstract

A novel, Gram-stain-positive, microaerophilic to aerobic, non-endospore-forming, no-motile and rod-shaped bacterium designated Q14^T was isolated from mangrove soil samples collected on chengmai, Hainan province, China. Strain Q14^T was able to grow at 10–40 °C (optimum 30 °C), pH 5.5–10.0 (optimum 6.5-8.0) and with 0.5–6% (w/v) NaCl (optimum 1%). The genomic DNA G+C content was 70.1%. The chemotaxonomic analysis showed that the predominant isoprenoid quinone was MK-12 and the major fatty acids were anteiso-$C_{15:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$. The major polar lipids of strain Q14^T were diphosphatidylglycerol, phosphatidylglycerol and one glycolipid. The strain Q14^T contained 2,4-diaminobutylic acid (A_2bu), alanine acid, glutamic acid and glycine in the peptidoglycans. The phylogenetic analysis and DNA–DNA hybridization, along with the phenotypic and chemotaxonomic characteristics, indicate that strain Q14^T as a novel species of the genus *Agromyces*, for which the name *Agromyces mangrovi* sp. nov. is proposed. The type strain is Q14^T (=MCCC 1K03191^T=KCTC 39814^T).

Introduction

The genus *Agromyces*, belonging to the family, was proposed by Gledhill and Casida [8] and the description of which was emended by Zgurskaya et al. [35]. At the time of writing, the genus consists of 30 recognized species, and these species were isolated from different environments: many kinds of soils [13, 33, 34, 36], plants [4, 25], fermented seafood [24], sea sediment [9] and wall of tomb [14]. These strains are yellow, non-spore-forming, irregular rod-shaped bacteria. The DNA G+C contents of these strains ranged from 65.3 to 73.4 mol%. All members of genus *Agromyces* have 2,4 diaminobutylic acid(A_2bu) in their peptidoglycan. The strains of *Agromyces* contained MK-12 as the predominant menaquinone with smaller amounts of MK-11 or MK-13 menaquinones. Most of the strains contained anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ as the major fatty acids. In this study, a novel species, isolated from mangrove soil of chengmai, Hainan province, is described based on the polyphasic taxonomy analysis.

Ruijun Wang and Can Chen have contributed equally to this study and are joint first authors.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Q14^T is KX170835 and the DPD number is TA00333.

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Materials and Methods

Strains and Culture Conditions

In February 2016, a study about microbial diversity in mangrove soil led to the isolation of a novel actinobacterium. The soil sample was collected in January, 2016 on mangrove soil of chengmai (19°91′N, 110°00′E), Hainan province, China. The sample was diluted using tenfold dilution series methods and spread on modified Gause's Synthetic Agar medium no. 1 [1] supplemented with 25 mg/L nalidixic acid and 50 mg/L cycloheximide [11, 28]. The plates were then incubated at 28 °C. After 7 days of incubation, some colonies were picked and one pale yellow-coloured colony named strain Q14^T, then the strain was subsequently purified on different mediums such as marine agar (MA; BD Difco) and Luria–Bertani agar (LB). The purified strain was preserved at 80 °C in the marine broth 2216 (MB) medium supplemented with 30% (v/v) glycerol. Strain Q14^T has been deposited at the MCCC (Marine Culture Collection of China) and the KCTC (Korean Collection for Type Cultures).

Reference Strains

Reference strains used in this study (*A. iriomotensis* DMS 26155^T, *A. subtropicus* DSM 26153^T and *A. ramosus* DSM 43045^T) were purchased from the DeutscheSammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and *A. binzhouensis* CGMCC47180^T was purchased from China General Microbiological Culture Collection Center (CGMCC) .

Phenotypic Characterization

Cell morphology and motility were observed by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) after cells were incubated on marine agar 2216 (MA) at 30 °C for 2 days. Gram reaction was tested by using the Gram staining method as Claus described [3]. The temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35, 37, 40, 45, and 50 °C. The pH range for growth was determined at different pH values (pH 4.5–10, at increments of 0.5 pH units) and supplemented with buffering agents 40 mM MES (pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5), and CAPSO (pH 9.0–10.0), respectively. A modified marine broth (MB) was used for NaCl tolerance test, in which NaCl was omitted (0%) or added to final concentration of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0% (w/v), respectively. Growth under anaerobic and microaerobic conditions was determined with microaerobic system (AnaeroPack-MicroAero) using the modified MB medium supplemented with 20 mM sodium thiosulphate, 5 mM sodium sulphite, 20 mM sodium sulphate, 5 mM sodium nitrite, 20 mM sodium nitrate [23].

Antibiotic sensitivity was tested on MA with antibiotic discs, each disc containing neomycin (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), rifampicin (5 μg), streptomycin (10 μg), kanamycin (30 μg), tetracycline (30 μg), ampicillin (10 μg), or polymyxin B (100 U), respectively. The strain was incubated at 30 °C for 6 h before the antibiotic discs were attached to the MA. The sensitivity to antibiotic was determined by the diameter of the inhibition (> 13 mm as susceptible, 10–12 mm as partly susceptible, < 10 mm as resistant) after 2 days.

Oxidase activity was determined using oxidase reagent (bioMérieux) and catalase activity was determined by observing bubble production in 3% (v/v) H_2O_2. Hydrolysis of casein and gelatin were tested on MA supplemented with 1% skimmed milk (Difco) and 1% gelatin, respectively. Degradation of starch was tested on MA supplemented with 0.2% soluble starch [29]. Hydrolysis of Tweens 20, 40, 60, and 80 was determined as described by Sun et al. [31]. MA containing 0.5% L-tyrosine was used to test the degradation of L-tyrosine. H_2S and indole production tests were assayed according to Zhang et al. [37]. The methyl red and Voges–Proskauer test was performed according to Lányi [19]. Other physiological and biochemical tests were performed using API ZYM (bioMérieux) systems according to the manufacturer's instructions. Acid production was tested using API 50CH kits with modified MOF medium [20] which contained (per litre distilled water): casitone 1 g, yeast extract 0.1 g, $(NH_4)_2SO_4$ 0.5 g, Tris buffer 0.5 g, phenol red 0.01 g, NaCl 13.75 g, $MgCl_2{\cdot}6H_2O$ 7.75 g, $MgSO_4{\cdot}7H_2O$ 2.0 g, $CaCl_2$ 0.5 g, KCl 1.0 g, $FeSO_4$ 0.001 g, adjusted to pH 7.5.

Chemotaxonomic Characterization

For cellular fatty acid analysis, cells of strain Q14^T, *A. binzhouensis* OAct353^T, *A. subtropicus* IY07-56^T and *A. ramosus* DSM 43045^T were obtained and freeze-dried after incubation in MB at 30 °C for 24 h [18] while *A. iriomotensis* IY07-20^T was incubated for 48 h because of slow growth. Fatty acids were then analysed according to the standard protocol of the Microbial Identification System (MIDI; Microbial ID). Isoprenoid quinones were analysed using reversed-phase HPLC [17]. The amino-acid composition of the cell-wall peptidoglycan was determined and analysed according to the method of Schleifer&Kandler [27]. The polar lipids were extracted and separated by two-dimensional TLC on silica gel plates (10 × 10 cm; Merck 5554) [22]. The solvent systems of the two dimensions were prepared as described by Jia et al. [12]. The plates were then

heated at 120 °C for 10–15 min after spraying with 50% (v/v) sulphuric acid ethanol solution. Other reagents such as ninhydrin and molybdenum blue (Sigma) were used to detect aminolipids and phospholipids, respectively. In addition, the silica gel plates were sprayed with 5% phosphomolybdic acid and heated at 160 °C for 10–15 min to identify the total polar lipids.

Determination of 16S rRNA Gene Sequence and Phylogenetic Analysis

The Quick Bacteria Genomic DNA Extraction Kit (DongSheng Biotech) was used to extract genomic DNA. The 16S rRNA gene was amplified by PCR using two universal primers, 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AGAAAGGAGGTGATCCAGCC-3′) [5], and the PCR products were then purified and cloned into pMD 19-T vector (TaKaRa) for sequencing. The 16S rRNA gene sequence was identified on the ExTaxon-e service [3, 15]. Multiple sequence alignment was accomplished via the CLUSTAL W program of the MEGA 5 package [32]. The neighbour-joining [26] maximum-likelihood [6] and maximum-parsimony [7] methods were used to reconstruct phylogenetic trees using the MEGA 5.0 software [32]. Bootstrap values of three phylogenetic trees were based on 1000 replicates. The algorithm of Kimura's two-parameter model [16] was chosen for the neighbour-joining method.

DNA Base Composition and DNA–DNA Hybridization

For G+C content analysis, the genomic DNA was extracted as previously described and treated with P1 nuclease and calf intestine alkaline phosphatase before the G+C content was determined by reversed-phase HPLC [21]. DNA–DNA hybridization values of strainQ14^T and reference strains were determined by using a Beckman DU800 spectrophotometer according to the method of Zhang et al. [38].

Results and Discussion

Phenotypic Characterization

Strain Q14^T grew at 10–40 °C (optimum 30 °C), pH 5.5–10.0 (optimum 6.5–8.0) and with 0.5–6% (w/v) NaCl (optimum 1%), and sensitive to rifampicin, neomycin, erythromycin, chloramphenicol and ampicillin, but resistant to kanamycin, streptomycin, polymyxin B, gentamicin and tetracycline. The physiological and biochemical characteristics of strain Q14^T in comparison to the reference strains are shown in Table 1. All strains were negative for H_2S production and the results of methyl red and Voges–Proskauer tests were negative. The hydrolysis of starch and esculin were positive in all strains. Strain Q14^T and reference strains shared numerous similarities, such as being positive for activities of esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin and acid phosphatase. However, some characteristics were found to discriminate strain Q14^T from the reference strains. Strain Q14^T could grow at pH 5.5, but reference strains could not. Strain Q14^T could hydrolyse Tween 20, in contrast to the four reference strains. In the API ZYM system, alkaline phosphatase, α-glucosidase and β-glucosidase were positive for strain Q14^T but negative for *A. binzhouensis* OAct353^T. Strain Q14^T showed different results for alkaline phosphatase, β-galactosidase and *N*-acetyl-β-glucosaminidase production from *A. iriomotensis* IY07-20^T. Unlike *A. subtropicus* IY07-56^T, α-galactosidase was negative for strain Q14^T. The results of *α*-glucosidase and *β*-glucosidase were positive for strain Q14^T, which were different from *A. ramosus* DSM 43045^T. As for the API 50 CH test, the results of D-mannitol and raffinose were negative in Q14^T but positive in *A. binzhouensis* OAct353^T. Acid is produced from D-arabinose in strain Q14^T, unlike *A. iriomotensis* IY07-20^T. Strain Q14^T could not produce acid from D-mannitol, D-lactose and raffinose, which were in contrast to *A. subtropicus* IY07-56^T. Strain Q14^T could produce acid from D-arabinose, D-mannose, L-rhamnose and L-fucose, but *A. ramosus* DSM 43045^T could not.

Chemotaxonomic Characterization

The fatty acid profiles of strain Q14^T and the reference strains are listed in Table 2.

The major fatty acids (≥ 5.0% of the total fatty acids) found in strain Q14^T were anteiso-$C_{15:0}$ (47.7%), iso-$C_{17:0}$ (26.7%), anteiso-$C_{17:0}$ (12.9%) and iso-$C_{14:0}$ (5.4%).The five strains had similarities in major components (≥ 5.0%), such as anteiso-$C_{15:0}$, iso-$C_{17:0}$. However, there were some characteristics that distinguished strain Q14^T from the reference strains. Iso-$C_{14:0}$ was a major fatty acid in strain Q14^T but not in *A. iriomotensis* IY07-20^T, *A. subtropicus* IY07-56^T and *A. ramosus* DSM 43045^T. Iso-$C_{15:0}$ was not a major acid in strain Q14^T compared with *A. ramosus* DSM 43045^T and antesio-$C_{17:0}$ was a major fatty acid in strain Q14^T but not in the closest phylogenetic neighbour *A.binzhouensis* OAct353^T. The main respiratory quinone detected in strain Q14^T was MK-12, as the four reference strains. MK-13 is present as a minor component, which different from *A.binzhouensis* OAct353^T. The major polar lipids of strain Q14^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphoglycolipid (PGL), phospholipid (L), three glycolipids (GL) and four lipids (Supplementary Fig. S2), a profile that was similar to reference strains of the genus *Agromyces*. Strain Q14^T contained three lipids (L1, L3 and L4) and one phosphoglycolipid (PGL), which distinguished

Table 1 Differential characteristics between strain Q14^T and related members of the genus *Agromyces*

Characteristic	1	2	3	4	5
Colony colour	Pale yellow	Yellow	Cream yellow	Pale yellow	Cream
Growth at					
10 °C	+	−	−	+	−
pH 5.5	+	−	−	−	−
5% (w/v) NaCl	+	+	−	−	−
Nitrate reduction	−	−	−	+	−
Catalase	+	−	−	+	−
Hydrolysis of					
Gelatin	+	−	−	+	+
Tyrosine	+	−	−	+	−
Skimmed milk	+	−	−	+	−
Tween 20	+	−	−	−	−
API ZYM results					
ALKALINE phosphatase	+	−	−	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	w
α-Galactosidase	−	−	−	+	−
β-Galactosidase	+	+	−	+	−
α-Glucosidase	+	−	+	+	−
β-Glucosidase	+	−	+	+	−
N-Acetyl-β-glucosaminidase	−	−	+	−	+
Acid production					
D-Arabinose	+	+	−	+	−
Galactose	−	−	−	+	+
D-Mannose	+	+	+	−	−
L-Rhamnose	+	+	+	+	−
D-Mannitol	−	+	+	+	+
D-Lactose	−	−	−	+	−
Inulin	−	−	+	−	+
Raffinose	−	+	+	+	+
L-Fucose	+	+	+	+	−
DNA G+C content (mol%)	70.1	69.6[a]	72.9[b]	71.1[c]	71.0[d]

Strains: *1* strain Q14^T (=MCCC 1K03191^T=KCTC 39814^T), *2 A. binzhouensis* OAct353^T, *3 A. iriomotensis* IY07-20^T, *4 A. subtropicus* IY07-56^T, *5 A. ramosus* DSM 43045^T

+ positive, − negative, *w* weakly positive

[a]Data from Chen et al. [2]

[b]Data from Hamada et al. [10]

[c]Data from Hamada et al. [10]

[d]Data from Gledhill et al. [8]

strain Q14^T from *A. iriomotensis* IY07-20^T. In contrast to *A. subtropicus* IY07-56^T, one phosphoglycolipid (PL1) was not found in strain Q14^T. One glycolipid (GL2) and two lipids (L1 and L4) were different between strain Q14^T and *A. ramosus* DSM 43045^T. In contrast to the closest phylogenetic neighbor *A.binzhouensis* OAct353^T, strain Q14^T contained diphosphatidylglycerol (DPG) as major polar lipids. Besides, different lipids also could distinguish strain Q14^T from *A.binzhouensis* OAct353^T. The peptidoglycan of strain Q14^T contains Ala, Glu, Gly and A_2bu. This result was similar to the reference strains [2, 10].

Genotypic Characterization and Taxonomic Conclusion

On the basis of 16S rRNA gene sequence similarity, strain Q14^T was a close relative of species of the genus *Agromyces*, sharing 99.7, 97.8, 97.7 and 97.3% similarity with the type strains of *A.binzhouensis* OAct353^T, *A. iriomotensis* IY07-20^T, *A. subtropicus* IY07-56^T and *A. ramosus* DSM 43045^T, respectively. Strain Q14^T formed a cluster with *A. binzhouensis* OAct353^T in the neighbour-joining(Fig. 1), maximum-likelihood(Supplementary Fig. S3) and

Table 2 Cellular fatty acid contents of strain Q14^T and the reference strains

Fatty acid	1	2[a]	3	4	5
iso-$C_{14:0}$	5.35	9.85	0.41	0.93	2.11
iso-$C_{15:0}$	4.89	4.92	2.06	1.85	7.43
anteiso-$C_{15:0}$	47.67	38.43	39.52	48.83	49.81
iso-$C_{16:0}$	0.83	36.24	0.78	0.40	1.15
iso-$C_{17:0}$	26.70	11.1	11.80	16.53	20.79
antesio-$C_{17:0}$	12.90	ND	44.35	31.02	17.22

Strains: *1* strainQ14^T(=MCCC 1K03191^T=KCTC 39814^T), *2 A. binzhouensis* OAct353^T, *3 A. iriomotensis* IY07-20^T, *4 A. subtropicus* IY07-56^T, *5 A. ramosus* DSM 43045^T. Fatty acids comprising less than 1% in all of four strains are not shown

ND not detected

[a]Data from Chen et al. [2]

maximum-parsimony(Supplementary Fig. S4) trees, indicating that strain Q14^T may represent a novel species of the genus *Agromyces*. Based on 16S rRNA gene sequences, the phylogenetic analysis revealed that strain Q14^T belonged to the genus *Agromyces*, and the most closely related strain was *A.binzhouensis* OAct353^T(99.7% similarity). The G+C content of strain Q14^T was 70.1 mol%, which was similar to the reference strains. DNA–DNA hybridization results showed that strain Q14^T was 51.9, 51.5, 50.9, and 44.5% related to *A.binzhouensis* OAct353^T, *A. iriomotensis* IY07-20^T, *A. subtropicus* IY07-56^T and *A. ramosus* DSM 43045^T, respectively. These results were significantly below the threshold value (70%) for determining bacterial species [30].

Based on the phenotypic, phylogenetic and chemotaxonomic properties presented in this study, strainQ14^T represents a novel species in the genus *Agromyces*, for which the name *Agromyces mangrove* sp. nov. is proposed.

Description of *Agromyces mangrovi* sp. nov.

Agromyces mangrovi (man.gro'vi. N.L. gen. n. mangrovi of a mangrove, referring to the isolation of the type strain from mangrove soil).

Cells are Gram-stain-positive, microaerophilic to aerobic, no-motile, non-endospore-forming and rod-shaped (1.5–1.8μm × 0.5–0.7 μm) without flagella. Colonies are 2 mm in diameter, circular, convex and pale yellow after growth on MA 30 °C for 2days. Growth occurs at 10–40 °C (optimum 30 °C), pH 5.5–10.0 (optimum 6.5–8.0) and with 0.5–6% (w/v) NaCl (optimum 1%). Sensitive to rifampicin, neomycin, erythromycin, chloramphenicol and ampicillin, but resistant to kanamycin, streptomycin, polymyxin B, gentamicin and tetracycline. Oxidase is negative and catalase is positive. Gelatin, starch, Tween 20, Tween 40, Tween 60, skimmed milk and L-tyrosine can be hydrolyzed, but Tween 80 cannot. Negative for indole production, H_2S production, methyl red and Voges–Proskauer test. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase are positive. In API 50 CH assays, acid is produced from glycerol, D-arabinose, L-arabinose, D-glucose, D-fructose, D-mannose, salicin, cellobiose, maltose, sucrose, melezitose, starch, glycogen, turanose and L-fucose. The major respiratory quinone is MK-12. The major fatty acids (≥ 5% of the total fatty acids) are iso-$C_{14:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$. The major polar lipids of strain Q14^T are diphosphatidylglycerol, phosphatidylglycerol, phosphoglycolipid, three glycolipids and four unidentified lipids. The peptidoglycan contains Ala, Glu, Gly and A_2bu. The G+C content of the genomic DNA of the type strain is 70.1 mol%.

The type strain, Q14^T (=MCCC 1K03191^T=KCTC 39814^T), was isolated from mangrove soil samples collected on chengmai, Hainan province, China.

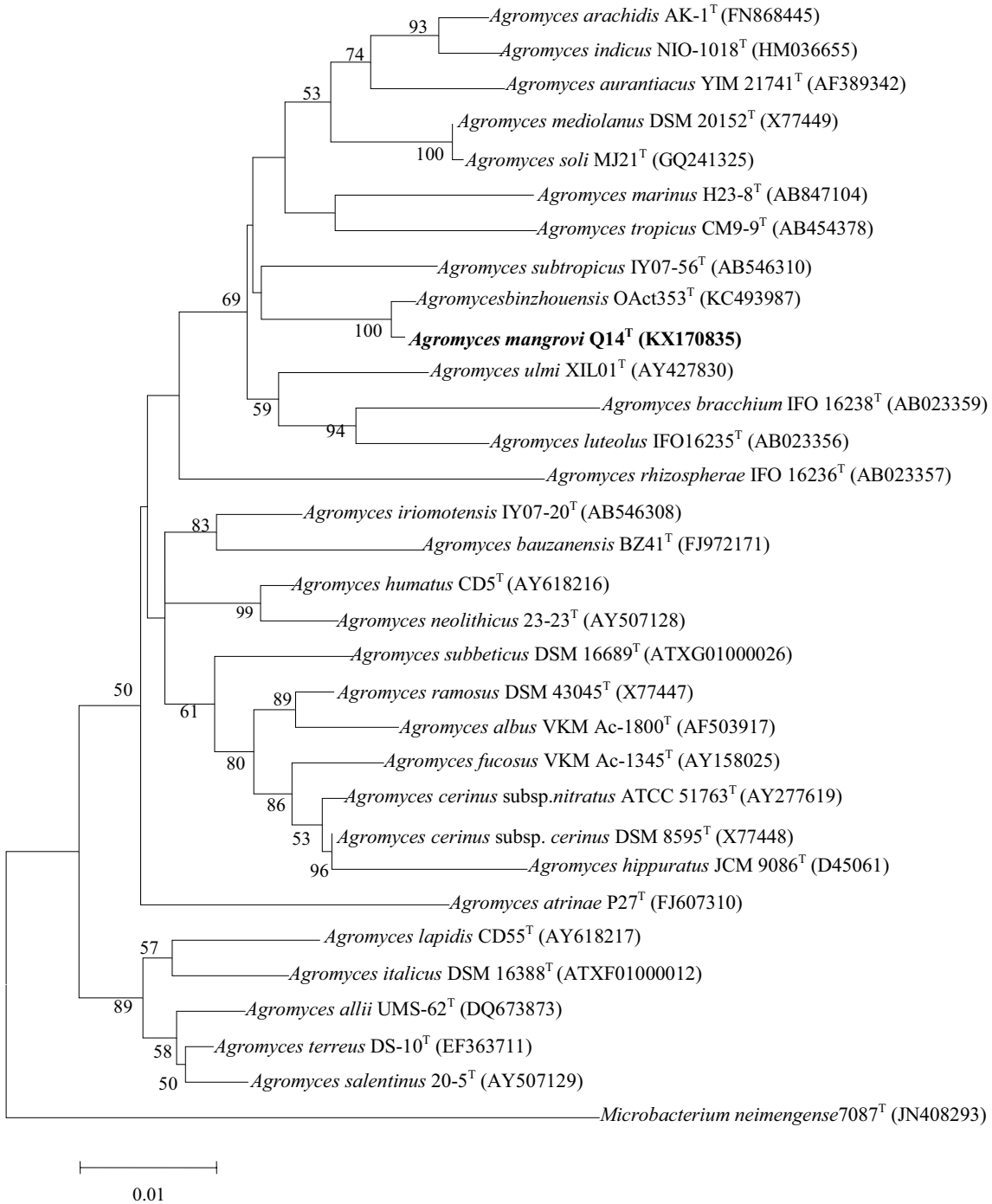

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationship of strain Q14^T with the related taxa. Bootstrap values were based on 1000 replicates; only showed values ≥ 50%. Bar, 0.01 substitutions per nucleotide position. *Microbacterium neimengense* 7087^T was used as an outgroup

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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