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ORIGINAL ARTICLE

Micromonospora endophytica sp. nov., an endophytic actinobacteria of Thai upland rice (*Oryza sativa*)

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An actinobacterial strain, DCWR9-8-2^T, was isolated from a leaf of Thai upland rice (*Oryza sativa*) collected in Chumporn province, Thailand. Strain DCWR9-8-2^T is Gram-stain-positive aerobic bacteria that produce single spores directly on the vegetative hypha. Cell wall peptidoglycan of this strain exhibits *meso*-diaminopimelic acid and glycine, the reducing sugars of whole-cell hydrolysate are arabinose, glucose, ribose, xylose and small amount of mannose. The phospholipid profiles in the membrane are comprised of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides. The major menaquinones are MK-9(H_4) and MK-10(H_6). The diagnostic cellular fatty acids are iso-$C_{16:0}$ and iso-$C_{15:0}$. The G+C content of the genomic DNA is 72.5 mol%. The result of 16S rRNA sequence analysis of the strain revealed that this strain was closely related to *Micromonospora auratinigra* TT1-11^T (99.25%). On the other hand, the result of *gyrB* gene sequence analysis revealed that this strain was closed to *M. eburnea* JCM 12345^T (96.30%). In addition, a combination of DNA–DNA hybridization results and some phenotypic properties supported that this strain should be judged as a novel species of the genus *Micromonospora*, for which the name *M. endophytica* sp. nov. is proposed. The type strain is DCWR9-8-2^T (= BCC 67267^T = NBRC 110008^T).
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INTRODUCTION

Orskov[1] is the first scientist that proposed the actinobacteria, namely *Micromonospora*. All members of this genus produced single non-motile spores directly on the substrate mycelium. This organism cannot produce aerial mycelium during its life cycle. Cell wall peptidoglycan of this organism contains glutamic acid, glycine, alanine and diaminopimelic acids. Peptidoglycan type (Schleifer and Kandler[2]) of *Micromonospora* is A1γ. The acyl type of cell wall muramic acid is glycolyl. Diagnostic reducing sugars of cell hydrolysates are xylose and arabinose. Characteristic phospholipids are phosphatidylethanolamine. The major cellular fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-C17:0 and 10-methyl-$C_{17:0}$. Mycolic acids are absent. The range of DNA G+C contents (mol%) is 71–73. *Micromonospora* is well known as antibiotic producers. Several antibiotics used in the present time, that is, gentamicin,[3] lomaiviticins,[4] lupinacidin,[5] maklamicin,[6] are produced by the members of this genus. Nowadays, 61 species have been characterized as members of the genus *Micromonospora*. Here, we described the taxonomic characterization of a novel endophytic *Micromonospora* strain DCWR9-8-2^T, which was isolated from a leaf of Thai upland rice.

MATERIALS AND METHODS

Strain DCWR9-8-2^T was isolated from a leaf of upland rice collected from Chumporn province, Thailand. A Leaf sample was rinsed with tap water for three times and was surface-sterilized with 70% ethyl alcohol for 5 min and subsequently with absolute ethyl alcohol for 1 min. A treated leaf was dried on sterilized filter paper and then was treated with 6% sodium hypochlorite in distilled water for 2 min. After that, the leaf tissue was rinsed with sterilized distilled water for five times and the surface-sterilized leaf was ground in sterilized distilled water. Two hundred microlitres of the plant solution was spread on starch casein agar (0.1% sodium caseinate, 1% soluble starch, 0.03% K_2HPO_4, 1.8% agar, pH 7.0–7.5) supplemented with 25 mg l^{-1} nalidixic acid and 100 mg l^{-1} nystatin and incubated at 30 °C for 21 days. The final rinsing water was spread on starch casein agar as the control plates. The colony of strain DCWR9-8-2^T was selected and purified on ISP 2 medium (International *Streptomyces* Project, ISP 2 medium).[7] Strain DCWR9-8-2^T was grown on starch casein agar medium at 30 °C for 21 days. Then, the colonies were observed by scanning electron microscopy (model LEO/1455VP). The scanning electron microscopic samples of strain DCWR9-8-2^T were prepared as described previously.[8]

Several standard methods were used for the phenotypic determination. Cultural properties were examined by using 14-day cultures grown at 30 °C on various agar media. The determining color designations was judged using the ISCC–NBS Color Charts standard sample no. 2106.[9] The determination of the

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carbon utilization of the strain was done using International *Streptomyces* Project medium no.9 (ISP9 medium) supplemented with a final concentration of 1% of the carbon sources. The basal medium recommended by Gordon *et al.*[10] were used for the examination of the decomposition of various compounds and acid production from carbon sources. The test for growth at various temperatures (10–50 °C), different sodium chloride concentration (0–12% NaCl) and pH 4–12 was examined on ISP 2 medium. The desired pH was adjusted using sterile solutions of citric acid/Na_2HPO_4 (for pH 4.0–5.0), Na_2HPO_4/NaH_2PO_4 buffer (for pH 6.0–8.0), $NaHCO_3/Na_2CO_3$ buffer (for pH 9.0–10.0), Na_2HPO_4/ NaOH buffer (pH 11.0) and KCl/NaOH buffer (for pH 12.0–13.0). The culture media and methods described by Arai[11] and Williams and Cross[12] were used for determination of starch hydrolysis, peptonization of milk, gelatin liquefaction and the reduction of nitrate.

Cells of strain DCWR9-8-2^T were grown in ISP 2 broth on rotary shaker (200 r.p.m.) at 30 °C for 5 days and used for chemotaxonomic analyses. Cell wall peptidoglycan was prepared by the methods of Kawamoto *et al.*,[13] and the isomer of diaminopimelic acid was analyzed by the method of Staneck and Roberts.[14] The acyl group in the peptidoglycan was performed as described by Uchida and Aida.[15] The reducing sugars of cell hydrolysate were determined by the method of Komagata and Suzuki.[16] The phospholipid of the cell membranes were analyzed by the method of Minnikin *et al.*[17] The analysis of cellular fatty acid profile was performed by gas liquid chromatography according to the instructions of the Microbial Identification System (MIDI, version 6.0, Newark, DE, USA) (Sasser;[18] Kämpfer and Kroppenstedt[19]) with the ACTIN1 MIDI database. Menaquinones were extracted by the method of Collins *et al.*[20] and were examined by HPLC equipped with a Cosmosil 5C_{18} column (4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan). The elution solvent was a mixture of methanol and 2-propanol (2:1, v/v). The presence of mycolic acids was investigated using the method of Minnikin *et al.*[21]

Genomic DNA of strain DCWR9-8-2^T used for polymerase chain reaction, the G+C content analysis and DNA–DNA hybridization was prepared according to the method of Tamaoka.[22] The G+C content (mol %) was analyzed using the HPLC method of Tamaoka and Komagata.[23] Lambda DNA (Invitrogen, USA) was used as the standard. DNA–DNA hybridization was determined as described by Ezaki *et al.*[24] DNA–DNA relatedness (%) was analyzed by the colorimetric method of Verlander.[25] The 16S rRNA gene fragment was amplified as described by Suriyachadkun *et al.*[26]

Amplification and sequencing of the *gyrB* gene was performed according to the previously described method.[27] The sequence of the 16S rRNA gene was obtained by using the universal primers.[28] The pairwise alignment and the values for sequence similarity of the 16S rRNA gene and the *gyrB* gene sequence of strain DCWR9-8-2^T were performed using the EzTaxon server[29] and CLUSTAL X program (Thompson *et al.*),[30] respectively. Partial sequences of the 16S rRNA gene and the *gyrB* gene of recognized *Micromonospora* species were obtained from the GenBank/EMBL/DDBJ databases for multiple alignment analyses using the CLUSTAL W programme, version 1.81.[31] Prior to the construction of a phylogenetic tree, the alignment of 16S rRNA gene and the *gyrB* gene sequences was manually verified and adjusted. The phylogenetic analysis was constructed using the neighbor-joining[32] and maximum-likelihood[33] methods in the MEGA 5 software.[34] The evolutionary distances of the neighbor-joining tree were calculated by using the Kimura two-parameter model.[35] The confidence values of branches in the tree were determined using bootstrap analyses[36] with 1000 repeats.

RESULTS AND DISCUSSION

Strain DCWR9-8-2^T exhibited a range of phenotypic and chemotaxonomic properties that was consistent with their classification in the genus *Micromonospora*.[37] The strain grew well on ISP 2, ISP 3 and ISP 6, moderately on nutrient agar, weakly on ISP 4, ISP 5, ISP 7, czapek's sucrose agar and glucose-asparagine agar (Table 1). Strain DCWR9-8-2^T formed branched substrate mycelia but aerial mycelia were not produced in all media test. Spores of this strain were borne singly on the substrate mycelia and the spore surface was smooth (Figure 1). The color of substrate mycelia on these media was pale orange yellow to deep orange yellow. The orange yellow soluble pigment was produced on ISP2 and ISP 3. The range of pH and temperature for growth of strain DCWR9-8-2^T were pH 5–10 and 20–45 °C, respectively. The maximum NaCl tolerance was 2% (w/v). Other phenotypic characteristics are given in Table 2 and in the species description. Chemotaxonomic properties of strain DCWR9-8-2^T were similar to those of members of the genus *Micromonospora*. This organism contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan; the acyl type of cell wall muramic acid was glycolyl. Arabinose, glucose, ribose, xylose and small amount of mannose were observed as reducing sugars in cell hydrolysates. The menaquinones in cell of strain DCWR9-8-2^T were MK-9(H_4) (27.0%) and MK-10(H_6) (25.9%); substantial amounts of MK-10(H_4) (15.3%), MK-9(H_2) (13.1%), MK-10(H_8) (9.6%), MK-10 (H_2) (8.8%) and MK-9(H_6) (0.2%) were also present. The polar lipids in cell membrane were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, five unidentified phospholipids and two unidentified lipids (Supplementary Figure S2). Mycolic acids were not observed. The predominant cellular fatty acids (≥5%) were iso-$C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$ and iso-$C_{17:0}$ (Supplementary Table S1). The DNA base compositions of strain was 72.5 mol%.

Table 1 Cultural characteristics of strain DCWR9-8-2^T on various media cultivated at 30 °C after 14 days

Media	*Growth*	*Color of colony*	*Soluble pigment*
Yeast extract-malt extract agar (ISP2)	Good	Vivid orange yellow	Orange yellow
Oatmeal agar (ISP3)	Good	Deep orange yellow	Orange yellow
Inorganic salt-starch agar (ISP4)	Poor	Orange yellow	—
Glycerol-asparagine agar (ISP5)	Poor	Pale orange yellow	—
Peptone-yeast extract iron agar (ISP6)	Good	Deep orange yellow	—
Tyrosine agar (ISP7)	Poor	Pale orange yellow	—
Czapek's sucrose agar	Poor	Pale orange yellow	—
Glucose-asparagine agar	Poor	Pale orange yellow	—
Nutrient agar	Moderate	Orange yellow	—

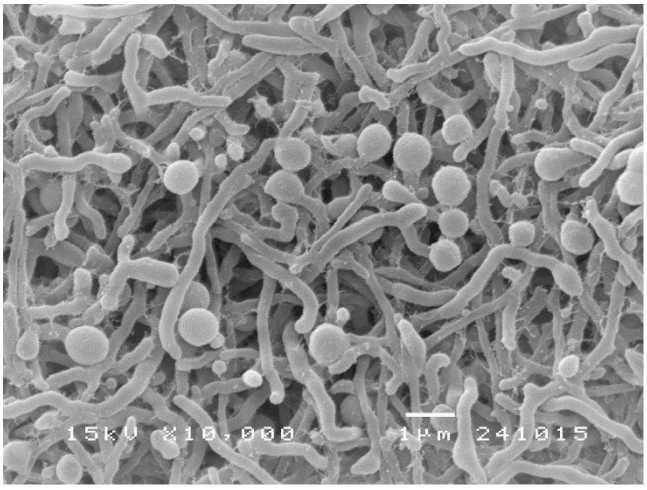

Figure 1 Scanning electron micrograph of strain DCWR9-8-2^T grown on starch casein agar medium for 21 days at 30 °C. Bar, 1 μm.

Table 2 Differential characteristics of strain DCWR9-8-2^T and *M. auratinigra* TT1-11^T

Characteristics	*Strain DCWR9-8-2^T*	*M. auratinigra TT1-11^T*
Cell wall amino acids	*Meso-diaminopimelic acid*	*Meso-diaminopimelic acid and 3-OH-meso-diaminopimelic acid*
Whole-cell sugar	Ara, Glu, Man, Rib, Xyl	Ara, Gal, Glu, Man, Rib, Xyl
Carbon utilization		
D-ribose	−	+
D-fructose	−	+
D-raffinose	−	+
D-melibiose	−	+
D-salicin	−	+
Acid production from		
D-glucose	−	+
D-mannose	−	+
D-galactose	−	+
D-cellobiose	−	+
D-ribose	−	+
D-fructose	−	+
D-raffinose	−	+
D-melibiose	−	+
Nitrate reduction	+	−

Abbreviations: +, positive; −, negative; Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rib, ribose; Xyl, xylose.
All phenotypic and chemotypic data were determined in this study.

The result of pairwise alignment analysis of the partial 16S rRNA gene sequence of strain DCWR9-8-2^T (1502 nt) obtained from the EzTaxon server[29] showed that strain DCWR9-8-2^T is a member of the genus *Micromonospora*. The highest 16S rRNA gene sequence similarity value was observed with *M. auratinigra* TT1-11^T (99.25%) followed by *M. echinospora* ATCC 15837^T (99.15%), *M. chaiyaphumensis* MS5-1^T (98.96%), *M. pattaloongensis* TJ2-2^T (98.91%) and *M. eburnea* LK2-10^T (98.62%). The neighbor-joining tree constructed with 16S rRNA gene sequences of all valid *Micromonospora* species confirmed the taxonomic position of this strain that belongs to the genus *Micromonospora* and forms a monophyletic clade with *M. auratinigra* TT1-11^T (Figure 2). Additionally, the maximum-likelihood tree also revealed that strain DCWR9-8-2^T is closely related to *M. auratinigra* TT1-11^T (Supplementary Figure S1). On basis of morphological, chemotaxonomic and phylogenetic data, this strain should be classified in the genus *Micromonospora*. Partial *gyrB* gene sequences of strain DCWR9-8-2^T and other members in the genus *Micromonospora* were used for the multiple alignment and the sequence analysis. The partial *gyrB* gene sequence of strain DCWR9-8-2^T displayed the highest sequence similarity (96.3%) with that of *M. eburnea* JCM12345^T. Consistently, the phylogenetic analysis of the *gyrB* gene sequences showed that strain DCWR9-8-2^T formed a cluster with *M. eburnea* JCM12345^T. This was also significantly supported by the 68% bootstrap value (Supplementary Figure S3). The discrepancy between the phylogenetic relationship of strain DCWR9-8-2^T based on the 16S rRNA and *gyrB* genes was similar to the results observed in previous studies. For example, the phylogenetic tree based on 16S rRNA gene sequences showed that *M. tulbaghiae* TVU1^T was phylogenetically related to *M. echinospora* DSM 43816^T and *M. rosaria* DSM 803^T, while the phylogenetic analysis of the *gyrB* gene revealed that it was clustered together with *M. aurantiaca* NBRC 16155^T and *M. chalcea* NBRC 13503^T.[38] Similarly, *M. rhizosphaerae* 211018^T was closely related to *M. olivasterospora* DSM 43868^T based on the phylogenetic analysis of the 16S rRNA gene sequences. However, the phylogenetic tree reconstructed from *gyrB* gene sequences indicated that its closest relative was *M. inositola* ATTC 21773^T.[39]

Distinct chemotypic and phenotypic properties between strain DCWR9-8-2^T and the closest phylogenetic relative, *M. auratinigra* TT1-11^T are shown in Table 2. In particular, strain DCWR9-8-2^T does not contain 3-OH-*meso*-diaminopimelic acid in its cell wall peptidoglycan while *M. auratinigra* TT1-11^T presents this diaminopimelic acid. On the other hand, the presence of galactose in the cell hydrolysate is the significant point that distinguishes strain DCWR9-8-2^T from *M. auratinigra* TT1-11^T. The phenotypic data clearly discriminated strain DCWR9-8-2^T from *M. auratinigra* TT1-11^T as strain DCWR9-8-2^T did not utilize D-fructose, D-melibiose, D-raffinose, D-ribose and salicin as the sole carbon sources, whereas *M. auratinigra* TT1-11^T could utilize these carbon sources. Furthermore, the result of acid production from several carbon sources including the reduction of nitrate test also confirmed the differentiation between strain DCWR9-8-2^T and *M. auratinigra* TT1-11^T. Additionally, the low DNA–DNA relatedness value (32.1% ± 0.2 and 26.3% ± 0.8) was observed between strain DCWR9-8-2^T, *M. auratinigra* TT1-11^T and *M. eburnea* JCM12345^T, respectively, below the 70% cut-off point suggested for the assignment of bacterial strains to the same genomic species.[40] All above the differential characteristics confirmed that strain DCWR9-8-2^T is a novel species of the genus *Micromonospora*, for which the name *M. endophytica* sp. nov. is proposed.

Description of *Micromonospora endophytica* sp. nov.

M. endophytica (en.do.phy'ti.ca. Gr. *endo* within; Gr. *phyton* plant; L. fem. suff. *-ica* adjectival suffix used with the sense of belonging to; N.L. fem. adj. *endophytica* within plant, endophytic, pertaining to the original isolation from plant tissue).

Gram-staining-positive, mesophilic actinobacteria that forms a single spore borne on the tip of the substrate mycelia. The orange yellow soluble pigment is observed on ISP2 and ISP3 media. Utilizes D-glucose, D-mannose, D-galactose, D-cellobiose, L-arabinose and D-xylose as sole carbon sources, weakly utilizes sucrose, but not glycerol, *myo*-inositol, L-rhamnose, D-ribose, D-fructose, D-raffinose, D-melibiose, mannitol, salicin and lactose. Acid production from L-arabinose and D-xylose. Peptonization of milk, hydrolysis of starch, gelatin liquefaction and nitrate reduction are positive. The maximum temperature for growth is 45 °C. The maximum NaCl concentration for growth is 2% (w/v). The pH range for growth is 5–10. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The acyl type of the cell wall is glycolyl. The predominant menaquinones is MK-9(H_4) and MK-10(H_6). The diagnostic whole-cell sugars are arabinose, glucose, ribose, xylose and small amount of mannose. The polar lipid profile contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, five unidentified phospholipids and two unidentified lipids but phosphatidylcholine is not detected. The major and sub-major fatty acids consists of iso-$C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$, $C_{17:1}$ ω8c, 10-methyl $C_{17:0}$, $C_{18:1}$ ω9c, $C_{17:0}$. Mycolic acids are absent. The G+C content of the DNA is 72.5 mol%. The type strain is DCWR9-8-2^T (= BCC 67267^T = NBRC 110008^T), which was isolated from a leaf of upland rice collected from Chumporn province, Thailand

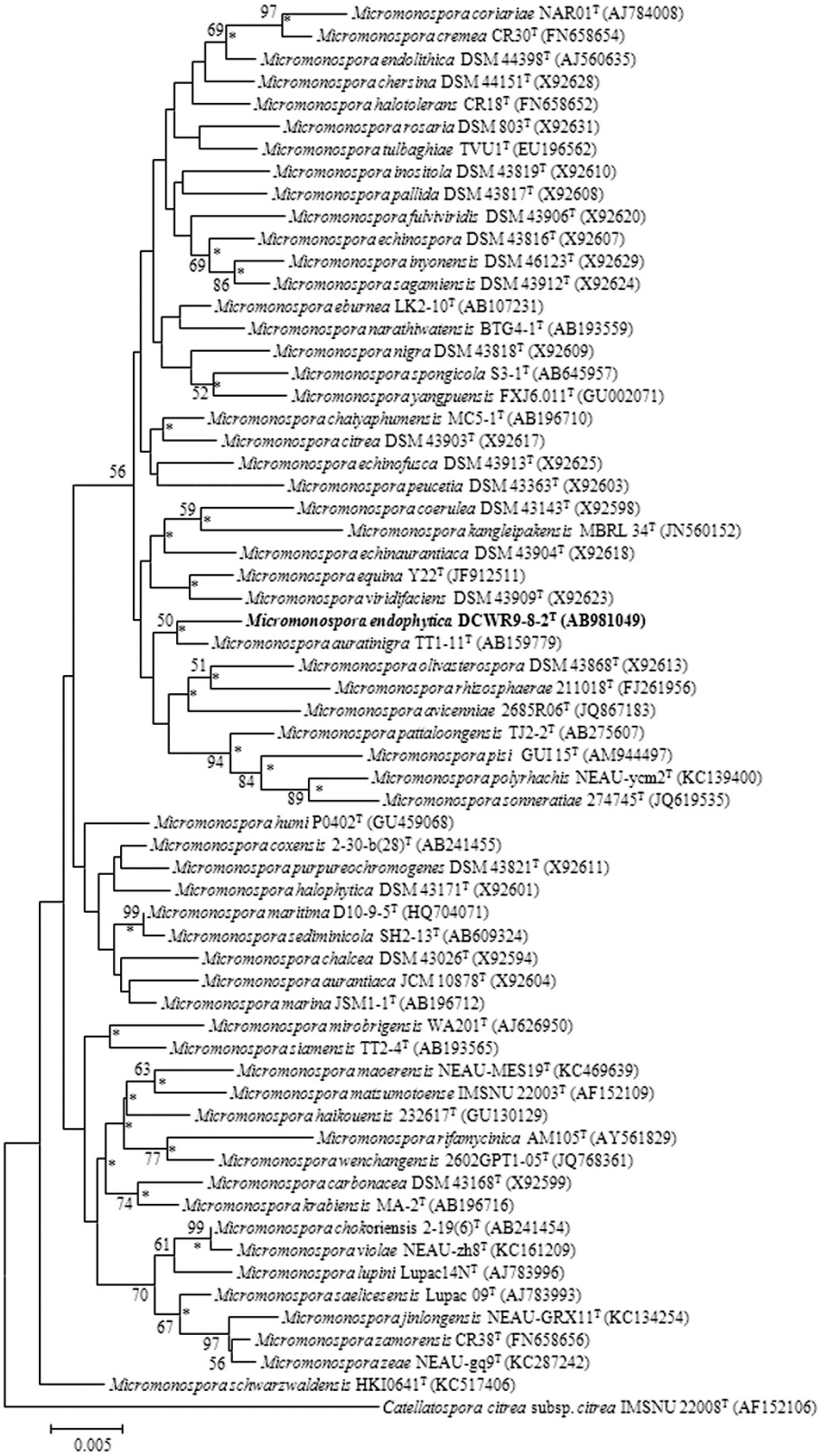

Figure 2 Phylogenetic tree obtained by neighbor-joining tree based on almost-complete 16S rRNA gene sequences showing the position of strain DCWR9-8-2^T among related species in the *Micromonospora* species. *Catellatospora citrea* subsp. Citrea IMSNU 22008^T was used as outgroup. Asterisks (*) indicating the branches of the tree that were also found using the maximum-likelihood methods. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values >50% are indicated. Bar, 0.005 substitutions per nucleotide position.

ACCESSION CODE:

The DDBJ accession number for the 16S rRNA gene sequence of strain DCWR9-8-2^T is AB981049.

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