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ORIGINAL ARTICLE

# *Micromonospora lycii* sp. nov., a novel endophytic actinomycete isolated from wolfberry root (*Lycium chinense* Mill)

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**A novel actinomycete, designated strain NEAU-gq11$^T$, was isolated from wolfberry root (*Lycium chinense* Mill) and characterized using a polyphasic approach. Strain NEAU-gq11$^T$ was observed to form rough-surfaced spores that borne singly on the substrate hyphae but aerial mycelia were not developed. The organism showed closest 16S rRNA gene sequence similarity to *Micromonospora saelicesensis* Lupac 09$^T$ (99.4%), and phylogenetically clustered with *M. chokoriensis* 2–19/6$^T$ (99.3%), '*M. zeae*' NEAU-gq9$^T$ (99.3%), *M. violae* NEAU-zh8$^T$ (99.1%), '*M. jinlongensis*' NEAU-GRX11$^T$ (99.1%), *M. zamorensis* CR38$^T$ (99.0%), *M. taraxaci* NEAU-P5$^T$ (98.9%) and *M. lupini* Lupac 14N$^T$ (98.6%). Phylogenetic analysis based on the *gyrB* gene sequences also indicated that the isolate clustered with the above type strains except *M. violae* NEAU-zh8$^T$. The cell-wall peptidoglycan consisted of *meso*-diaminopimelic acid and glycine. The major menaquinones were MK-9($H_8$), MK-10($H_6$) and MK-10($H_2$). The phospholipid profile contained diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The major fatty acid was iso-$C_{16:0}$. Furthermore, some physiological and biochemical properties and low DNA–DNA relatedness values enabled the strain to be differentiated from members of closely related species. Therefore, it is proposed that strain NEAU-gq11$^T$ represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora lycii* sp. nov. is proposed. The type strain is NEAU-gq11$^T$ (= CGMCC 4.7149$^T$ = DSM 46703$^T$).**
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## INTRODUCTION

*Micromonospora* is the type genus of the family *Micromonosporaceae* that was first described by Ørskov[1] for actinomycetes that formed single, non-motile spores directly on the substrate mycelium, but no aerial mycelia were produced. Up to now, the genus *Micromonospora* comprises 65 species including recently described *M. taraxaci* NEAU-P5$^T$,[2] *M. costi* CS1-12$^T$,[3] '*M. palomenae*' NEAU-CX1$^T$,[4] and '*M. harpali*' NEAU-JC6$^T$.[4] Furthermore, the genus *Micromonospora* has gradually been recognized as an important source of secondary metabolites.[5] Many antibiotics, such as gentamicin, netamicin, lomaiviticins A and B, tetrocarcin A, diazepinomicin and lupinacidins A and B have been isolated from this genus.[5–7] Therefore, new species in the genus *Micromonospora* remains a focus of efficient research for the discovery of new bioactive compounds. During the investigation of exploring potential sources of endophytic actinobacteria with novel natural products in the root of wolfberry (*Lycium chinense* Mill), a total of 14 isolates of endophytic actinomycetes were isolated from wolfberry root sample. Out of 14 isolates, *Micromonospora* was the dominant genus ($n=8$, 57.1% of isolates), followed by *Streptomyces* spp. ($n=6$, 42.9%). In this study, we performed polyphasic taxonomy on *Micromonospora* strain NEAU-gq11$^T$, and proposed that the novel isolate belongs to a new species of the genus, for which the name *Micromonospora lycii* sp. nov. is proposed.

## MATERIALS AND METHODS

### Isolation and cultivation

Strain NEAU-gq11$^T$ was isolated from wolfberry root (*L. chinense* Mill) collected from Harbin, Heilongjiang province, north China (45°45′ N, 126°41′ E). The root sample was processed as described by Wang *et al.*[8] and placed on a plate of humic acid-vitamin agar (HV)[9] supplemented with cycloheximide (50 mg l$^{-1}$) and nalidixic acid (50 mg l$^{-1}$). After 2 weeks of aerobic incubation at 28 °C, colonies were transferred and purified on International *Streptomyces* Project (ISP) medium 3[10] and maintained as glycerol suspensions (20%, v/v) at −80 °C.

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### Morphological and physiological characteristics

Gram staining was carried out by using the standard Gram reaction method. Morphological characteristics were observed by light microscopy (Nikon ECLIPSE E200, Tokyo, Japan) and scanning electron microscopy (Hitachi S-3400N, Tokyo, Japan) after cultivation on ISP 3 medium at 28 °C for 3 weeks. Cultural characteristics were determined after being growth for 3 weeks at 28 °C on SA 1 agar,[11] N-Z amine agar,[12] Bennett's agar[13] and ISP media 2–7.[10] ISCC-NBS color charts Standard Samples No 2106[14] was used to determine the color of colonies and soluble pigments. The growth temperature range (4, 10, 15, 20, 28, 35, 37, 40 and 45 °C) was determined on ISP 3 medium after culturing for 2 weeks. Tolerance of various pH (3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) and NaCl (0, 1, 2, 3, 4 and 5%, w/v) were determined in GY medium[15] in shake flasks (250 r.p.m.) at 28 °C for 7 days. Hydrolysis of Tweens (20, 40 and 80) were tested as described by Smibert and Krieg.[16] Reduction of nitrate, degradation of gelatin, cellulose and urea, hydrolysis of starch and aesculin, coagulation of milk and production of catalase and $H_2S$ were examined as described by Gordon *et al.*[17] Utilization of sole carbon sources was tested on ISP 9 medium.[10] Utilization of amino acids as nitrogen sources was tested as described by Williams *et al.*[18] The reference strains *M. saelicesensis* Lupac 09$^T$, *M. chokoriensis* 2–19/6$^T$, *M. zamorensis* CR38$^T$ and *M. lupini* Lupac 14N$^T$ were obtained from Japan Collection of Microorganisms and DSMZ (German Collection of Microorganisms and Cell Cultures), '*M. zeae*' NEAU-gq9$^T$,[19] '*M. jinlongensis*' NEAU-GRX11$^T$,[20] *M. violae* NEAU-zh8$^T$,[21] and *M. taraxaci* NEAU-P5$^T$,[2] were from our laboratory. These strains were cultured under the same conditions for comparative analyses.

### Chemotaxonomic analysis

Biomass for chemical studies was obtained by growing the strain in GY medium in shake flasks (250 r.p.m.) at 28 °C for 7 days. Cells were collected by centrifugation, then washed with distilled water and freeze-dried. Phospholipids in cells were extracted and identified using the method of Minnikin *et al.*[22] Whole-cell sugar composition was analyzed according to the method of Lechevalier and Lechevalier.[23] For the analysis of the amino acids in peptidoglycan, the cell walls were prepared according to the procedures described by McKerrow *et al.*[24] and analyzed by a HPLC method using Agilent TC-$C_{18}$ Column (250 × 4.6 mm i.d. 5 μm) with a mobile phase consisting of acetonitrile: 0.05 mol $l^{-1}$ phosphate buffer pH 7.2 (15:85, v/v) at a flow rate of 0.5 ml $min^{-1}$. Peak detection was performed by using an Agilent G1321A fluorescence detector (Agilent Technologies, Santa Clara, CA, USA) with a 365 nm excitation and 455 nm longpass emission filters.[24] Menaquinones were extracted from freeze-dried biomass and purified according to Collins.[25] The extracts were analyzed by a HPLC – UV method using an Agilent Extend-$C_{18}$ Column (150 × 4.6 mm, i.d. 5 μm), typically at 270 nm. The mobile phase was acetonitrile: 2-propanol (60:40, v/v) and the flow rate was set to 1.0 ml $min^{-1}$ and the run time was 60 min. The injection volume was 20 μl, and the chromatographic column was controlled at 40 °C.[20] Mycolic acids were checked by the acid methanolysis method as described previously.[26] Biomass for fatty acids analysis was obtained in Trypticase Soy Broth at 28 °C for 14 days and detected by GC–MS using the method of Gao *et al.*[20]

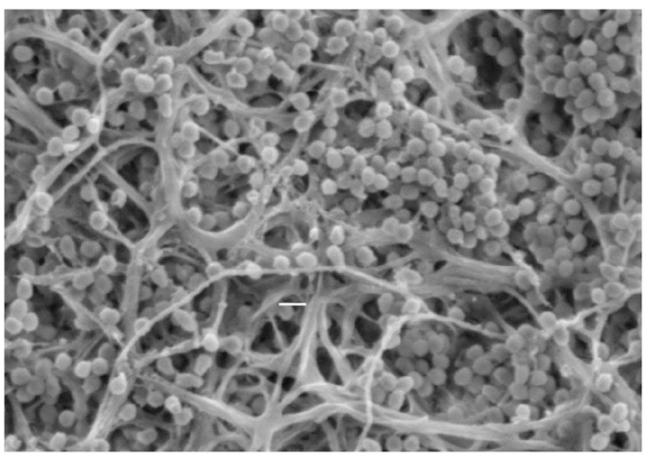

**Figure 1** Scanning electron micrograph of strain NEAU-gq11$^T$ grown on ISP 3 agar at 28 °C for 3 weeks. Bar, 1 μm.

### Phylogenetic analysis

Genomic DNA of strain NEAU-gq11$^T$ was extracted as described previously by Lee *et al.*[27] and PCR amplification of 16S rRNA gene was carried out using the method of Loqman *et al.*[28] The PCR product was purified and cloned into the pMD19-T vector (Takara, Dalian, China) and sequenced using an Applied Biosystems DNA sequencer (model 3730XL, Shanghai, China) and software provide by the manufacturer. Almost full-length 16S rRNA gene sequence (1473 nt) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using Clustal X 1.83. The alignment was manually verified and adjusted before the construction of phylogenetic trees. Phylogenetic trees were generated with the neighbor-joining,[29] maximum-parsimony[30] and maximum-likelihood[31] algorithms using Molecular Evolutionary Genetics Analysis software version 5.05.[32] The stability of the clades in the trees was appraised by bootstrap analysis with 1000 replicates.[33] A distance matrix was generated using the Kimura's two-parameter model.[34] All positions containing gaps and missing data were eliminated from the data set (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated on the basis of pairwise alignment using the EzTaxon-e server.[35] PCR amplification of the *gyrB* gene was carried out using primers GYF1 and GYR3B.[36] Sequencing and phylogenetic analysis was performed as described above. The G+C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel and Marmur,[37] with *Escherichia coli* JM109 as the reference strain. DNA–DNA hybridization was carried out between strains NEAU-gq11$^T$, *M. saelicesensis* Lupac 09$^T$, *M. chokoriensis* 2–19/6$^T$, '*M. zeae*' NEAU-gq9$^T$, '*M. jinlongensis*' NEAU-GRX11$^T$, *M. violae* NEAU-zh8$^T$, *M. zamorensis* CR38$^T$, *M. taraxaci* NEAU-P5$^T$ and *M. lupini* Lupac 14N$^T$ in a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian) as described previously.[38,39] The DNA concentration was adjusted spectrophotometrically at 260 nm to around 1 using 0.1 × saline sodium citrate. The renaturation rates of sheared DNA were determined at 70 °C.

## RESULTS AND DISCUSSION

### Morphological and physiological characteristics

Morphological observation of a 21-day-old culture of strain NEAU-gq11$^T$ grown on ISP 3 agar revealed it had the typical characteristics of genus *Micromonospora*. Substrate mycelium was well-developed without fragmentation. Spores (0.5 × 0.7 μm) were borne singly on the substrate mycelia and the spore surface was rough (Figure 1). Good growth was observed on N-Z amine, Bennett's, SA 1, ISP 2, ISP 3 and ISP 4 agar and moderate growth was observed on ISP 5, ISP 6 and ISP 7 agar. Aerial mycelia were not detected on any of the tested media. The color of colonies was grayish yellowish brown on N-Z amine agar, dark grayish yellowish brown on Bennett's agar and SA 1 agar, brilliant orange yellow on ISP 2 agar, dark reddish orange on ISP 3 agar, deep orange yellow on ISP 4 agar and ISP 5 agar, grayish yellow on ISP 6 agar and moderate yellow on ISP 7 agar. No diffusible pigment was detected. Growth of strain NEAU-gq11$^T$ occurred in the pH range 6–12 and 0–3% NaCl (w/v), with optimum growth at pH 7.0 and 0% NaCl (w/v). The temperature range for growth was 10–37 °C, with the optimum temperature being 28 °C. Detailed physiological and biochemical properties are presented in the species description.

### Chemotaxonomic characteristics

Chemotaxonomic characteristics of strain NEAU-gq11$^T$ also supported its classification as a member of the genus *Micromonospora*. Cells of strain NEAU-gq11$^T$ were observed to contain *meso*-diaminopimelic acid and glycine as diagnostic amino acids.

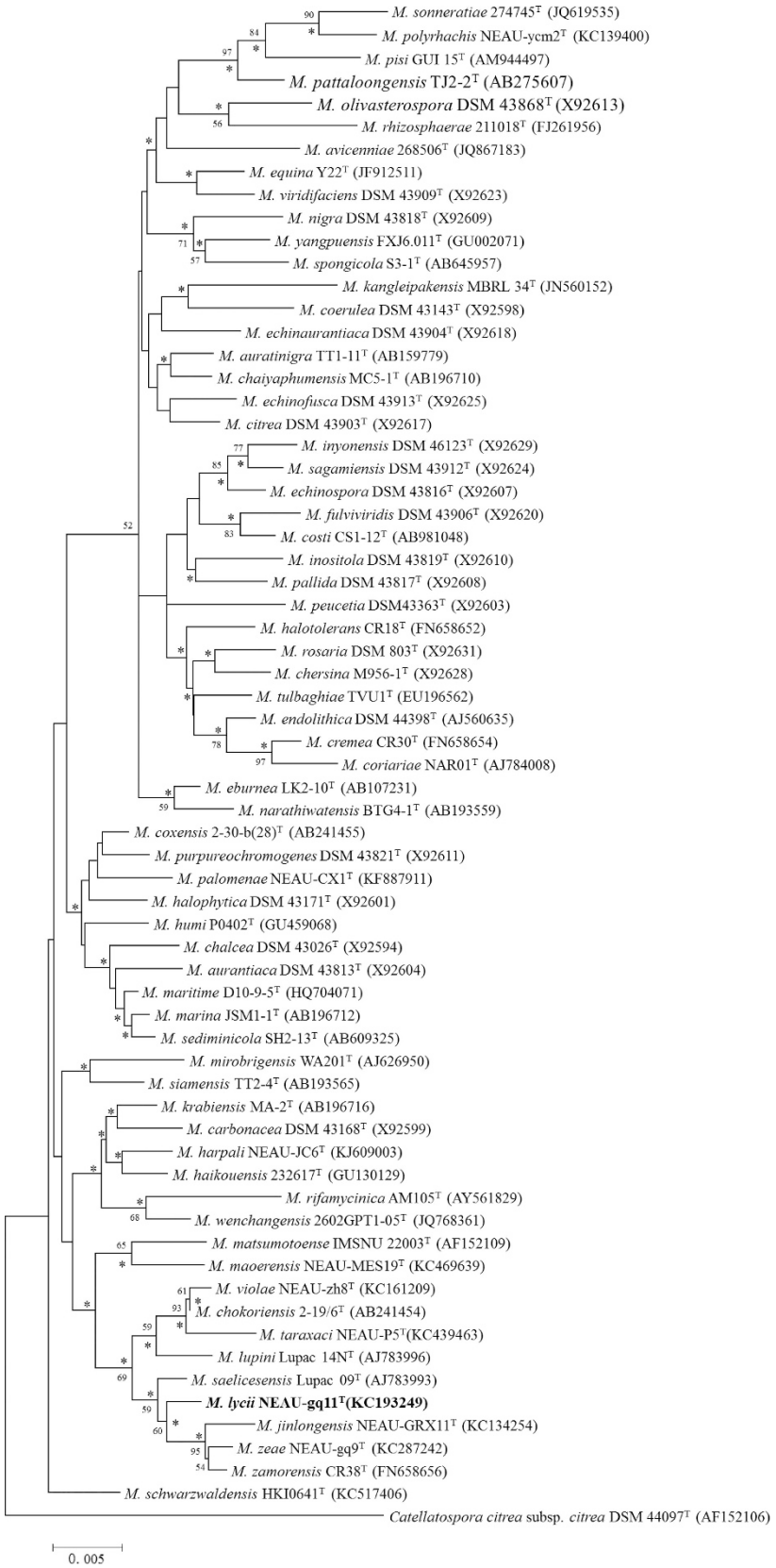


**Figure 2** Neighbour-joining tree based on 16S rRNA gene sequence (1354 nt) showing relationship between strain NEAU-gq11$^T$ and members of the genus *Micromonospora*. The out-group used was *Catellatospora citrea* subsp. *citrea* DSM 44097$^T$. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. Asterisks indicate branches also recovered in the maximum-parsimony and maximum-likelihood trees. Bar, 0.005 nucleotide substitutions per site.

**Table 1 Differential characteristics of strain NEAU-gq11$^T$ and the closely related species**

| *Characteristics* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* |
|---|---|---|---|---|---|---|---|---|---|
| Spore ornamentation | Rough | Smooth[a] | Smooth | Rough to nodular[b] | Smooth | Smooth | Smooth[c] | Smooth | Smooth[a] |
| Maximum NaCl tolerance (%, w/v) | 3 | 3 | 3 | 2 | 3 | 2 | 3 | 2 | 3 |
| Cellulose decomposition | + | + | − | + | − | − | − | + | + |
| Production of urease | + | + | − | + | − | + | + | + | + |
| Hydrolysis of starch | − | − | − | − | + | − | − | + | − |
| Liquefaction of gelatin | + | + | + | + | + | + | + | + | − |
| *Use as sole carbon source:* | | | | | | | | | |
| D-maltose | + | − | + | + | + | + | − | + | + |
| D-mannose | + | + | + | + | − | + | − | + | + |
| D-sorbitol | − | − | − | − | + | − | − | + | − |
| D-fructose | + | + | + | + | − | + | − | − | + |
| Inositol | + | − | − | − | + | − | − | − | − |
| L-rhamnose | + | + | + | + | + | − | + | − | + |
| D-mannitol | + | − | + | + | + | − | − | + | − |
| D-ribose | + | − | − | − | − | + | − | + | − |
| *Use as sole nitrogen source:* | | | | | | | | | |
| Creatine | + | + | + | + | − | − | + | + | + |
| L-tyrosine | + | + | + | + | − | + | + | − | + |
| L-threonine | + | + | + | − | + | − | + | + | + |
| L-glutamic acid | + | + | − | − | + | + | − | + | − |
| L-glutamine | + | + | + | + | + | + | + | − | + |

Strains: 1, NEAU-gq11$^T$; 2, *M. saelicesensis* Lupac 09$^T$; 3, *M. zeae* NEAU-gq9$^T$; 4, *M. chokoriensis* 2–19/6$^T$; 5, *M. jinlongensis* NEAU-GRX11$^T$; 6, *M. violae* NEAU-zh8$^T$; 7, *M. zamorensis* CR38$^T$; 8, *M. taraxaci* NEAU-P5$^T$; 9, *M. lupini* Lupac 14N$^T$. (+), positive; (–), negative. Data are from this study except where marked.
[a]Data from Trujillo *et al.*[12]
[b]Data from Ara and Kudo.[44]
[c]Data from Carro *et al.*[41]

Whole-cell hydrolysates were found to contain rhamnose, xylose and glucose. The menaquinones detected were MK-9($H_8$) (41.2%), MK-10 ($H_6$) (34.8%), MK-10($H_2$) (13.6%), MK-10($H_4$) (6.5%) and MK-9 ($H_4$) (3.9%). The phopholipid profile was found to consist of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol (Supplementary Figure S1). The major cellular fatty acid was identified as iso-$C_{16:0}$ (45.15%) (Supplementary Table S1). Mycolic acids were not detected. The G+C content of the DNA was 71.1 ± 0.25 mol%.

**Phylogenetic characteristics**

The almost complete 16S rRNA gene sequence (1473 nt) of strain NEAU-gq11$^T$ was determined and deposited as KC193249 in the GenBank/EMBL/DDBJ databases. Comparative 16S rRNA gene sequence analysis showed that strain NEAU-gq11$^T$ was phylogenetically related to members of the genus *Micromonospora*. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain NEAU-gq11$^T$ clustered with *M. saelicesensis* Lupac 09$^T$ (99.4%), '*M. zeae*' NEAU-gq9$^T$ (99.3%), *M. chokoriensis* 2–19/6$^T$ (99.3%), '*M. jinlongensis*' NEAU-GRX11$^T$ (99.1%), *M. violae* NEAU-zh8$^T$ (99.1%), *M. zamorensis* CR38$^T$ (99.0%), *M. taraxaci* NEAU-P5$^T$ (98.9%) and *M. lupini* Lupac 14N$^T$ (98.6%), which was supported by a bootstrap value of 69% in the neighbour-joining tree (Figure 2) and also recovered with the maximum-parsimony and maximum-likelihood algorithms (Supplementary Figures S2 and S3). Partial sequence of *gyrB* gene was obtained (1162 nt) and deposited as KJ609000 in the GenBank/EMBL/DDBJ databases. The similarities of *gyrB* nucleotide sequence between strains NEAU-gq11$^T$, *M. saelicesensis* Lupac 09$^T$, '*M. zeae*' NEAU-gq9$^T$, *M. chokoriensis* 2–19/6$^T$, '*M. jinlongensis*' NEAU-GRX11$^T$, *M. violae* NEAU-zh8$^T$, *M. zamorensis* CR38$^T$, *M. taraxaci* NEAU-P5$^T$ and *M. lupini* Lupac 14N$^T$ were 97.1, 96.0, 94.9, 95.7, 92.3, 95.4 and 94.2%, respectively. Phylogenetic analysis of *gyrB* nucleotide sequence supported that strain NEAU-gq11$^T$ was placed in the genus *Micromonospora*, near to *M. saelicesensis* Lupac 09$^T$, '*M. zeae*' NEAU-gq9$^T$, *M. chokoriensis* 2–19/6$^T$, '*M. jinlongensis*' NEAU-GRX11$^T$, *M. zamorensis* CR38$^T$, *M. taraxaci* NEAU-P5$^T$ and *M. lupini* Lupac 14N$^T$ by a 68% bootstrap value but far away from *M. violae* NEAU-zh8$^T$ (Supplementary Figure S4). Other studies have also reported similar results that the topology of phylogenetic trees based on *gyrB* gene sequences differed from those based on 16S rRNA gene sequence data.[40–42]

DNA–DNA hybridization was employed to further clarify the relatedness between strains NEAU-gq11$^T$, *M. saelicesensis* Lupac 09$^T$, '*M. zeae*' NEAU-gq9$^T$, *M. chokoriensis* 2–19/6$^T$, '*M. jinlongensis*' NEAU-GRX11$^T$, *M. violae* NEAU-zh8$^T$, *M. zamorensis* CR38$^T$, *M. taraxaci* NEAU-P5$^T$ and *M. lupini* Lupac 14N$^T$; the levels of DNA–DNA relatedness between them were 65.4 ± 1.4, 64.0 ± 2.7, 43.5 ± 1.3, 57.5 ± 2.4, 57.4 ± 1.2, 62.4 ± 1.8, 45.9 ± 2.4 and 41.3 ± 2.0%, respectively. These values were below the threshold value of 70% recommended by Wayne *et al.*[43] for assigning strains to the same genomic species.

Besides the genotypic evidence above, the strain could also be distinguished from the seven related *Micromonospora* type strains by morphological, physiological and biochemical characteristics as summarized in Table 1, such as the clearly different colony colors on Bennett's, SA 1, ISP 2 and ISP 3 media after being incubated at 28 °C for 3 weeks (Supplementary Figure S5) and the differences in production of urease, hydrolysis of starch, decomposition of cellulose and patterns of carbon and nitrogen utilization (Table 1). In addition, the fatty acid composition of strain NEAU-gq11$^T$ was similar to those of their closely related species such as the presence of anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:0}$, $C_{17:1}$ ω7c and $C_{18:0}$, but differed based on the presence

or proportions of 10-methyl $C_{16:0}$, $C_{17:1}$ ω5c, iso-$C_{18:0}$, $C_{18:1}$ ω5c and $C_{18:1}$ ω7c (Supplementary Table S1). Therefore, strain NEAU-gq11$^T$ represents a novel species of the genus *Micromonospora*, for which the name *Micromonospora lycii* sp. nov. is proposed. It is quite interesting that several *Micromonospora* species isolated from different endophytic root origins are clustering together after 16S rRNA and *gyrB* gene sequencing and worthy of further research.

**Description of *M. lycii* sp. nov.**

*M. lycii* (ly'ci.i. L. n. Lycium, name of a plant, and also a botanical generic name (Lycium); L. gen. n. lycii of Lycium, referring to the isolation of the organism from *L. chinense* Mill).

Gram staining positive and aerobic actinomycete that forms well-developed and branched substrate mycelium but no aerial hypha. Good growth is observed on N-Z amine, Bennett's, SA 1, ISP 2, ISP 3 and ISP 4 agar; moderate growth is observed on ISP 5, ISP 6 and ISP 7 agar. Colonies are orange yellow color series. No diffusible pigment is detected on any of the tested media. Spores ($0.5 \times 0.7$ μm) are single, oval with rough surface. Growth occurs at pH values between 6 and 12, the optimum being pH 7.0. Tolerates up to 3% NaCl and grows at temperatures between 10 and 37 °C, with an optimum temperature of 28 °C. Positive for liquefaction of gelatin, hydrolysis of aesculin, cellulose and Tween 40, production of catalase and urease and negative for reduction of nitrate, coagulation of milk, hydrolysis of starch, Tweens (20 and 80) and production of $H_2S$. L-arabinose, D-fructose, D-galactose, D-glucose, inositol, lactose, D-maltose, D-mannitol, D-mannose, L-rhamnose, D-raffinose, D-ribose, D-sucrose and D-xylose are used as sole carbon sources but D-sorbitol is not. L-alanine, L-arginine, L-asparagine, L-aspartic acid, creatine, L-glutamine, L-glutamic acid, L-serine, L-threonine and L-tyrosine are used as sole nitrogen sources but glycine is not. Cell wall contains *meso*-diaminopimelic acid and glycine, and the characteristic whole-cell sugars are rhamnose, xylose and glucose. The phopholipids include diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The major menaquinones are MK-9($H_8$), MK-10($H_6$) and MK-10($H_2$). The major cellular fatty acid is iso-$C_{16:0}$. Mycolic acids are absent. The G+C content of the DNA is $71.1 \pm 0.25$ mol%. The type strain, NEAU-gq11$^T$ ( = CGMCC 4.7149 $^T$ = DSM 46703 $^T$), was isolated from a root of wolfberry (*L. chinense* Mill) collected from Harbin, Northeast China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* gene sequences of strain NEAU-gq11$^T$ are KC193249 and KJ609000, respectively.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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Quotation marks indicate these strains are invalidly described species.

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