

Streptomyces castaneus sp. nov., a novel actinomycete isolated from the rhizosphere of *Peucedanum praeruptorum* Dunn

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Abstract During an investigation of microbial diversity in medicinal herbs, a novel actinomycete, strain NEAU-QHHV11^T was isolated from the rhizosphere of *Peucedanum praeruptorum* Dunn collected from Xianglu Mountain in Heilongjiang Province, northeast China and characterized using a polyphasic approach. The organism was found to have typical characteristics of the genus *Streptomyces*. Phylogenetic analysis based on 16S rRNA gene sequence also indicated that strain NEAU-QHHV11^T belongs to the genus *Streptomyces* and was most closely related to *Streptomyces graminilatus* NBRC 108882^T (98.7 % sequence similarity) and *Streptomyces turgidiscabies* NBRC 16080^T (98.7 % sequence similarity). The results of DNA–DNA hybridization and some phenotypic characteristics indicated that strain NEAU-QHHV11^T could be distinguished from its close phylogenetic relatives.

Thus, strain NEAU-QHHV11^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces castaneus* sp. nov. is proposed. The type strain is NEAU-QHHV11^T (=CGMCC 4.7235^T = DSM 100520^T).

Keywords *Streptomyces castaneus* sp. nov. · *Peucedanum praeruptorum* Dunn · Polyphasic taxonomy · 16S rRNA gene

Introduction

The genus *Streptomyces* was first described by Waksman and Henrici (1943) and now consists of more than 700 species with validly published names (<http://www.bacterio.net/streptomyces.html>). Members of the genus are aerobic, Gram-positive bacteria which form an extensively branched substrate mycelium with aerial hyphae which differentiate into chains of spores and DNA with high G+C content (Lechevalier and Lechevalier 1970; Kämpfer 2012; Manfio et al. 1995). Indeed, *Streptomyces* is the largest genus of the phylum *Actinobacteria* (Kämpfer and Labeda 2006; Lidders and Kämpfer 2007) and constitutes the most predominant component of micro-organisms found in soils, sediments and decaying vegetation (Labeda et al. 2012). *Peucedanum praeruptorum* Dunn is one of the most popular traditional medicinal herbs which has been widely used in traditional Chinese medicine for over 1500 years and officially listed in the Chinese Pharmacopoeia (Liu et al. 2004). As part of a programme to discover actinomycetes with novel antibiotic production properties present in medicinal plants, strain NEAU-QHHV11^T was isolated from the rhizosphere of *P. praeruptorum* Dunn. In this study, we performed a polyphasic taxonomy study on this strain and propose a novel species of the genus *Streptomyces*, *Streptomyces castaneus* sp. nov.

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Materials and methods

Isolation and maintenance of the organism

Strain NEAU-QHHV11^T was isolated from the rhizosphere soil of *P. praeruptorum* Dunn collected from Xian-glu Mountain in Heilongjiang Province, northeast China (45°45′N127°29′E) in June 2014. The strain was isolated using the standard dilution plate method and grown on humic acid–vitamin agar (Hayakawa and Nonomura 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After 21 days of aerobic incubation at 28 °C, the isolate was transferred and purified on the International *Streptomyces* Project (ISP) medium 3 (Shirling and Gottlieb 1966) and maintained as glycerol suspensions (20 %, v/v) at -80 °C. The type strains of *Streptomyces graminilatus* NBRC 108882^T and *Streptomyces turgidiscabies* NBRC 16080^T were purchased from the NITE Biological Resource Center (NBRC) and cultured under the same conditions for comparative analysis.

Morphological, cultural and physiological characteristics

Morphological characteristics were observed by light microscopy (Nikon ECLIPSE E200) and electron microscopy (Hitachi S-3400N) using cultures grown on ISP3 medium at 28 °C for 21 days. Samples for scanning electron microscopy were prepared using the method of Guan et al. (2015). Cultural characteristics were determined on ISP media 2–7 (Shirling and Gottlieb 1966) after 14 days at 28 °C. The ISCC–NBS colour charts were used to determine the designations of colony colours (Kelly 1964). Growth at different temperatures (4, 10, 15, 20, 28, 32, 37, 40 and 45 °C) was determined on ISP3 medium after incubation for 14 days. Growth tests for pH range (pH 3–12) and NaCl tolerance (0, 1, 2, 3, 4, 5, 6 and 7 % NaCl, w/v) were tested in ISP2 medium at 28 °C for 14 days on a rotary shaker. Production of catalase, esterase and urease was tested as described by Smibert and Krieg (1994). The utilization of sole carbon and nitrogen sources, decomposition of urea and cellulose, hydrolysis of starch and aesculin, reduction of nitrate, coagulation and peptonization of milk, liquefaction of gelatin and production of H₂S were examined as described previously (Gordon et al. 1974; Yokota et al. 1993).

Chemotaxonomic characterization

Biomass for chemical studies was prepared by growing strain NEAU-QHHV11^T in tryptic soy broth (TSB; Lee and Whang 2014) in shake flasks at 28 °C for 7 days. Cells were harvested by centrifugation, then washed with distilled water and freeze-dried. The isomer of diaminopimelic

acid (DAP) in the cell wall hydrolysates was derivatized and analysed by a HPLC method (McKerrow et al. 2000) using an Agilent TC-C18 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. An Agilent G1321A fluorescence detector was used for peak detection with 365 nm excitation and 455 nm long pass emission filters. Whole-cell sugars were analysed according to the procedures developed by Lechevalier and Lechevalier (1980). Polar lipids were examined by two-dimensional TLC and identified using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985). Extracts were analysed by a HPLC–UV method (Wu et al. 1989) using an Agilent Extend-C18 Column (150 × 4.6 mm, i.d. 5 µm), typically at 270 nm. The mobile phase was acetonitrile–propyl alcohol (60:40, v/v). The presence of mycolic acids was checked by the acid methanolysis method as described previously (Minnikin et al. 1980). To determine cellular fatty acid compositions, strain NEAU-QHHV11^T was cultivated in TSB medium in shake flasks at 28 °C for 7 days. Fatty acid methyl esters were extracted from the biomass as described by Gao et al. (2014) and were analysed by GC–MS using the method of Xiang et al. (2011).

DNA preparation, amplification and determination of 16S rRNA sequence

Biomass for molecular biological studies was prepared by growing the organism in TSB medium in shake flasks at 28 °C for 7 days. Extraction of chromosomal DNA and PCR amplification of the 16S rRNA gene sequence were carried out according to the procedure developed by Kim et al. (2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced using an Applied Biosystems DNA sequencer (model 3730XL). An almost full-length 16S rRNA gene sequence of strain NEAU-QHHV11^T (1517 bp) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using Clustal X1.83 software (Thompson et al. 1997). Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987) and maximum likelihood (Felsenstein 1981) algorithms using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.06 (Tamura et al. 2013). The stability of the topology of the phylogenetic tree was assessed using the bootstrap method with 1000 repetitions (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated

on the basis of pairwise alignments using the EzTaxon-e server (Kim et al. 2012).

DNA base composition and DNA–DNA hybridization

The G+C content of the genomic DNA was determined by the thermal denaturation (T_m) method (Mandel and Marmur 1968) with *Escherichia coli* JM109 DNA as the control. The DNA–DNA relatedness tests between strain NEAU-QHHV11^T and *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T were carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983), using 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). The concentration and purity of DNA samples were determined by measuring the optical density at 260, 280 and 230 nm. The DNA samples used for hybridization were diluted to OD₂₆₀ around 1.0 using 0.1 × SSC (saline sodium citrate buffer) and then broken using a JY92-II ultrasonic cell disruptor (ultrasonic time 3 s, interval time 4 s, 90 times). The DNA renaturation rates were determined in 2 × SSC at 70 °C. The experiments were performed with three replications, and the DNA–DNA relatedness value was expressed as mean of the three values.

Results and discussion

EzTaxon-e analysis of the 16S rRNA gene sequence (1517 bp) revealed that strain NEAU-QHHV11^T belongs to the genus *Streptomyces*, with the highest 16S rRNA gene sequence similarity to *S. graminilatus* NBRC 108882^T (98.7 %) and *S. turgidiscabies* NBRC 16080^T (98.7 %). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain NEAU-QHHV11^T formed a separate branch with *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T in the neighbour-joining tree (Fig. 1) and a similar topology was also recovered with the maximum likelihood algorithm (Fig. S4). DNA–DNA hybridization was employed to further clarify the relatedness between strain NEAU-QHHV11^T and *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T. The levels of DNA–DNA relatedness between them were 49.7 ± 0.5 and 39.1 ± 0.9 %, respectively. These values are below the threshold value of 70 % recommended by Wayne et al. (1987) for assigning strains to the same genomic species. The G+C content of the genomic DNA of strain NEAU-QHHV11^T was 71.5 ± 0.3 mol %.

The morphological characteristics observation of a 3-week-old culture of strain NEAU-QHHV11^T grown on

ISP3 medium revealed that it had the typical characteristics of the genus *Streptomyces*. Aerial mycelium and substrate mycelium were well developed without fragmentation. Long spore chains were observed, and the spores were found to be cylindrical, smooth and non-motile (Fig. S1). Cultural characteristics of strain NEAU-QHHV11^T are shown in Table S1. Good growth was observed on ISP2, ISP3, ISP4 and ISP7 media; moderate growth was observed on ISP5 and ISP6 media. The colour of the colonies varied from yellowish white to deep purplish red. Purplish red soluble pigments were observed on ISP3 medium (Fig. S2), which could apparently distinguish the isolate from *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T. Melanoid pigments were not observed on ISP6 or ISP7 media. Growth of strain NEAU-QHHV11^T was observed at 10–32 °C (optimum 28 °C), pH range 5–11 (optimum pH 7) and in the presence of 0–4 % NaCl (w/v). Hydrolysis of starch and Tween 20 were positive, which could also differentiate strain NEAU-QHHV11^T from its closely related strains. Other physiological and biochemical characteristics of strain NEAU-QHHV11^T compared with *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T are listed in Table 1.

Strain NEAU-QHHV11^T contained LL-diaminopimelic acid as the cell wall diamino acid, ribose and glucose as whole-cell sugars and MK-9(H₈) (51.6 %) and MK-9(H₁₀) (38.6 %) as the predominant menaquinones, with smaller amounts of MK-9(H₆) (6.7 %), MK-9(H₉) (1.8 %) and MK-9(H₂) (1.3 %). The polar lipids were found to be diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and an unidentified phospholipid (Supplementary Fig. S3). The major cellular fatty acids were determined to be composed of iso-C_{16:0} (27.8 %), C_{17:0} cyclo (14.0 %), anteiso-C_{15:0} (9.2 %), iso-C_{15:0} (8.9 %), anteiso-C_{17:0} (7.7 %), C_{16:0} (5.9 %) and C_{16:1}ω5c (5.1 %), and minor amounts of C_{16:1}ω7c (4.7 %), iso-C_{17:0} (4.2 %), C_{18:1}ω2c (3.0 %), iso-C_{14:0} (2.4 %), C_{15:0} (1.9 %), C_{18:0} (1.9 %) and C_{17:1}ω9c (1.5 %) were also present. Strain NEAU-QHHV11^T contained major amounts of iso- and anteiso-fatty acids, which are in accord with the fatty acid composition of *Streptomyces* spp. However, the presence of C_{17:0} cyclo as major fatty acids is rare in the genus *Streptomyces* (Kämpfer 2012).

In conclusion, it is evident from the genotypic and phenotypic data that strain NEAU-QHHV11^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces castaneus* sp. nov. is proposed.

Description of *Streptomyces castaneus* sp. nov

Streptomyces castaneus (cas.ta'ne.us. L. masc. adj. castaneus, chestnut coloured).

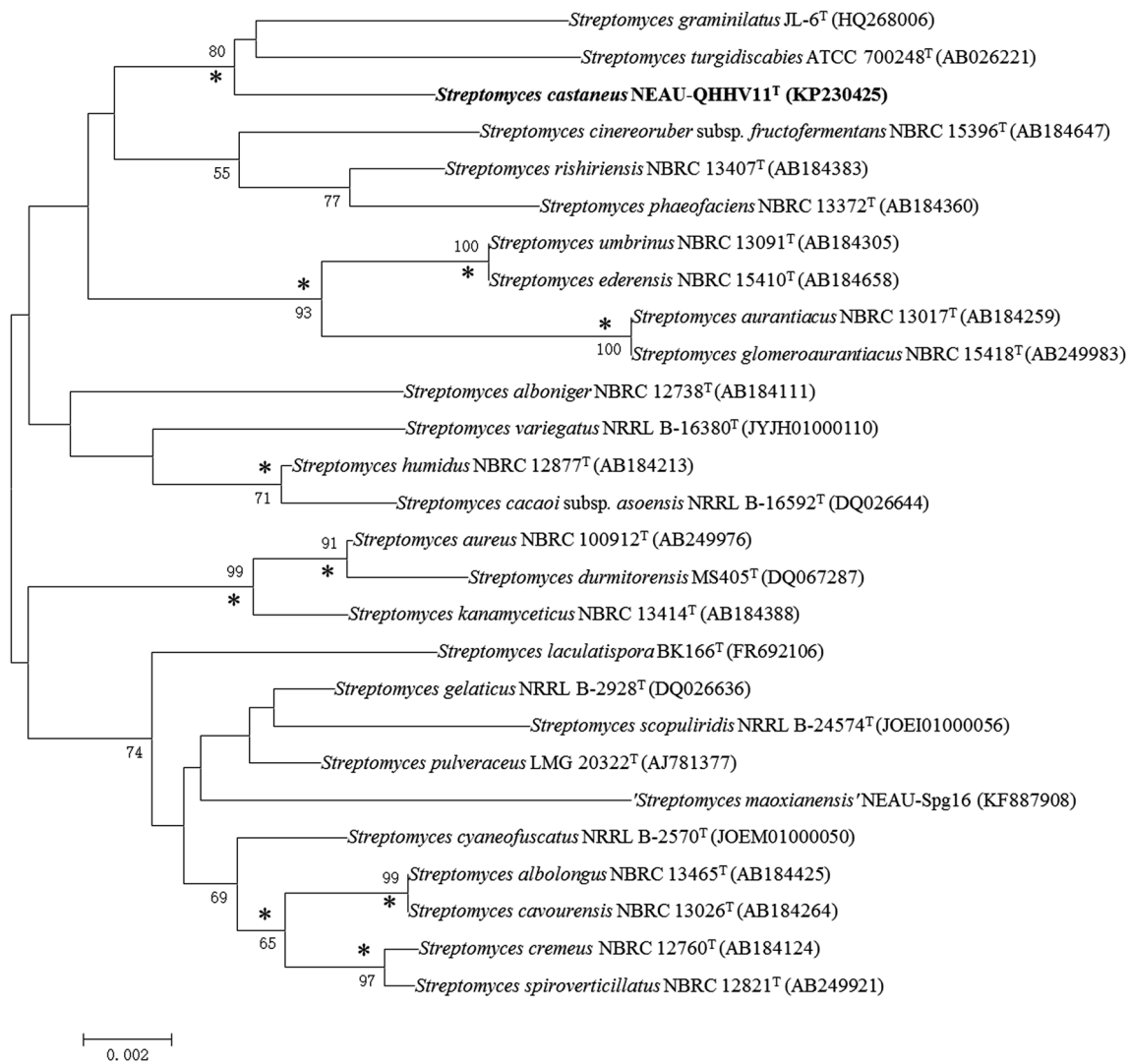


Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences (1421 bp, by omitting unaligned regions) showing the relationship between strain NEAU-QHHV11^T and related taxa. Asterisks indicate branches of the tree that were also recovered using the maximum

likelihood method. Bootstrap values >50 % (based on 1000 replications) are shown at branch points. Bar, 0.002 substitutions per nucleotide position

Aerobic, Gram-staining positive, non-motile actinobacteria that forms well-developed substrate mycelium and aerial hyphae. Long spore chains appear as straight, and spores are smooth, cylindrical and non-motile. Grows well on ISP 2, ISP 3, ISP 4 and ISP 7 media, moderately on ISP 5 and ISP 6 media. Purplish red soluble pigment is observed on ISP3 medium and grows at 15–32 °C, pH range 5–11 and in the presence of 0–4 % NaCl (w/v) with optimum at 28 °C and pH 7. Positive for liquefaction of gelatin, coagulation and peptonization of milk, production of esterase, decomposition of urea, hydrolysis of starch and aesculin and negative for decomposition of cellulose, reduction of nitrate and production of H₂S. D-Galactose, D-glucose, *meso*-inositol, lactose, D-maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-sorbitol and

D-sucrose are utilized as sole carbon sources, while L-arabinose, D-fructose, D-ribose and D-xylose are not metabolized. L-Alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-proline, L-serine and L-threonine are utilized as sole nitrogen sources, while creatine and L-tyrosine are not metabolized. The cell wall contains LL-diaminopimelic acid as diagnostic diamino acid, and the whole-cell hydrolysates contain ribose and glucose. The major menaquinones are MK-9(H₈) and MK-9(H₁₀). The polar lipids profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and an unidentified phospholipid. The major fatty acids are C_{16:0}, C_{17:0} cyclo, anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:0} and C_{16:1}ω5c (>5 %). The DNA G+C content of the type strain is 71.5 mol %.

Table 1 Differential characteristics of strains NEAU-QHHV11^T, *S. graminilatus* NBRC 108882^T and *S. turgidiscabies* NBRC 16080^T

Characteristics	NEAU-QHHV11 ^T	<i>S. graminilatus</i> NBRC 108882 ^T	<i>S. turgidiscabies</i> NBRC 16080 ^T
Hydrolysis of tween 20	+	–	–
Hydrolysis of starch	+	–	–(+) ^b
Utilization of			
D-Galactose	+	–	+
Lactose	+	–	–(+) ^b
D-Maltose	+	–	–(+) ^b
L-Rhamnose	+	–(+) ^a	+
D-Sorbitol	+	+	–

Conflicting results from other studies are indicated in parentheses

Data are from this study except where marked. ^aData from Lee and Whang (2014); ^bData from Miyajima et al. (1998)

+, positive; –, negative

The type strain is NEAU-QHHV11^T (=CGMCC 4.7235^T = DSM 100520^T), which was isolated from the rhizosphere soil of *Peucedanum praeruptorum* Dunn collected from Xianglu Mountain in Heilongjiang Province, northeast China. The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain NEAU-QHHV11^T is KP230425.

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