Actinoplanes lutulentus sp. nov., isolated from mucky soil in China

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A novel actinomycete, designated strain NEAU-GRX6^T, was isolated from mucky soil collected from a stream of Jinlong Mountain in Harbin, Heilongjiang Province, north China, and characterized using a polyphasic approach. The isolate formed irregular sporangia containing motile sporangiospores on the substrate mycelium. The whole-cell sugars were xylose, glucose and galactose. The predominant menaquinones were MK-9(H_6), MK-10(H_4) and MK-9(H_4). The major fatty acids were $C_{16:0}$, $C_{15:0}$, $C_{18:1}\omega9c$, $C_{17:1}\omega7c$ and $C_{18:0}$. The phospholipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. The DNA G+C content was 67 mol%. 16S rRNA gene sequence similarity studies showed that strain NEAU-GRX6^T belonged to the genus *Actinoplanes*, being most closely related to *Actinoplanes palleronii* IFO 14916^T (97.80 % similarity) and *Actinoplanes missouriensis* NBRC 102363^T (97.76 %). However, the low observed levels of DNA–DNA relatedness allowed the isolate to be differentiated from the above-mentioned species of the genus *Actinoplanes*. Moreover, strain NEAU-GRX6^T could also be distinguished from *A. palleronii* IFO 14916^T and *A. missouriensis* NBRC 102363^T by phenotypic characteristics. Therefore, it is proposed that strain NEAU-GRX6^T represents a novel species of the genus *Actinoplanes*, for which the name *Actinoplanes lutulentus* sp. nov. is proposed. The type strain is strain NEAU-GRX6^T (=CGMCC 4.7090^T=DSM 45883^T).

The genus *Actinoplanes*, belonging to the family *Micromonosporaceae*, was described by Couch (1950). *Actinoplanes philippinensis* is the type species. Members of the genus develop spherical, cylindrical, digitate, lobate, bottle- or flask-shaped or very irregular sporangia that contain motile sporangiospores with tufts of polar flagella at the ends of sporangiophores on the substrate mycelium. The first detailed phenotypic and chemotaxonomic analysis of the genus was provided by Goodfellow *et al.* (1990), who determined the chemotaxonomic and phenotypic characteristics of species of the genus *Actinoplanes* and reported that chemical and numerical taxonomic data supported the integrity of the genus. A comprehensive phylogenetic analysis of the genus has been given by Tamura & Hatano (2001). At the time of writing, the genus *Actinoplanes* comprised 36 species, including recently described '*Actinoplanes hulinensis*' (Shen *et al.*, 2013) and *Actinoplanes siamensis* (Suriyachadkun *et al.*, 2013). During the investigation of potential sources of novel species and novel natural products, strain NEAU-GRX6^T was isolated from mucky soil collected from a stream of Jinlong Mountain in Harbin, Heilongjiang Province, north China. In this study, the taxonomic status of this strain is reported based on phylogenetic, chemotaxonomic and phenotypic evidence. It is proposed that strain NEAU-GRX6^T should be classified as representing a novel species of the genus *Actinoplanes*.

Strain NEAU-GRX6^T was isolated from mucky soil collected from a stream of Jinlong Mountain in Harbin, Heilongjiang Province, north China (45° 30′ N 127° 06′ E). Mucky soils are mineral–organic or mineral soils that contain less than 20 % organic matter (mucky material), or that have a horizon with over 20 % organic matter (muck) but less than 30 cm thick. The strain was isolated using the standard dilution plating method and grown on humic acid-vitamin (HV) agar (Hayakawa & 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, colonies

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Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NEAU-GRX6^T is KC134255.

Two supplementary figures and a supplementary table are available with the online version of this paper.

were transferred and purified on oatmeal agar [International *Streptomyces* Project (ISP) 3 medium] (Shirling & Gottlieb, 1966) and maintained as glycerol suspensions (20 %, v/v) at −80 °C.

Morphology of the sporangia was observed by scanning electron microscopy (Hitachi S-3400N) using cultures grown on ISP3 agar for 21 days. Spore motility was assessed by light microscopic observation (Nikon ECLIPSE E200) of cells suspended in phosphate buffer (pH 7.0, 1 mM). Cultural characteristics were determined by growth on tap-water agar (Gordon *et al.*, 1974), M8 agar (Castiglione *et al.*, 2008), sucrose-nitrate agar (Waksman medium 1), yeast extract-starch agar (JCM medium 61), modified Bennett's (MB) agar (Jones, 1949), Czapek's agar (Waksman, 1967) and ISP media 2–7 (Shirling & Gottlieb, 1966) at 28 °C for 14 days. The ISCC-NBS colour charts (Kelly, 1964) were used to determine the names and designations of colony colours. Growth at 4, 10, 15, 18, 22, 28, 30, 32, 37 and 40 °C was determined on ISP3 agar after incubation for 14 days. Growth at pH 4, 5, 6, 7, 8, 9 and 10 was assessed by using the buffer system described by Xie *et al.* (2012), and NaCl tolerance was determined in GY medium (Jia *et al.*, 2013) supplemented with 0–6 % (w/v) NaCl at 28 °C for 7 days on a rotary shaker. Production of catalase, esterase and urease was tested as described by Smibert & Krieg (1994). Utilization of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, peptonization of milk, liquefaction of gelatin and production of H_2S were examined as described previously (Gordon *et al.*, 1974; Yokota *et al.*, 1993).

Freeze-dried cells used for chemotaxonomic analysis were obtained from cultures grown in GY medium on a rotary shaker for 4 days at 28 °C. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid (DAP) in the peptidoglycan were derivatized according to McKerrow *et al.* (2000) and analysed by HPLC using an Agilent TC-C_{18} column (250 × 4.6 mm; i.d. 5 μm) with a mobile phase consisting of 0.05 mol phosphate buffer l^{-1} (0.2 M NaH_2PO_4/0.2 M Na_2HPO_4 at 28 : 72, v/v), pH 7.2/acetonitrile (85 : 15, v/v) at a flow rate of 0.5 ml min^{-1}. Peak detection used an Agilent G1321A fluorescence detector with a 365 nm excitation filter and 455 nm longpass emission filter. The *N*-acyl group of muramic acid in the peptidoglycan was determined by the method of Uchida *et al.* (1999). Whole-organism sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). Phospholipids in cells were extracted 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 an 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/propyl alcohol (60 : 40, v/v), the flow rate was 1.0 ml min^{-1} and the run time was 60 min. The injection volume was 20 μl, and the chromatographic column was maintained at 40 °C (Wu *et al.*, 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin *et al.*, 1980). Cellular fatty acids were analysed by GC-MS using the method of Xiang *et al.* (2011).

Extraction of chromosomal DNA and PCR-mediated amplification of the 16S rRNA gene were carried out using a standard procedure (Kim *et al.*, 2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced by using an Applied Biosystems DNA sequencer (model 3730XL) and software provided by the manufacturer. An almost full-length 16S rRNA gene sequence (1508 nt) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL_X 1.83 software. Phylogenetic trees were generated with the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms using MEGA software version 5.05 (Tamura *et al.*, 2011). The stability of clades in the trees was appraised using a bootstrap value with 1000 repeats (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps or missing data were eliminated from the dataset (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated on the basis of pairwise alignment using the EzTaxon-e server (Kim *et al.*, 2012). The G+C content of the genomic DNA was determined by the thermal denaturation (T_m) method as described by Mandel & Marmur (1968), and *Escherichia coli* JM109 (50.4 mol% G+C) was used as the reference strain. DNA–DNA relatedness tests between isolate NEAU-GRX6^T and *Actinoplanes palleronii* IFO 14916^T and *Actinoplanes missouriensis* NBRC 102363^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).

Morphological observation of a 21-day-old culture of strain NEAU-GRX6^T grown on ISP3 agar revealed that it had characteristics typical of the genus *Actinoplanes*. Strain NEAU-GRX6^T was observed to produce branched, non-fragmenting substrate hyphae which bore irregular sporangia (5.7–6.5 μm). The spore vesicles released motile spores (0.7 × 0.8 μm). The motile spores were oval and smooth-surfaced (Fig. 1). The novel isolate showed good growth on M8, MB, ISP3 and ISP6 agars, moderate growth on JCM medium 61, tap-water, Czapek's, ISP2, ISP4 and ISP7 agars and poor growth on Waksman medium 1 and ISP5 agars (Table 1). The colour of colonies on different media was strong orange (ISP2 and ISP6), vivid orange (M8, MB and ISP3), brilliant orange (JCM and ISP7), pale yellow (Waksman medium 1), yellowish white (Czapek's, tap-water and ISP4) or white (ISP5) (Table 1). Aerial mycelium was absent on these media. Soluble pigment was not produced in any medium tested (Table 1). Growth of strain NEAU-GRX6^T occurred at pH 6.0–9.0 and 0–4 % NaCl (w/v), with optimum growth at pH 8.0. The temperature

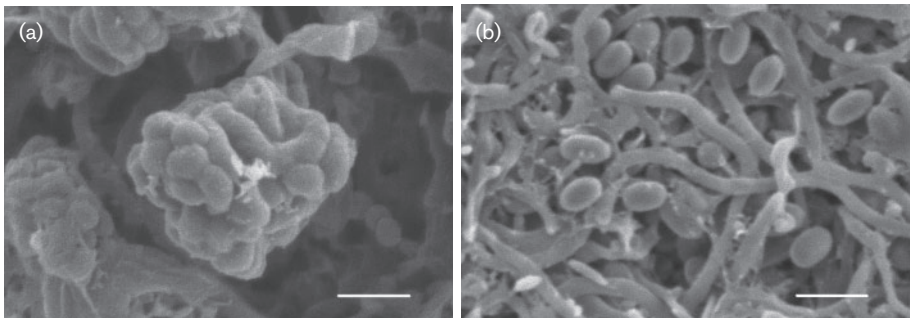

Fig. 1. Scanning electron micrographs of strain NEAU-GRX6^T grown on ISP3 agar for 21 days at 28 °C. Bars, 2 μm. (a) Sporangia; (b) spore vesicles releasing motile spores.

range for growth was 15–32 °C, with optimum growth at 28 °C. Detailed physiological and biochemical properties are presented in the species description and in Table 2.

Cell-wall hydrolysates of strain NEAU-GRX6^T contained glycine and *meso*-DAP, indicating that the strain had wall chemotype II (Lechevalier & Lechevalier, 1970). The whole-cell sugars were xylose, glucose and galactose. The acyl type of the cell-wall polysaccharides was glycolyl. Mycolic acids were not detected. The predominant menaquinones were MK-9(H_6) (40.8 %), MK-10(H_4) (33.7 %) and MK-9(H_4) (25.5 %). The phospholipid profile consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, one unknown glucosamine-containing phospholipid and three unknown phospholipids (Fig. S1, available in the online Supplementary Material), corresponding to phospholipid type PII (Lechevalier *et al.*, 1977). The cellular fatty acid profile was composed of $C_{16:0}$ (28.15 %), $C_{15:0}$ (20.93 %), $C_{18:1}\omega9c$ (13.17 %), $C_{17:1}\omega7c$ (8.36 %), $C_{18:0}$ (8.26 %), $C_{16:1}\omega7c$ (6.00 %), $C_{17:0}$ (5.63 %), anteiso-$C_{17:0}$ (4.79 %), iso-$C_{14:0}$ (2.39 %), 10-methyl $C_{17:0}$ (1.46 %) and $C_{14:0}$ (0.84 %).

The almost-complete 16S rRNA gene sequence (1508 nt) of strain NEAU-GRX6^T was used for phylogenetic analysis together with those of members of family *Micromonosporaceae*. The 16S rRNA gene sequence of strain NEAU-GRX6^T revealed the highest similarities to *A. palleronii* IFO

Table 1. Colonial characteristics of strain NEAU-GRX6^T and the most closely related type strains of the genus *Actinoplanes*

Strains: 1, NEAU-GRX6^T; 2, *A. palleronii* IFO 14916^T; 3, *A. missouriensis* NBRC 102363^T. All data are from this study.

Characteristic	1	2	3
Growth			
ISP2	Moderate	Good	Good
ISP3	Good	Good	Good
ISP4	Moderate	Good	Good
ISP5	Poor	Moderate	Moderate
ISP6	Good	Moderate	Good
ISP7	Moderate	Good	Good
Substrate mycelium			
ISP2	Strong orange	Brilliant orange	Strong orange yellow
ISP3	Vivid orange	Brilliant orange	Light orange yellow
ISP4	Yellowish white	Brilliant orange	Brilliant orange yellow
ISP5	White	Light yellow green	Light orange yellow
ISP6	Strong orange	Greyish greenish yellow	Light orange yellow
ISP7	Brilliant orange	Light yellow green	Strong orange
Soluble pigment			
ISP2	None	None	None
ISP3	None	None	None
ISP4	None	None	None
ISP5	None	None	None
ISP6	None	Light olive grey	None
ISP7	None	None	None

Table 2. Differential phenotypic characteristics of strain NEAU-GRX6^T and the most closely related type strains of the genus *Actinoplanes*

Strains: 1, NEAU-GRX6^T; 2, *A. palleronii* IFO 14916^T; 3, *A. missouriensis* NBRC 102363^T. All data are from this study.

Characteristic	1	2	3
Hydrolysis of starch	+	−	−
Nitrate reduction	−	+	+
Urea hydrolysis	+	−	−
Liquefaction of gelatin	+	+	−
Production of catalase	+	+	−
Growth at 37 °C	−	+	+
NaCl tolerance (%, w/v)	0–4	0–3	0–3
Utilization of:			
L-Alanine	−	−	+
L-Arabinose	−	−	+
Creatine	−	+	+
D-Fructose	−	+	+
Glycine	−	−	+
Inositol	+	−	−
Raffinose	−	−	+
D-Xylose	+	+	−

14916^T (97.80 %) and *A. missouriensis* NBRC 102363^T (97.76 %). Phylogenetic analysis based on 16S rRNA gene sequences of all type strains of the genus *Actinoplanes* and representative of the nearest genera *Micromonospora* and *Dactylosporangium* showed that the isolate fell into the clade of the genus *Actinoplanes* adjacent to *A. missouriensis* NBRC 102363^T (Fig. 2). This was also shown in the maximum-likelihood tree (Fig. S2). To establish the precise taxonomic position of strain NEAU-GRX6^T, DNA–DNA hybridizations were performed between the novel isolate and *A. palleronii* IFO 14916^T and *A. missouriensis* NBRC 102363^T; the levels of DNA–DNA relatedness between them were 41.1 ± 1.5 and 44.4 ± 0.7 %, respectively. These values were below the threshold value of 70 % recommended by Wayne *et al.* (1987) for assignment of strains to the same species.

Strain NEAU-GRX6^T shares many chemotaxonomic characteristics with other species of the genus *Actinoplanes*, such as the presence of *meso*-DAP in the whole-cell peptidoglycan, the absence of mycolic acids, MK-9(H_6) and MK-9(H_4) as the predominant menaquinone and phosphatidylethanolamine as the diagnostic phospholipid (Couch, 1950; Goodfellow *et al.*, 1990; Tamura & Hatano, 2001; Kämpfer *et al.*, 2007; Ara *et al.*, 2010). However, the whole-cell sugar pattern (absence of arabinose) of strain NEAU-GRX6^T differentiates it from other species of the genus *Actinoplanes*, which contain arabinose as a characteristic sugar. In addition, the fatty acid profile of strain NEAU-GRX6^T was clearly different from those of the most closely related type strains (Table S1). The major fatty acid of strain NEAU-GRX6^T was determined to be $C_{16:0}$, similar to closely related neighbours. This might be because of the GY medium used to culture cells for cellular fatty acid analysis. Another study in our laboratory also obtained a similar result (Zhang *et al.*, 2014). Furthermore, the isolate could be clearly distinguished from *A. palleronii* IFO 14916^T and *A. missouriensis* NBRC 102363^T based on phenotypic characteristics, as summarized in Tables 1 and 2. Strain NEAU-GRX6^T had different colonial characteristics on various media compared with the most closely related species. The novel strain could not reduce nitrate or grow at 37 °C, while the most closely related species could. The isolate could hydrolyse starch and urea, whereas *A. palleronii* IFO 14916^T and *A. missouriensis* NBRC 102363^T could not. Other phenotypic characteristics that differentiated *A. palleronii* IFO 14916^T, *A. missouriensis* NBRC 102363^T and NEAU-GRX6^T included liquefaction of gelatin, production of catalase, NaCl ranges for growth and patterns of carbon and nitrogen source utilization.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU-GRX6^T represents a novel species of the genus *Actinoplanes*, for which we propose the name *Actinoplanes lutulentus* sp. nov.

Description of *Actinoplanes lutulentus* sp. nov.

Actinoplanes lutulentus (lu.tu.len′tus. L. masc. adj. *lutulentus* muddy, turbid, used to refer to mucky soil, where the type strain was found).

Aerobic, Gram-stain-positive actinomycete that forms well-developed substrate mycelium that carries irregular sporangia on ISP3 agar. The spore vesicles release motile, oval, smooth-surfaced spores. Grows well on M8, MB, ISP3 and ISP6 agars, moderately well on JCM medium 61, tapwater, Czapek's, ISP 2, ISP 4 and ISP 7 agars and poorly on Waksman medium 1 and ISP 5 agars. No diffusible pigment is detected on any medium tested. Colonies are white to orange with age on most tested media. Positive for production of catalase, esterase and urea, hydrolysis of aesculin and starch and liquefaction of gelatin; negative for decomposition of cellulose, reduction of nitrate, peptonization of milk and production of H_2S. D-Galactose, D-glucose, inositol, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, sucrose and D-xylose are utilized as sole carbon sources but L-arabinose, D-fructose, raffinose and D-ribose are not. L-Arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, L-serine and L-threonine are utilized as sole nitrogen sources but L-alanine, creatine, glycine and L-tyrosine are not. Tolerates up to 4 % NaCl and grows at 15–32 °C, with an optimum temperature of 28 °C. Growth occurs at initial pH 6.0–9.0, the optimum being pH 8.0. Cell-wall hydrolysates contain glycine and *meso*-DAP. Whole-cell sugars are xylose, glucose and galactose. The acyl type of cell-wall peptidoglycan is glycolyl. Mycolic acids are absent. The major menaquinones are MK-9(H_6), MK-10(H_4) and MK-9(H_4). The phospholipid profile consists of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, one unknown glucosamine-containing phospholipid and three

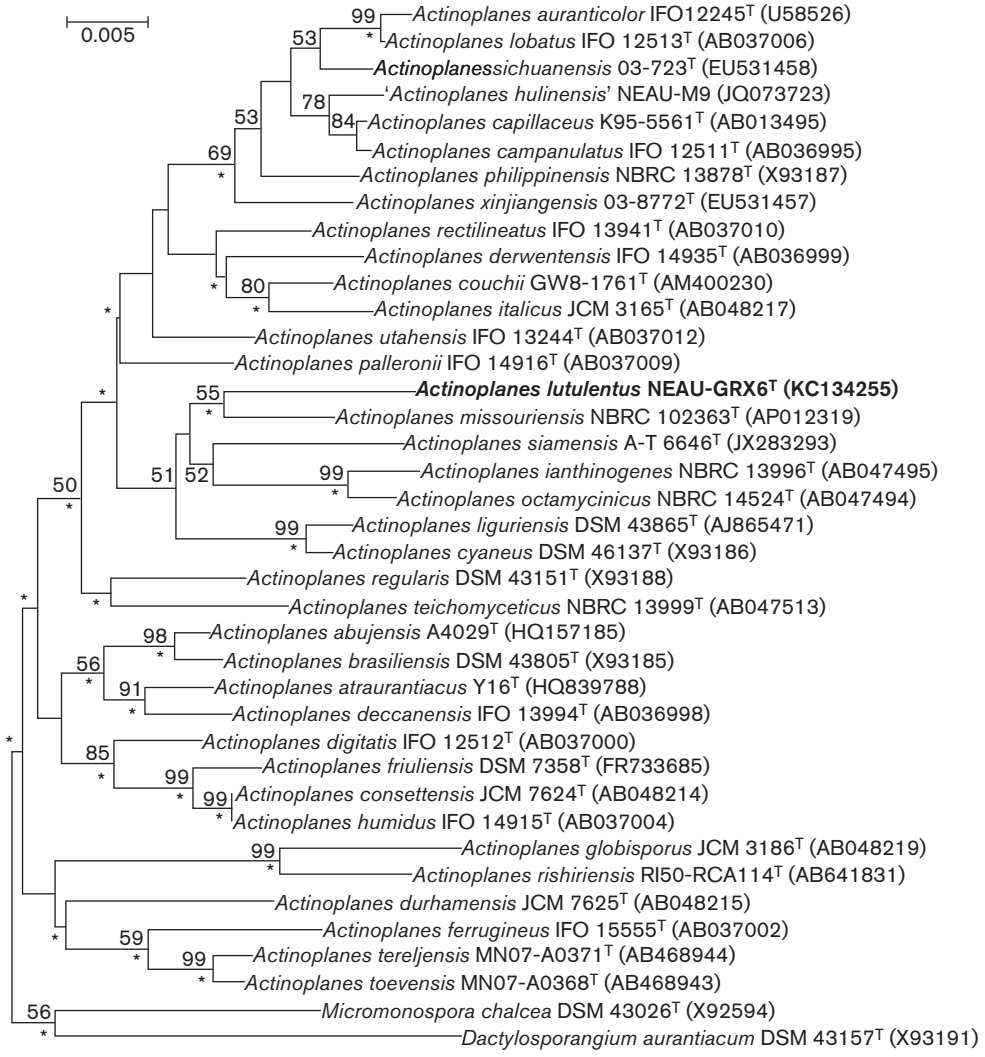

Fig. 2. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences (1508 nt) showing the relationship between strain NEAU-GRX6^T and all species of the genus *Actinoplanes* and representatives of the nearest genera *Micromonospora* and *Dactylosporangium*. Asterisks (*) indicate branches of the tree that were also found using the maximum-likelihood method. Numbers at branch points indicate bootstrap percentages (based on 1000 replicates); only values $\geqslant$50 % are indicated. Bar, 0.005 substitutions per nucleotide position.

unknown phospholipids. The major cellular fatty acids are $C_{16:0}$, $C_{15:0}$, $C_{18:1}\omega9c$, $C_{17:1}\omega7c$ and $C_{18:0}$.

The type strain is NEAU-GRX6^T (=CGMCC 4.7090^T= DSM 45883^T), which was isolated from mucky soil collected from Jinlong Mountain in Harbin, Heilongjiang Province, north China. The G+C content of the DNA of the type strain is 67 mol%.

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