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Jishengella endophytica gen. nov., sp. nov., a new member of the family *Micromonosporaceae*

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A novel endophytic actinomycete, designated strain 202201^T, was isolated from an *Acanthus illicifolius* root collected from the mangrove reserve zone in Hainan Province, China. Phylogenetic analysis based on 16S rRNA gene sequences suggested that strain 202201^T fell within the family *Micromonosporaceae*. The strain formed an extensively branched substrate mycelium, which carried uneven warty-surfaced spores. Cell walls of strain 202201^T contained *meso*-diaminopimelic acid and xylose, mannose, arabinose, ribose and glucose were detected as whole-cell sugars. The acyl type of the cell-wall polysaccharides was glycolyl. The major menaquinones were MK-9(H_4), MK-9(H_6), MK-9(H_8) and MK-10(H_4). The polar lipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol mannoside and phosphatidylserine. The major cellular fatty acids were 10-methyl-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{17:1}\omega8c$. The DNA G+C content was 72.3 mol%. On the basis of the morphological and chemotaxonomic characteristics, phylogenetic analysis and characteristic patterns of 16S rRNA gene signature nucleotides, strain 202201^T (=CGMCC 4.5597^T =DSM 45430^T) represents a novel species of a new genus within the family *Micromonosporaceae*, for which the name *Jishengella endophytica* gen. nov., sp. nov. is proposed.

The family *Micromonosporaceae* was originally proposed by Krasil'nikov (1938) on a morphological basis and was emended by Goodfellow *et al.* (1990) and Koch *et al.* (1996). Based on phylogenetic clustering of 16S rRNA gene sequences and the presence of taxon-specific 16S rRNA signature nucleotides, Stackebrandt *et al.* (1997) and Zhi *et al.* (2009) subsequently amended the family again. At the time of writing, the family *Micromonosporaceae* comprised 26 genera.

In the course of investigating endophytic actinomycetes from mangrove plants (Hong *et al.*, 2009), strains designated 202201^T, 161111 and 161612 were isolated from the roots of *Acanthus illicifolius*, *Xylocarpus granatum* and *Sonneratia paracaseolaris*, respectively. These mangrove plants were collected from the mangrove reserve zone in Hainan Province, China. The 16S rRNA gene sequences of strains 202201^T, 161111 and 161612 formed a lineage within the family *Micromonosporaceae* that was adjacent to, but distinct from, the genus *Verrucosispora* (Fig. 1). In this study we propose that these strains represent a novel species of a novel genus within the family *Micromonosporaceae*.

Healthy root samples of mangrove plants were washed in running tap water to remove adhered epiphytes and soil debris. After drying under sterile conditions, the root surfaces were sterilized by sequential immersion in 75 % (v/v) ethanol for 5 min and 0.1 % mercury bichloride for 15 min. The surface-treated roots were washed five times in sterile 1 % Tween 80 to remove the mercury bichloride. A 5 gram sample of air-dried sterilized roots was mixed with 45 ml sterilized 50 % (v/v) seawater, mill ground and then spread over the surface of raffinose–histidine (RH) plates (10.0 g raffinose, 1.0 g L-histidine, 0.5 g $MgSO_4 . 7H_2O$, 0.01 g $FeSO_4 . 7H_2O$, 1.0 g K_2HPO_4, 20.0 g agar, 500 ml seawater, 500 ml H_2O; pH 7.0–7.4) (Williams *et al.*, 1984) supplemented with 50 μg ml^{-1} potassium dichromate. The plates were then incubated at 28 °C for 30 days. The efficacy of the surface-sterilization technique was evaluated by incubating the remaining surface-sterilized root material on RH medium at 28 °C for 21–30 days.

Cultural characteristics of strain 202201^T were determined by growth on tap-water agar (Gordon *et al.*, 1974),

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 202201^T is EU560726.

One supplementary figure and two supplementary tables are available with the online version of this paper.

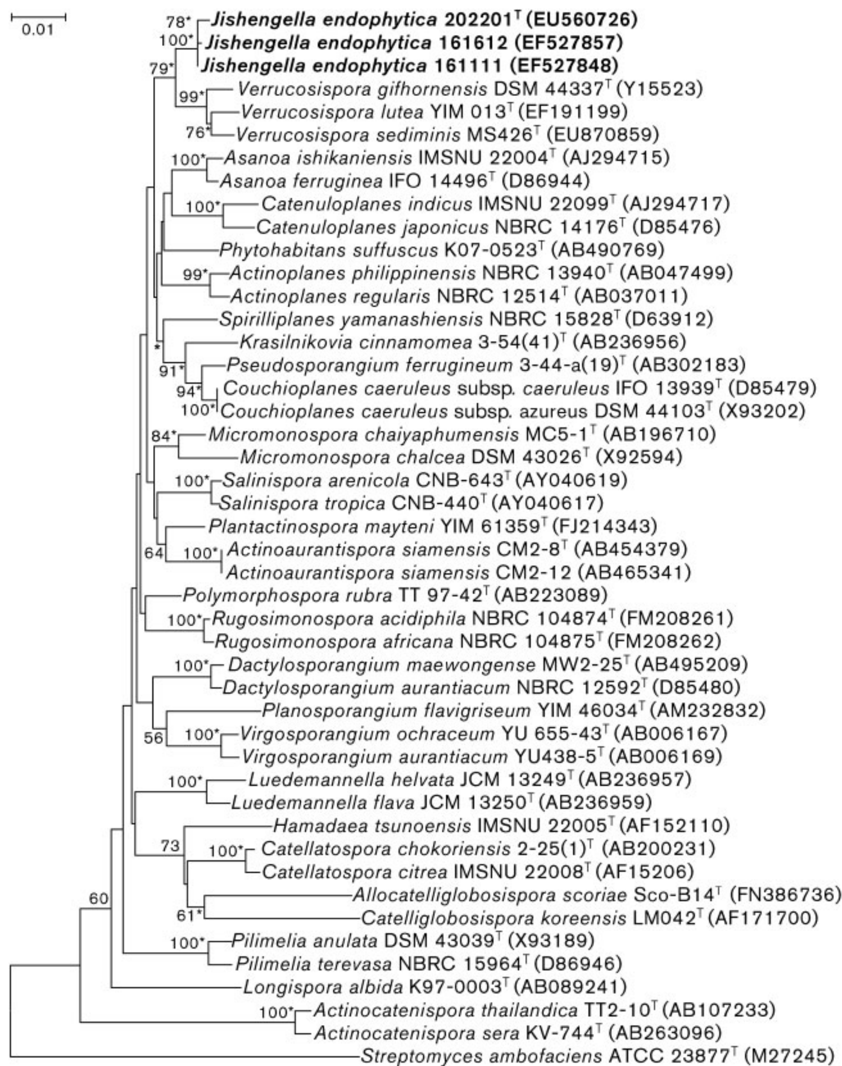

Fig. 1. Neighbour-joining (Saitou & Nei, 1987) phylogenetic tree derived from nearly complete 16S rRNA gene sequences (1409 nt) showing the relationship between strains 202201^{T}, 161111 and 161612 and type species of recognized genera of the family *Micromonosporaceae*. *Streptomyces ambofaciens* ATCC 23877^{T} was used as an outgroup. Numbers at nodes indicate bootstrap levels >50 % based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.01 substitutions per nucleotide position. Asterisks (*) indicate branches of the tree that were also recovered using the maximum-likelihood (Felsenstein, 2008) and maximum-parsimony methods (Fitch, 1971).

Czapek's agar (Raper & Fennell, 1965), GYM (glucose–yeast–malt) agar (Shirling & Gottlieb, 1966), ATCC 172 agar (http://www.lgcstandards-atcc.org), M8 agar (Castiglione *et al.*, 2008), Modified Bennett (MB) agar (Jones, 1949) and International *Streptomyces* Project (ISP) media 1–7 (Shirling & Gottlieb, 1966) at 28 °C for 14–21 days. ISCC–NBS colour charts were used to designate colony colours (Kelly, 1964). The morphologies of spores and mycelia were observed after incubation on ISP 2 medium at 28 °C for 14–21 days by using light microscopy (80i; Nikon) and scanning electron microscopy (Quanta 200; FEI). The Gram reaction was performed according to Gregersen (1978) by using KOH for cell lysis. Growth at a range of temperatures was determined on ISP 2 medium after incubation for 14–21 days. Tolerance of NaCl and pH range for growth were determined by incubation at 28 °C for 14–21 days. Carbon-source utilization was tested using ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with one of a range of designated carbon sources (1 % final concentration). The utilization of amino acids as nitrogen sources was tested as described by Williams *et al.* (1983). Production of melanoid pigments was determined using tyrosine agar (ISP 7).

Biomass for molecular systematic studies and freeze-dried cells for chemotaxonomic studies were obtained by growth in yeast extract–malt extract broth (ISP 2) or TSB (trypticase soy broth), for fatty acids, at 28 °C for 14 days on a rotary shaker. Cell-wall amino acids and whole-cell sugars were analysed using the procedure of Lechevalier & Lechevalier (1980). The *N*-acyl group of the muramic acid in the peptidoglycan was determined according to Uchida & Aida (1977). Phospholipids in cells were extracted and identified by using the method of Minnikin *et al.* (1984). Fatty acids were extracted and methylated according to the methods of Sasser (1990) and analysed by GC (Oliver & Colwell, 1973). Menaquinones were determined using the procedures of Minnikin *et al.* (1984) and analysed by HPLC (Shimadzu).

Genomic DNA of strains 202201^{T}, 161111 and 161612 was extracted as described by Pospiech & Neumann (1995). The DNA G+C content was determined by HPLC

(Mesbah *et al.*, 1989). DNA–DNA relatedness experiments were carried out by using a modified nylon membrane hybridization method based on PCR-mediated amplification. Chromosomal DNA of the three strains was digested with *Sau*3AI and fragments of 0.2–2 kb were collected. *Sau*3AI-digested DNA was ligated with the appropriate linker using T4 DNA ligase (TaKaRa) according to a method modified from Wassill *et al.* (1998). The linker-modified target DNA was labelled using a PCR DIG Probe Synthesis kit (Roche). Subsequent DNA–DNA hybridization experiments were carried out as previously described by Cardinali *et al.* (2000). The filter was then processed with phosphatase-labelled anti-Dig antibody and signals were produced with the chromogenic substrate NBT/BCIP, as described in the manufacturer's protocol (Roche), and quantified densitometrically.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima *et al.* (1999). The 16S rRNA gene sequence of strain 202201^T was aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL_X software (Thompson *et al.*, 1997). The alignment was manually verified and adjusted prior to the construction of phylogenetic trees. Phylogenetic trees were generated with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms using MEGA version 4.0 (Tamura *et al.*, 2007). For construction of the maximum-likelihood tree, the PHYLIP package version 3.68 (Felsenstein, 2008) was used. The stability of the clades in the trees was estimated by bootstrap analysis (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). *Streptomyces ambofaciens* ATCC 23877^T was used as an outgroup. Sequence similarities among the 50 most closely related strains were determined using the EzTaxon server 2.1 (Chun *et al.*, 2007).

For similarity and phylogenetic analyses, the almost complete 16S rRNA gene sequences of strains 202201^T, 161111 and 161612 were compared with those of members of the family *Micromonosporaceae*. Based on 16S rRNA gene sequence analysis, the phylogenetic position of the isolates was within the confines of all recognized type strains in the family *Micromonosporaceae* and formed a distinct monophyletic clade adjacent to the genus *Salinispora* with weak bootstrap support of <50 % (Supplementary Fig. S1, available in IJSEM Online). However, the trees constructed using only two representative species of each of the recognized genera of the family *Micromonosporaceae* showed that the isolates had a close association with the genus *Verrucosispora* with a bootstrap value of 79 % (Fig. 1). The 16S rRNA gene sequence similarities of isolate 202201^T to strains 161111 and 161612 were 99.7 % and 99.8 %, respectively, with DNA–DNA relatedness values of 84.7 ± 8.5 % and 81.5 ± 7.6 %, respectively. Therefore, strains 202201^T, 161111 and 161612 should be classified as members of the same species. Sequence similarities between strain 202201^T and *Micromonospora olivasterospora* DSM 43868^T, *Micromonospora pattaloongensis* TJ2-2^T, *Micromonospora auratinigra* TT1-11^T, *Verrucosispora gifhornensis* DSM 44337^T, *Micromonospora eburnea* LK2-10^T and *Verrucosispora sediminis* MS426^T were 98.7 %, 98.6 %, 98.3 %, 98.3 %, 98.0 % and 98.0 %, respectively. Despite these high 16S rRNA gene sequence similarities, the genus *Micromonospora* was not supported by the trees as a close phylogenetic relative, whereas the genus *Verrucosispora* did appear to be closely related to the novel isolates (Fig. 1).

The position of strain 202201^T in the family *Micromonosporaceae* was also supported by the presence of a complete set of family-specific signature nucleotides (Zhi *et al.*, 2009) in its 16S rRNA gene sequence. However, when the signature nucleotide positions of strain 202201^T were compared with those of its closest relatives, there were several nucleotide pair differences from the genera *Verrucosispora*, *Micromonospora* and *Salinispora* (Supplementary Table S1).

The cell wall of the novel isolate contained *meso*-diaminopimelic acid and the whole-cell sugars were xylose, mannose, arabinose, ribose and glucose. The acyl type of the cell-wall polysaccharides was glycolyl. Mycolic acids were not detected. The predominant menaquinones were MK-9(H_4) (65.66 %), MK-9(H_6) (20.60 %), MK-9(H_8) (7.15 %) and MK-10(H_4) (4.76 %); small amounts of MK-10(H_6) (1.42 %) and MK-10(H_8) (0.41 %) were also present. The characteristic phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol mannoside and phosphatidylserine but phosphatidylcholine, lysophosphatidylcholine, phosphatidylglycerol and phosphatidylinositol were not present, corresponding to phospholipid type PII as described by Lechevalier *et al.* (1977). The major cellular fatty acids (>10 %) were 10-methyl-$C_{17:0}$ (25.38 %), iso-$C_{15:0}$ (15.07 %), iso-$C_{16:0}$ (11.29 %) and $C_{17:1}\omega 8c$ (11.02 %), with small amounts of $C_{15:0}$ (9.63 %), $C_{17:0}$ (3.09 %) and $C_{18:0}$ (3.06 %) (Supplementary Table S2), corresponding to fatty acid type 3a as described by Kroppenstedt (1985). The DNA G+C content of strain 202201^T was 72.3 mol% (Table 1).

Cells of strain 202201^T were Gram-reaction-positive, aerobic and non-acid-fast and formed well-developed branched substrate hyphae ~0.6 μm in diameter on all tested media (except ISP 3). Aerial hyphae and sporangia were not present. Spores were borne singly on the substrate mycelium with a diameter of ~0.8 μm. The spore surface appeared unevenly warty (Fig. 2). The colour of the substrate mycelium was vivid orange to dull orange. No soluble pigment was produced. Good growth was observed on ISP 1, ISP 2, ISP 4, ISP 6, MB, ATCC 172, Czapek's, GYM and M8 agar and moderate growth was observed on ISP 5, ISP 7 and tap-water agar but no growth was observed on ISP 3 medium. Colonies were orange and became increasingly brownish with age on most tested media. Cells of strain 202201^T grew well in 0–2 % (w/v)

Table 1. Characteristics of strain 202201^{T} and genera in the family *Micromonosporaceae*

Taxa: 1, strain 202201^{T}; 2, *Actinoaurantispora*; 3, *Actinocatenispora*; 4, *Actinoplanes*; 5, *Allocatelliglobosispora*; 6, *Asanoa*; 7, *Catellatospora*; 8, *Catelliglobosispora*; 9, *Catenuloplanes*; 10, *Couchioplanes*; 11, *Dactylosporangium*; 12, *Hamadaea*; 13, *Krasilnikovia*; 14, *Longispora*; 15, *Luedemannella*; 16, *Micromonospora*; 17, *Phytohabitans*; 18, *Pilimelia*; 19, *Plantactinospora*; 20, *Planosporangium*; 21, *Polymorphospora*; 22, *Pseudosporangium*; 23, *Rugosimonospora*; 24, *Salinispora*; 25, *Spirilliplanes*; 26, *Verrucosispora*; 27, *Virgisporangium*. Data for reference genera were taken from Ørskov (1923), Couch (1950), Kane (1966), Thiemann *et al.* (1967), Asano & Kawamoto (1986), Yokota *et al.* (1993), Rheims *et al.* (1998), Kudo *et al.* (1999), Tamura *et al.* (1994, 1997, 2001, 2006), Lee & Hah (2002), Matsumoto *et al.* (2003), Maldonado *et al.* (2005), Thawai *et al.* (2006, 2010), Ara & Kudo (2006, 2007a, b), Ara *et al.* (2008a, b), Wiese *et al.* (2008), Monciardini *et al.* (2009), Qin *et al.* (2009), Lee & Lee (2011), Inahashi *et al.* (2010). +, Present; −, absent; ND, no data available; *m*-DAP, *meso*-diaminopimelic acid; L-Lys, L-lysine; Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Spore motility	−	−	−	+	−	−	−	−	+	+	+	−	−	−
Spore vesicle	−	−	−	+	−	−	−	−	−	−	+	−	−	−
Diagnostic acid(s)	*m*-DAP	*m*-DAP	*m*-DAP	*m*-DAP	3-OH-DAP	*m*-DAP	*m*-DAP3O	*m*- and 3-OH-DAP	L-Lys	L-Lys	*m*-DAP	*m*-DAP	*m*-DAP	*m*-DAP
Diagnostic sugar(s)	Xyl, Man, Ara, Rib, Glu	Xyl, Man, Gal	Ara, Gal, Xyl	Ara, Xyl	Glu, Rha, Rib, Xyl, Ara, Gal, Man	Ara, Gal, Xyl	Ara, Gal, Xyl or only Xyl	Rha, Man, Xyl, Gal, Glu	Xyl	Ara, Gal, Xyl	Ara, Xyl	Xyl, Gal, Man, Rib, Ara, Rha	Gal, Ara, Xyl	Ara, Gal, Xyl
Fatty acid type*	3a	3b	3b	2d	3b	2d	3b	3b	2c	2c	3b	3b	2d	2d
Major menaquinones	$9(H_4, H_6, H_8)$, $10(H_4)$	$9(H_4)$, $10(H_6)$	$9(H_4,H_6)$	$9(H_4)$, $10(H_4)$	$10(H_4)$, $9(H_4)$, $10(H_6)$	$10(H_6,H_8)$	$9(H_4,H_6)$, $10(H_4)$	$10(H_4)$	$9(H_8)$, $10(H_8)$	$9(H_4)$	$9(H_4,H_6,H_8)$	$9(H_4)$	$9(H_6,H_4,H_8)$	$10(H_{4,6})$
Phospholipid type†	II	II	II	II	II	II	II	II	III	II	II	II	II	II
DNA G+C content (mol%)	72	72	72	72–73	70.1	71–72	70–71	70	71–73	70–72	71–73	70	71	70

Taxa	15	16	17	18	19	20	21	22	23	24	25	26	27
Spore motility	+	−	−	+	−	−	−	−	−	−	+	−	+
Spore vesicle	−	−	−	+	+	−	−	−	−	−	−	−	+
Diagnostic acid(s)	m-DAP	*m*-DAP	m-DAP, L-Lys	*m*-DAP	*m*-DAP	*m*-DAP	*m*-DAP	*m*- and 3-OH-DAP	3-OH-DAP	*m*-DAP	*m*-DAP	*m*-DAP	*m*-DAP
Diagnostic sugar(s)	Gal, Man, Rha, Rib, Xyl, Ara	Ara, Xyl	Gal, Glu, Man, Rib, Xyl	Ara, Xyl	Ara, Xyl	Ara, Gal, Xyl	Xyl	Ara, Gal, Glu, Man, Xyl	Ara, Gal, Xyl	Ara, Gal, Xyl	Ara, Xyl	Man, Xyl	Ara, Gal, Man, Rha, Xyl
Fatty acid type*	2d	3b	2d	2d	3b	2d	2a	2d	2c	3a	2d	2d	2d
Major menaquinones	$9(H_6,H_{4,2,8})$	$10(H_{4,6})$, $9(H_{4,6})$	$9(H_6)$, $10(H_{4,6})$	$9(H_2,H_4)$	$9(H_4)$, $10(H_4)$	$10(H_{6,8,4})$	$9(H_{4,6})$, $10(H_{4,6})$	$9(H_6)$	$9(H_8, H_6)$	$9(H_4)$	$10(H_4)$	$9(H_4)$	$10(H_{4,6,8})$
Phospholipid type†	II	II	II	II	II	II	II	II	II	II	II	II	II
DNA G+C content (mol%)	71	71–72	73	ND	71.4	69.7	71	73.6	72–73	70–73	69	70	71

*According to the classification of Kroppenstedt (1985).

†According to the classification of Lechevalier *et al.* (1981).

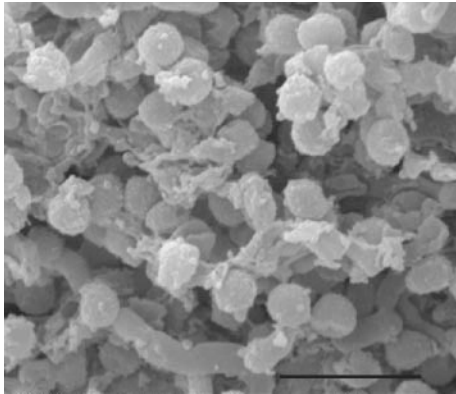

Fig. 2. Scanning electron micrograph of substrate mycelium of strain 202201^T grown on ISP 2 agar for 21 days at 28 °C. Bar, 2 μm.

NaCl but failed to grow at concentrations of 3 % (w/v) NaCl or above. Growth was observed at 4–45 °C and pH 4–11 (optimum pH 7).

Cell morphology of strain 202201^T was similar to that of members of the genus *Verrucosispora* but was clearly different from members of the genus *Salinispora*, whose spore surfaces are not warty (Maldonado *et al.*, 2005). The major fatty acids, the major menaquinone, the whole-cell sugars and signature nucleotides of strain 202201^T differed from those of the closest phylogenetic neighbours of the genus *Verrucosispora*. In addition, members of the genus *Micromonospora* were not supported as close phylogenetic relatives by the trees, despite the high 16S rRNA gene sequence similarities between strain 202201^T and *M. olivasterospora* DSM 43868^T, *M. pattaloongensis* TJ2-2^T (Thawai *et al.* 2008), *M. auratinigra* TT1-11^T, *V. gifhornensis* DSM 44337^T, *M. eburnea* LK2-10^T and *V. sediminis* MS426^T. Furthermore, the menaquinone components, fatty acid composition and signature nucleotides of the novel isolates were different from members of the genus *Micromonospora*. In conclusion, using a combination of phenotypic, chemotaxonomic and phylogenetic properties, strain 202201^T was clearly distinguished from all recognized members of the genera in the family *Micromonosporaceae*. On the basis of morphological, chemotaxonomic and physiological characteristics, as well as the signature nucleotide pattern of the 16S rRNA gene, strain 202201^T was readily distinguishable from closely related members of the genera *Verrucosispora*, *Salinispora* and *Micromonospora*. Strains 202201^T, 161111 and 161612, therefore, represent a novel species of a new genus in the family *Micromonosporaceae*, for which the name *Jishengella endophytica* gen. nov., sp. nov. is proposed.

Description of *Jishengella* gen. nov.

Jishengella (Ji.sh.eng.ell′a. N.L. fem. n. *Jishengella* from Jisheng, named after Jisheng Ruan, the Chinese microbiologist).

Aerobic, Gram-reaction-positive and non-acid-fast actinomycetes that form extensively branched substrate mycelia ~0.6 μm in diameter, which carry unevenly warty–surfaced spores ~0.8 μm in diameter. Spores are non-motile. Cell walls contain *meso*-diaminopimelic acid and xylose, mannose, arabinose, ribose and glucose are the whole-cell sugars. The acyl type of the cell-wall polysaccharide is glycolyl. Mycolic acids are absent. The major menaquinones are MK-9(H_4), MK-9(H_6), MK-9(H_8) and MK-10(H_4). Phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol mannoside and phosphatidylserine are present, corresponding to phospholipid type PII. The major cellular fatty acids are 10-methyl-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and $C_{17:1}\omega 8c$, corresponding to fatty acid type 3a. The DNA G+C content is ~72–73 mol%. The type species of the genus is *Jishengella endophytica*.

Description of *Jishengella endophytica* sp. nov.

Jishengella endophytica (en.do.phy′ti.ca. Gr. pref. *endo* within; Gr. n. *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 tissues).

Displays the following morphological, chemotaxonomic and general characteristics in addition to those given in the genus description. Colonies are vivid orange–dull orange on tap-water agar, Czapek's agar, GYM, ATCC 172, M8 and MB agar and ISP 1–2 and ISP 4–7 media. Nitrate is not reduced to nitrite. Positive for peptonization of milk, hydrolysis of starch, gelatin liquefaction and cellulose decomposition but negative for formation of melanin and H_2S production. Grows optimally at 27–36 °C and in 0–2 % (w/v) NaCl. D-Xylose, D-fructose, sucrose, D-arabinose, D-galactose and D-glucose are utilized; glycerol, lactose, raffinose, L-rhamnose and D-ribose are not utilized. L-Arginine, L-histidine, L-hydroxyproline and L-serine are used as sole nitrogen sources. The major polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylinositol mannoside and phosphatidylserine. The fatty acid profile (>3 %) comprises 10-methyl-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:1}\omega 8c$, $C_{15:0}$, $C_{17:0}$ and $C_{18:0}$.

The type strain is 202201^T (=CGMCC 4.5597^T =DSM 45430^T), isolated from surface-sterilized roots of *Acanthus illicifolius* collected from the mangrove reserve zone in Hainan Province, China. The DNA G+C content of the type strain is 72.3 mol%.

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