Description of *Verrucosispora qiuiae* sp. nov., isolated from mangrove swamp sediment, and emended description of the genus *Verrucosispora*

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A *Micromonospora*-like strain, RtIII47^T, was isolated from a mangrove swamp in Sanya, Hainan Province, China. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the strain had a close association with the genus *Verrucosispora* and shared the highest sequence similarity with *Verrucosispora lutea* YIM 013^T (98.0 %). The strain also showed high 16S rRNA gene sequence similarities to *Micromonospora olivasterospora* DSM 43868^T (97.9 %), *Plantactinospora mayteni* YIM 61359^T (97.9 %), *Salinispora tropica* CNB-440^T (97.8 %), *Micromonospora peucetia* DSM 43363^T (97.7 %), *Micromonospora auratinigra* TT1-11^T (97.7 %), *Verrucosispora sediminis* CGMCC 4.3550^T (97.6 %) and *Salinispora arenicola* CNH-643^T (97.5 %). Phylogenetic analysis based on the *gyrB* gene sequence supported the conclusion that strain RtIII47^T should be assigned to the genus *Verrucosispora*. DNA–DNA relatedness between strain RtIII47^T and the most closely related type strain, *V. lutea* YIM 013^T, was less than 40 %. Chemotaxonomic results confirmed the taxonomic position of the isolate in the genus *Verrucosispora*, and revealed differences at the species level in polar lipids, whole-cell sugars and DNA G+C content. A combination of physiological and biochemical tests also distinguished this strain from other *Verrucosispora* species. Based on genotypic and phenotypic observations, strain RtIII47^T (=CGMCC 4.5826^T =NBRC 106684^T) is proposed as the type strain of a novel species, *Verrucosispora qiuiae* sp. nov. An emended description of the genus *Verrucosispora* is also provided.

The genus *Verrucosispora*, which was established by Rheims *et al.* (1998) as a member of the family *Micromonosporaceae*, has drawn much attention in recent years because it has proved to be a source of potential antitumour compounds such as abyssomicin, gifhornenolones and proximicins (Bister *et al.*, 2004; Keller *et al.*, 2007; Riedlinger *et al.*, 2004; Schneider *et al.*, 2008; Shirai *et al.*, 2010). At the time of writing, this genus comprised three species with validly published names, *Verrucosispora gifhornensis*, *Verrucosispora lutea* and *Verrucosispora sediminis*, with type strains isolated from peat bog, mangrove sediment and deep-sea sediment, respectively (Rheims *et al.*, 1998; Liao *et al.*, 2009; Dai *et al.*, 2010). Marine environments seem to be a good source of both novel species of this genus and unique natural products.

During the course of selective isolation of actinomycetes present in mangrove soil in China, hundreds of *Micromonospora*-like actinomycetes were isolated (Qiu *et al.*, 2008). Strain RtIII47^T is one of them, and showed morphological characteristics typical of the family *Micromonosporaceae*. In this paper, we report the taxonomic characterization and classification of this isolate, and propose that it represents a novel species of the genus *Verrucosispora*.

Strain RtIII47^T was isolated from mangrove swamp sediment in Sanya, Hainan Province, China. The sediment sample was dried at room temperature, suspended in sterile distilled water and diluted in series; the suspensions were then heated in an oven at 100 °C for 60 min. The heat-treated suspensions were plated on oatmeal agar plates [International *Streptomyces* Project medium 3 (ISP 3); Shirling & Gottlieb, 1966] supplemented with nalidixic acid, cycloheximide, nystatin (each at 50 mg l^{-1}) and novobiocin (25 mg l^{-1}). The plates were then incubated at 28 °C for 3–4 weeks.

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PIM, phosphatidylinositol mannoside; PL, unknown phospholipid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain RtIII47^T and the *gyrB* gene sequences of strain RtIII47^T, *Verrucosispora lutea* YIM 013^T, *Verrucosispora sediminis* CGMCC 4.3550^T, *Verrucosispora gifhornensis* JCM 10457^T, *Jishengella endophytica* CGMCC 4.5597^T and *Plantactinospora mayteni* YIM 61359^T are EU427445, HQ199219, HM134795, HQ199220, HM134796, JN207849 and JN207850, respectively.

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

Morphological characteristics were observed by light microscopy (Zeiss Axioskop) and scanning electron microscopy (FEI QUANTA 200) using cultures grown on ISP 2 medium at 28 °C for 14 days. Cultural characteristics of strain RtIII47^T were recorded after growth at 28 °C for 14 days on various agar media: ISP 2, ISP 3, ISP 5, ISP 7 (Shirling & Gottlieb, 1966), potato dextrose agar (PDA; Summerell *et al.*, 2003), Sauton's agar (Mordarska *et al.*, 1972) and Gause inorganic agar (Gause *et al.*, 1983). Phenotypic characteristics were examined using several standard methods: tolerance to NaCl (0–20 %, w/v) for growth was determined on ISP 2 agar for 14–21 days at 28 °C; catalase, nitrate reduction and substrate degradation were determined by the methods of Goodfellow (1971) and Williams & Cross (1971); carbon-source utilization was tested according to Gordon & Mihm (1957); and utilization of amino acids as nitrogen sources was tested as described by Williams *et al.* (1983).

Freeze-dried cells used for chemotaxonomic analyses were obtained from cultures grown in ISP 2 broth in flasks on a rotary shaker at 125–180 r.p.m. and 28 °C for 1 week. The isomer of diaminopimelic acid and the whole-cell sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983). The *N*-acyl type of muramyl residue in the cell-wall peptidoglycan was tested using the method of Uchida *et al.* (1999). Polar lipids were extracted, examined by two-dimensional TLC and identified using procedures described previously (Minnikin *et al.*, 1979). Menaquinones were isolated according to the methods of Groth *et al.* (1997). Biomass for quantitative fatty acid analysis was prepared by scraping growth from tryptic soy agar plates (Difco, BD) that had been incubated for 7 days at 28 °C. The fatty acids were extracted, methylated and analysed using the standard MIDI system (Microbial Identification, Sherlock version 6.0) (Sasser, 1990) and an Agilent 6890 GC. The resulting profiles were identified using the database TSBA6, version 6.0.

Genomic DNA was extracted as described by Chun & Goodfellow (1995). 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 RtIII47^T was multiply aligned with related sequences obtained from the GenBank/EMBL/DDBJ databases using MEGA version 4.0 (Kumar *et al.*, 2008). The alignment was verified manually and adjusted prior to the construction of phylogenetic trees. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA 4.0 (Kumar *et al.*, 2008). Confidence values for branches of phylogenetic trees were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 replications. Values for sequence similarity among the closest strains were calculated manually after pairwise alignment using the EzTaxon sever (Chun *et al.*, 2007). PCR amplification of the *gyrB* gene (encoding gyrase B) and sequencing of the PCR products were carried out using primers GYF1/GYR1B and GYF3/GYR3B, as described by Garcia *et al.* (2010), and the resultant sequences were subjected to phylogenetic analyses using the same methods used for 16S rRNA gene sequences.

The DNA G+C content was determined by the thermal denaturation (T_m) method (Mandel & Marmur, 1968) using a Beckman DU-800 spectrophotometer. DNA–DNA relatedness between strain RtIII47^T and *V. lutea* YIM 013^T was determined using the modified fluorometric microwell method described by Rong & Huang (2010).

Strain RtIII47^T grew well on all media tested except ISP 5 and formed bittersweet to xanthine orange (Ridgway, 1912) substrate hyphae. It produced branched substrate hyphae bearing single spores with warty surfaces (Fig. S1, available in IJSEM Online). No aerial mycelium was formed. Growth occurred at 0–10 % (w/v) NaCl, with good growth at 0–5 %. Other physiological characteristics are given in Table 1 and in the species description.

The cell-wall diamino acid of strain RtIII47^T was *meso*-diaminopimelic acid. The major whole-cell sugars were glucose, mannose, xylose and ribose. The acyl type of the muramyl residue was glycolyl. The predominant menaquinone was MK-9(H_4) (84.8 %), with minor amounts of MK-9(H_6) (11.2 %) and MK-9(H_2) (4.0 %). The phospholipids consisted of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol mannoside (PIM) and an unknown phospholipid (PL) (Fig. S2), phospholipid type PII according to Lechevalier *et al.* (1977). The cellular fatty acids of strain RtIII47^T were iso-$C_{16:0}$ (38.1 %), 10-methyl $C_{17:0}$ (12.9 %), iso-$C_{15:0}$ (11.4 %), $C_{17:0}$ (8.3 %), anteiso-$C_{17:0}$ (7.1 %), $C_{17:1}\omega 8c$ (6.4 %), iso-$C_{17:0}$ (2.4 %), $C_{18:0}$ (2.4 %), anteiso-$C_{15:0}$ (2.0 %), $C_{17:1}\omega 9c$ (1.9 %), 10-methyl $C_{16:0}$ (1.9 %), $C_{16:0}$ (1.5 %), iso-$C_{18:0}$ (1.4 %), iso-$C_{14:0}$ (1.1 %), $C_{18:1}\omega 9c$ (0.9 %) and 10-methyl $C_{18:0}$ (0.4 %). This fatty acid profile was similar to that of *V. lutea* YIM 013^T in our parallel test (Table S1). The G+C content of the DNA was 72.0 mol%. These chemotaxonomic results were largely consistent with those of members of the genus *Verrucosispora*, but revealed differences at the species level in polar lipids, whole-cell sugars and DNA G+C content.

An almost-complete 16S rRNA gene sequence (1484 nt) was obtained for strain RtIII47^T and compared with those deposited in public databases. A preliminary comparison indicated that it belonged to the family *Micromonosporaceae*. Phylogenetic analysis based on the 16S rRNA gene sequence (Fig. 1) showed that the strain formed a distinct line at the periphery of recognized members of the genera *Verrucosispora* and *Jishengella*, sharing the highest sequence similarity of 98.0 % with *V. lutea* YIM 013^T. The strain also shared over 97.5 % sequence similarity with *Micromonospora olivasterospora* DSM 43868^T (97.9 %), *Plantactinospora mayteni* YIM 61359^T (97.9 %), *Salinispora tropica* CNB-440^T (97.8 %), *Micromonospora peucetia* DSM 43363^T (97.7 %), *Micromonospora auratinigra* TT1-11^T (97.7 %), *V. sediminis* CGMCC 4.3550^T (97.6 %) and *Salinispora arenicola* CNH-643^T (97.5 %). The sequence similarities to other type strains of the family *Micromonosporaceae* were less than 97.5 %, and

Table 1. Phenotypic properties that distinguish strain RtIII47^T from recognized species of the genus *Verrucosispora*

Strains: 1, RtIII47^T; 2, *V. lutea* YIM 013^T; 3, *V. sediminis* CGMCC 4.3550^T; 4, *V. gifhornensis* JCM 10457^T. All physiological data and fatty acid data for *V. lutea* YIM 013^T were obtained in this study. Spore morphological characteristics and other chemotaxonomic data for recognized species are from previous studies (Rheims *et al.*, 1998; Liao *et al.*, 2009; Dai *et al.*, 2010). +, Positive; w, weakly positive; −, negative.

Characteristic	1	2	3	4
Spore arrangement	Single	Single, pairs, clusters	Single or clusters	Single, pairs, clusters
Spore-surface ornamentation	Warty	Smooth	Warty	Smooth, warty, hairy
Aerial mycelium	Absent	Sparse	Sparse	Absent
NaCl range (%, w/v)	0–10	0–7	0–6	0–4
Polar lipids*	PE, DPG, PIM, PL	PE, DPG, PIM, PI, PL	PE, DPG, PIM, PI, PL	PE, DPG, PIM, PS, PL
Whole-cell sugars†	Glc, Man, Xyl, Rib	Glc, Xyl, Man, Rib	GlcN, Glc, Man	Man, Xyl, Rib
Major fatty acids (>10 %)‡	i-$C_{16:0}$, i-$C_{15:0}$, 10-Me $C_{17:0}$	i-$C_{16:0}$, i-$C_{15:0}$, $C_{17:1}\omega 8c$, 10-Me $C_{17:0}$	$C_{17:0}$, i-$C_{16:0}$, i-$C_{15:0}$	i-$C_{16:0}$, i-$C_{15:0}$, ai-$C_{17:0}$
DNA G+C content (mol%)	72.0	69.3	66.8	70.0
Nitrite produced from nitrate	−	+	+	−
Utilization as carbon source:				
Trehalose	+	−	+	+
L-Rhamnose	+	−	−	−
D-Mannitol	+	−	+	+
D-Fructose	+	+	−	−
Glycerol	−	−	+	+
D-Ribose	+	−	+	−
Sucrose	+	−	+	+
Inositol	+	−	+	−
Utilization as nitrogen source:				
L-Alanine	+	−	+	w
L-Phenylalanine	−	−	+	+
Decomposition of:				
Adenine	+	−	+	w
Casein	−	−	+	−
Starch	−	+	+	+
Tween 80	−	+	+	+

*DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PS, phosphatidylserine; PL, unknown phospholipid.
†Glc, Glucose; GlcN, glucosamine; Man, mannose; Xyl, xylose; Rib, ribose.
‡ai, Anteiso-branched; i, iso-branched; Me, methyl.

that to *Jishengella endophytica* CGMCC 4.5597^T was only 96.6 %. To evaluate the phylogenetic position of strain RtIII47^T further, *gyrB* gene sequences of strain RtIII47^T (1126 nt), *Verrucosispora* type strains (1111–1118 nt), *J. endophytica* CGMCC 4.5597^T (1113 nt) and *P. mayteni* YIM 61359^T (482 nt, due to the failure of PCR amplification with primers GYF3/GYR3B) were also obtained and compared with those of members of the family *Micromonosporaceae* deposited in public databases. Phylogenetic analysis based on the *gyrB* gene (Fig. 2) confirmed that strain RtIII47^T should be assigned to the genus *Verrucosispora*, being most closely related to *V. lutea* YIM 013^T, with a sequence similarity of 95.3 %. *gyrB* gene sequence similarities between strain RtIII47^T and *V. sediminis* CGMCC 4.3550^T and *V. gifhornensis* JCM 10457^T were 93.8 and 93.1 %, respectively. *J. endophytica* JCM 10457^T also fell into the genus *Verrucosispora* in the *gyrB* gene tree, being most closely related to *V. gifhornensis* JCM 10457^T, with a sequence similarity of 94.3 %, and showing 93.2–93.5 % sequence similarity to the other two *Verrucosispora* type strains and strain RtIII47^T.

DNA–DNA relatedness between strain RtIII47^T and the closest type strain, *V. lutea* YIM 013^T, was 37.9 ± 2.1 %. According to the revised 16S rRNA gene sequence similarity threshold range of 98.7–99 % (Stackebrandt & Ebers, 2006), and a recent study that showed that, between *Verrucosispora* type strains, even 16S rRNA gene sequence similarity as high as 99.1 % corresponded to a low DNA–DNA relatedness of less than 60 % (Dai *et al.*, 2010), hybridizations between strain RtIII47^T and the other two *Verrucosispora* type strains were not performed. Based on the unique 16S rRNA gene sequence, the low *gyrB* gene sequence similarities and the level of

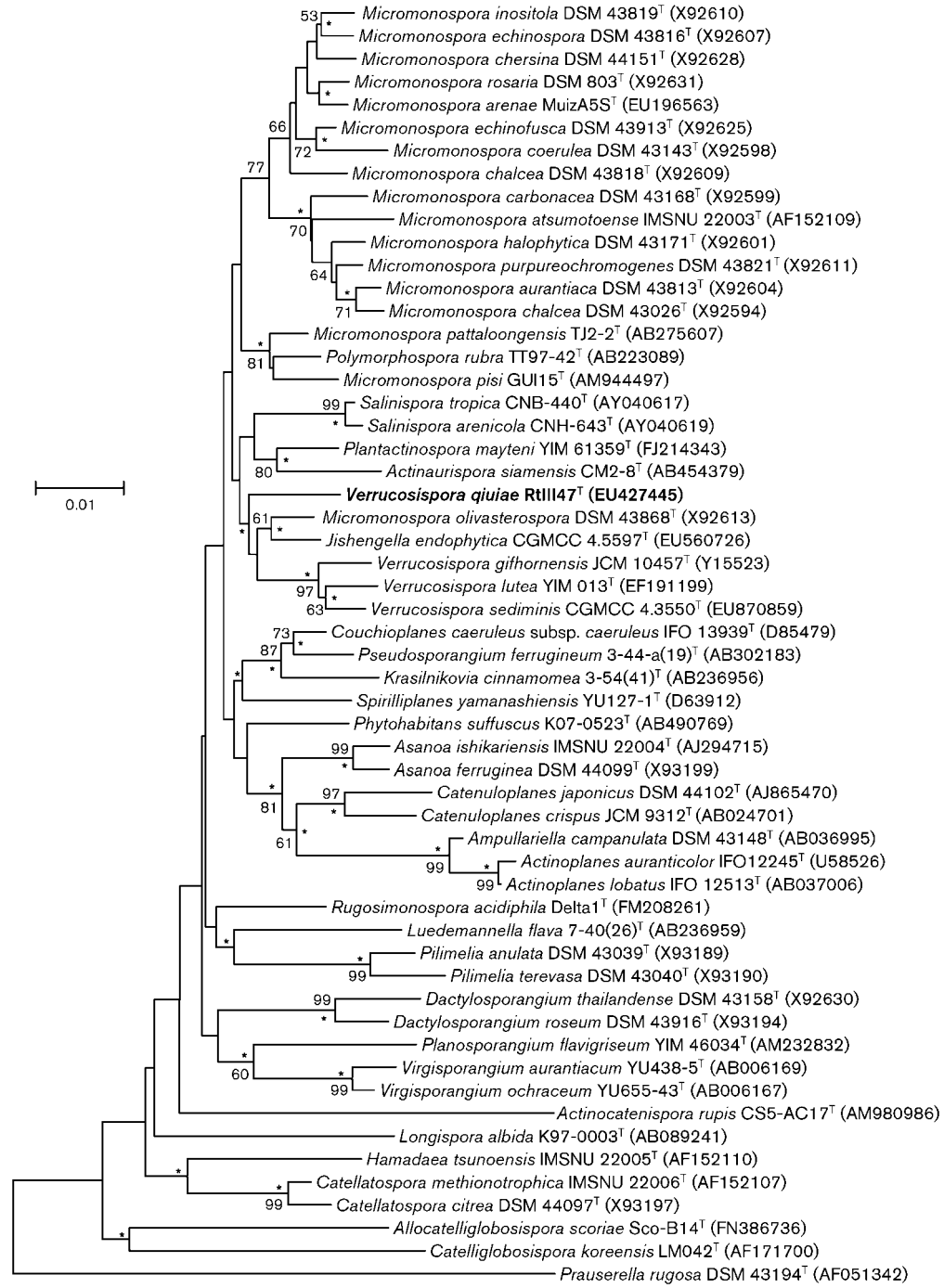

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing relationships between strain RtIII47^T, recognized species of the genus *Verrucosispora* and other representatives of the family *Micromonosporaceae*. Asterisks indicate branches recovered with both neighbour-joining and maximum-parsimony methods. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analysis of 1000 replicates (Saitou & Nei, 1987); only values >50 % are given. Bar, 0.01 substitutions per nucleotide position.

DNA–DNA hybridization, we propose that strain RtIII47^T is significantly different from recognized *Verrucosispora* species. In fact, a number of distinct phenotypic characteristics shown in Table 1 clearly distinguish strain RtIII47^T from its closest phylogenetic relatives. Therefore, strain RtIII47^T represents a novel species of the genus, for which the name *Verrucosispora qiuiae* sp. nov. is proposed.

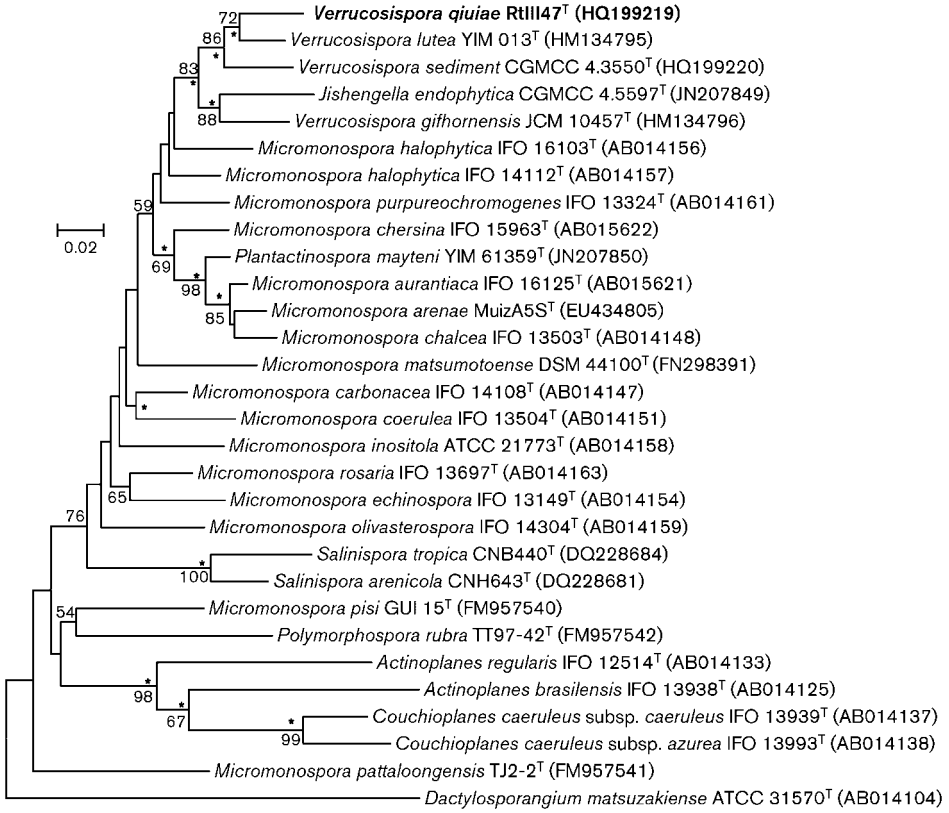

Fig. 2. Neighbour-joining phylogenetic tree based on *gyrB* gene sequences of strain RtIII47^T, recognized species of the genus *Verrucosispora* and other related members of the family *Micromonosporaceae*. Sequences were >1000 nt, except that of *P. mayteni* YIM 61359^T (482 nt). Asterisks indicate branches that were also found using the maximum-parsimony method (Fitch, 1971). Numbers at nodes indicate bootstrap percentages based on 1000 replicates; only values >40 % are given. Bar, 0.02 substitutions per nucleotide position.

Emended description of the genus *Verrucosispora* Rheims *et al.* 1998

The description of the genus *Verrucosispora* is as given previously (Rheims *et al.*, 1998), but with the following amendments. The diagnostic whole-cell sugar is mannose. The main phospholipids are PE, DPG and PIM. The major fatty acids (>10 %) are iso-$C_{16:0}$ and iso-$C_{15:0}$. The DNA G+C content is 66.8–72.0 mol%.

Description of *Verrucosispora qiuiae* sp. nov.

Verrucosispora qiuiae (qi.u.i′a.e. N.L. fem. gen. n. *qiuiae* of Qiu, in honour of Danheng Qiu, for her devotion to the investigation of *Micromonospora*-like actinomycetes).

Aerobic, Gram-reaction-positive actinomycete. Forms branched, bittersweet to xanthine orange substrate hyphae, but no aerial mycelium. Single spores are formed on substrate hyphae, and the spore surface is warty. Grows well on ISP 2, ISP 3, PDA, Sauton's agar and Gause inorganic agar, but grows weakly on ISP 5 agar. No soluble pigments are produced on ISP 7 agar. Does not reduce nitrate to nitrite. Utilizes L-rhamnose, D-glucose, D-xylose, trehalose, D-galactose, D-fructose, D-ribose, mannitol, D-arabinose, *myo*-inositol and sucrose as sole carbon sources, but not glycerol. L-Alanine is used as a sole nitrogen source, but L-phenylalanine is not. Negative for catalase and hydrolysis of starch and Tween 80. Hydrolyses adenine and guanine, but not hypoxanthine, xanthine or L-tyrosine. The maximum NaCl concentration for growth is 10 % (w/v), with an optimum at 0–5 %. The cell wall contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-9(H_4). The characteristic whole-cell sugars are mannose and xylose. The phospholipid profile is composed of DPG, PE, PIM and PL. The major (>10 %) fatty acids are iso-$C_{16:0}$, 10-methyl $C_{17:0}$ and iso-$C_{15:0}$.

The type strain, RtIII47^T (=CGMCC 4.5826^T =NBRC 106684^T), was isolated from sediment from a mangrove swamp in Sanya, Hainan Province, China. The DNA G+C content of the type strain is 72.0 mol%.

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