

Author version: *Antonie van Leeuwenhoek*, vol.102; 2012; 345-352

***Agromyces indicus* sp. nov., isolated from mangroves sediment in Chorao Island, Goa, India**

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NIO-1018^T is HM036655.

Abstract

A Gram-positive, non-motile, rod-shaped bacterium strain NIO-1018^T isolated from mangrove sediment sample of the Chorao Island, Goa, India, was subjected to a detailed polyphasic taxonomic study. The strain designated as NIO-1018^T matched with most of the phenotypic and chemotaxonomic properties of the genus *Agromyces* and represents novel species. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NIO-1018^T fell within the cluster comprising species of the genus *Agromyces*, clustering with *Agromyces soli* (98.1%), *Agromyces flavus* (97.9%), *Agromyces aurantiacus* (97.7%) and *Agromyces ulmi* (97.3%). The predominant menaquinone was MK-12, and the major cellular fatty acids were anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0} and iso-C_{15:0}. The polar lipids consisted of diphosphatidylglycerol, and phosphatidylglycerol. The genomic DNA G + C content of strain NIO-1018^T was 71.8 mol%. The combination of phylogenetic analysis, DNA-DNA relatedness, phenotypic characteristics and chemotaxonomic data supported the view that strain NIO-1018^T represents a novel species of the genus *Agromyces*, for which the name *Agromyces indicus* sp. nov. is proposed. The type strain is NIO-1018 (=JCM 17573^T=CCTCC AB2011122^T)

Key words: *Agromyces indicus* sp.nov., Polyphasic, 16S rRNA, Mangrove sediment.

Introduction

The genus *Agromyces* was proposed by Gledhill & Casida (1969) for the soil organism *Agromyces ramosus*, to accommodate filamentous, branching, fragmenting, nutritionally fastidious and catalase-negative actinomycetes. Zgurskaya *et al.*, (1992) emended the genus, for having characterized chemotaxonomically by menaquinone MK-12 and a peptidoglycan with 2, 4-diamino-n-butyric acid (DAB) as the principal amino acid. Species of the genus includes both non-filamentous-filamentous, microaerophilic-aerobic, catalase and oxidase variable actinomycetes (Takeuchi and Hatano, 2001; Li *et al.*, 2003; Ortiz-Martinez *et al.*, 2004; Jurado *et al.*, 2005 a, b, c; Jung *et al.*, 2007; Yoon *et al.*, 2008; Zhang *et al.*, 2010; Park *et al.*, 2010; Thawai *et al.*, 2011; Chen *et al.*, 2011). Currently, *Agromyces* genus harbors twenty four species (<http://www.bacterio.cict.fr/collections.html>). In the present paper we describe a Gram-positive, rod-shaped strain, designated NIO-1018^T, was isolated from a mangrove sediment sample in Chorao Island, Goa province, India. Based on phenotypic, chemotaxonomic and phylogenetic analyses, the isolate was affiliated with the genus *Agromyces*.

Materials and Methods

Isolation and cultivation of bacterial strain

Strain NIO-1018^T, isolated on International Streptomyces Project (ISP4-Inorganic Salts Starch) agar medium after about 2 weeks incubation at 28°C, originates from a sediment sample collected from intertidal region of mangroves at Chorao Island in Goa, India (GPS coordinates 15°32'34"N and 73°55'15" E). The strain was maintained on ISP 2 agar slants (yeast extract-malt extract) and ISP 5 (glycerol-asparagine) at 4°C and as glycerol suspensions (20%, v/v) at -80°C. Biomass for chemical and molecular-systematic studies was obtained following growth in shake flasks (about 200 r.p.m.) of ISP2 broth, supplemented with the vitamin mixtures of the HV medium (Hayakawa & Nonomura, 1987) at 28°C for 2 weeks.

Polyphasic taxonomic approach

Morphological features were determined on ISP 2 agar after 3 weeks at 28°C. Strain NIO-1018^T had morphological characteristics typical of the genus *Agromyces*, observed with light microscopy (BH2; Olympus) and by transmission electron microscopy (H-7650; Hitachi). Cells were rod-shaped, irregular, non-motile fragments. Cell dimensions were 0.4–0.9 X 1.2–1.5µm. Cultural characteristics were

determined after a week at 28°C by methods used in the ISP (Shirling & Gottlieb, 1966). ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and Bennett agar seeded with the strain NIO-1018^T were used for observation of the colony and cultural characteristics. Cultures were incubated at 28°C and properties were recorded **after seven days**. Color determinations were made by comparing the pure cultures with color chips from the ISCC-NBS Color Charts Standard Samples no. 2106 (Kelly, 1964). Media and procedures used for examination of physiological and biochemical features and carbon-source utilization were those described by Shirling & Gottlieb (1966) and Williams et al (1989). Growth was tested over a series of temperature (4, 10, 20, 28, 30, 32, 37, 40, 42 and 45 °C), NaCl concentrations (1, 3, 5, 7 to 10 %) (At intervals of 1 %) (w/v) on ISP 2 medium and pH (pH 4.0–11.0) (at intervals of 0.5 pH units) in ISP 2 broth medium using the buffer system: pH 4.0–5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0: 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0–10.0: 0.1 M NaHCO₃/0.1 M Na₂CO₃. The utilization of sugars and other compounds listed in API 20E kits (bioMérieux) as carbon sources was assessed according to the manufacturer's instructions. Acid production from carbohydrates was determined using API 50CH kits (bioMérieux), with API 50CHB as inoculation medium. Fingerprints of enzymic activities were attained using API ZYM test strips (bioMérieux) according to the manufacturer's instructions. Nitrogen assimilation was assessed using the following basal liquid medium (**g l⁻¹**): D-glucose 1, MgSO₄·7H₂O 0.05, NaCl 0.05, FeSO₄·7H₂O 0.01, K₂HPO₄ 0.01. Catalase activity was determined by production of bubbles after the addition of a drop of 3 % H₂O₂. Oxidase activity was determined using API oxidase reagent. Hydrolysis of urea was determined on peptone-glucose agar (**g l⁻¹**): peptone 1g, glucose 1g, NaCl 5g, KH₂PO₄ 2g, containing 2% (w/v) urea and 0.001% (w/v) phenol red. Hydrolysis of starch was determined on peptone-beef extract agar containing 0.2% (w/v) soluble starch by flooding of the plates with iodine solution. Hydrolysis of casein was tested on casein agar by observation of clear zones around the colonies. The incubation period for hydrolysis of urea, starch and casein was 4 d at 28°C. Gelatin hydrolysis was determined by incubation for 3 weeks at 28°C on peptone-gelatin medium (**L⁻¹**) (peptone 5g, gelatin 120g). Milk coagulation and peptonization was determined using 20% (w/v) skimmed milk as medium incubation for a week at 28°C. For the various biochemical analyses listed in Table 1 and in the species description, the culture was grown at 30°C in nutrient broth and the tests were performed as described by Lanyi (1987) and Smibert & Krieg (1994). In addition, the ability of strain NIO-1018^T to utilize carbon compounds as sole carbon and energy sources was determined in medium [1.05% (w/v) K₂HPO₄, 0.45% (w/v) KH₂PO₄, 0.1% (w/v) (NH₄)₂SO₄ and 1.5% (w/v) agarose] supplemented with 0.5% (w/v) of each filter sterilized carbon compound. Assimilation of various amino

acids as sole nitrogen sources was determined in a medium containing 1% (w/v) D-glucose, 0.05% (w/v) MgSO₄·7H₂O, 0.05% (w/v) NaCl, 0.001% (w/v) Fe₃SO₄·7H₂O, 0.1% (w/v) K₂HPO₄ and 1.5% (w/v) agarose, supplemented with the amino acid (0.2%,w/v) being tested. Nutrient agar with antibiotic discs (Hi-Media) was used for checking the sensitivity of strain NIO-1018^T to different antibiotics.

Chemotaxonomy

The peptidoglycan structure of the cell wall of strain NIO-1018^T was determined by using established procedures (Schleifer & Kandler, 1972; Schleifer, 1985; MacKenzie, 1987). Sugar analysis of the purified cell wall followed procedures described by Staneck & Roberts (1974). Polar lipids were extracted, analyzed by two-dimensional TLC (Minnikin et al., 1984), spraying with molybdenum-Blue and drying in incubator at 80°C, and mark the reaction sites with soft pencil before executing for a second spraying treatment with ninhydrin and plate was TLC developed at 120-160°C. Menaquinones were isolated according to Minnikin et al., (1984) and separated by HPLC. For fatty acids analysed, cells of strain NIO-1018^T were cultured on tryptic soy agar (TSA, Difco) at 28 °C for 4d. Cellular fatty acids analysis was performed as described by Sasser (1990) according to MIDI protocol by gas chromatography with flame ionization detector (GC-FID) using the Microbial Identification Software (MIDI Sherlock aerobic method and TSBA library version 4.0; Newark, DE, USA). For the measurement of chromosomal DNA G+C content, the genomic DNA of the strain was extracted, purified and degraded enzymatically into nucleosides; the DNA G+C content was determined using reverse-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions and re-extracted in n-hexane-water (1:1, v/v). The crude quinone in n-hexane was purified using Sep-Pak Vac silica cartridges (Waters) and then analyzed by HPLC, as described previously (Hiraishi et al., 1996).

Determination of 16S rRNA gene sequence, phylogenetic analysis and genomic relatedness

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Li et al (2007). The DNA G+C content of strain NIO-1018^T was determined using the thermal denaturation method (Marmur & Doty, 1962). PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li et al., (2007). Multiple alignments with sequences from the most closely related actinobacteria and calculations of sequence similarity were carried out using CLUSTAL_X (Thompson et al., 1997). A phylogenetic tree

(Fig. 1) was constructed by means of the neighbour-joining method of Saitou & Nei (1987), from *Knuc* values (Kimura, 1980), using MEGA, version 3.1 (Kumar et al., 2004). Alignment positions with insertions or deletions were excluded from the calculations. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. DNA-DNA hybridization was carried out according to the thermal renaturation method (De Ley et al., 1970), using a UV-1700 spectrophotometer (SHIMADZU) equipped with a DCW-2008 thermo bath.

Results and discussion

Phenotypic characterization

Colonies of strain NIO-1018^T were yellow on ISP 2, ISP 3, ISP 4, and ISP 5 agar, cream on ISP 6, and deep yellow on Bennett agar, lack of aerial mycelia. No diffusible pigment was observed. Young cultures produced branching substrate hyphae which broke up into rod-like fragments (Fig. 2). Aerial mycelium was absent. Cells were Gram-positive, short rods with 0.3-0.5 × 0.6-2.0 μm in size. Growth was observed at 10–40°C, and pH 5.0-9.5 with 0-3% (w/v) NaCl. Optimum growth was observed at 28-30°C, and pH 7.0-7.5, with 0-1% (w/v) NaCl. The detailed physiological and biochemical characteristics of the strain NIO-1018^T were given in the species description and table 1.

Chemotaxonomic characterization

Strain contained 2,4-diaminobutyric acid (DAB), glutamic acid, glycine and alanine in their cell walls. This composition is consistent with the B2γ-type peptidoglycan, which has been reported for all strains currently representing the genus *Agromyces*. Cell-wall sugars found in strains NIO-1018^T were rhamnose, glucose, galactose and xylose. Rhamnose and glucose have been reported for most species of the genus *Agromyces*. The major fatty acids found for strain NIO-1018^T were anteiso-C_{15:0} (36.8%), iso-C_{16:0} (25.6%) and anteiso-C_{17:0} (19.4%). This result is in agreement with other *Agromyces* species described (Suzuki *et al.* 1996; Takeuchi & Hatano, 2001; Li *et al.* 2003; Rivas et al., 2004). HPLC analysis of the menaquinones revealed major amounts of MK-12, a result that is also reported for many *Agromyces* species (Takeuchi & Hatano, 2001; Dorofeeva et al., 2003). The isolate NIO-1018^T contained the menaquinones MK-12, -11, and -10, (80:13:7). Polar lipids were studied for strain NIO-1018^T and were composed of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), two

unidentified phospholipid (PL) and one unknown glycolipid (GL)(Supplementary Fig.3). The genomic DNA G+C content of the strain NIO-1018^T was 71.8 mol%.

Molecular characterization

The almost complete 16S rRNA gene sequences (1454bp) of all strains were compared by sequence similarity calculations after a neighbour-joining analysis. The results of these calculations indicated that the closest relatives of strain NIO-1018^T were *Agromyces soli* (98.1%), *Agromyces flavus* (97.9%), *Agromyces aurantiacus* (97.7%) and *Agromyces ulmi* (97.3%) with 25, 30, 32, and 38 nucleotide differences respectively. The levels (in triplicate) of DNA–DNA hybridization between strain NIO-1018^T and *Agromyces soli*, *Agromyces flavus*, *Agromyces aurantiacus* and *Agromyces ulmi* were 29.0 ± 0.7 %, 23.6 ± 0.6 %, 19.7 ± 0.5 %, and 16.0 ± 0.4 % (Average value ± Standard deviation), respectively, which are all significantly below 70 %, the cut-off point recommended by Wayne et al (1987) for the recognition of genomic species, and indicating that it is not related to them at the species level (Stackebrandt & Goebel, 1994).

Conclusion

The phenotypic properties of strain NIO-1018^T and the 16S rRNA gene sequences comparison results supported to classify the novel isolate as a member of the genus *Agromyces*. Thus, on the basis of the phenotypic, genotypic and phylogeny combined with the DNA–DNA hybridization results, distinctly distinguish strain NIO-1018^T from other *Agromyces* members. This isolate represents a novel species within the genus, for which *Agromyces indicus* sp. nov. is proposed.

Description of *Agromyces indicus* sp. nov.

Agromyces indicus (in`di.cus. L. masc. adj. indicus pertaining to India, Indian).

Cells are Gram-positive, aerobic, non-motile. Catalase-positive while oxidase-negative. Colony color is pale yellow on ISP 2, ISP 3, ISP 4 and ISP 5, cream on ISP 6, whereas deep yellow on Bennett agar. Cells are rod-shaped, irregular, non-motile fragments. Cell dimensions are 0.4–0.9 X 1.2–1.5µm. Aerial mycelium is absent. Cell biomass and growth were enhanced by the addition of vitamins. Diffusible pigment is not observed. The temperature range for growth is 10–40 °C and good growth is observed at 28–30°C. Cells can grow in presence of 0-3 % NaCl. The pH range for growth is 5.0–9.5 and the

optimum is pH 7.0-7.5. N-acetyl-glucosamine, amygdalin, arbutin, L-arabinose, D-cellobiose, esculin, ferric citrate, D-fructose, D-galactose, β -gentiobiose, D-glucose, glycerol, inulin, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-ribose, salicin, sucrose D-tagatose, trehalose and D-xylose can be used as sole carbon sources for energy and growth, but not D-adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, gluconate, glycogen, inositol, D-lyxose, α -methyl D-glucoside, 2-ketogluconate, 5-ketogluconate, melezitose, α -methyl D-mannoside, β -methyl-D-xyloside, rhamnose, D-sorbitol, L-sorbose, starch, D-turanose, L-xylose, and xylitol. Weakly positive for maltose and lactose. Positive for hydrolysis of esculin, β -galactosidase and gelatin. Negative for arginine dehydrogenase, indole production, nitrate reduction and urease. The peptidoglycan amino acids are 2,4-diaminobutyric acid, alanine, glutamic acid and glycine, and cell-wall sugars are rhamnose, glucose and galactose. The predominant menaquinone is MK-12. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, two unidentified phospholipids and one unknown glycolipid. The cellular fatty acids profile is **anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0}, iso-C_{15:0}, anteiso-C_{15:1}, iso-C_{14:0}, iso-C_{17:0}, and C_{16:0}**. The DNA G+C content of the type strain is 71.8 mol%. The type strain is NIO-1018^T (JCM 17573^T=CCTCC AB2011122^T), isolated from an intertidal region of mangroves at Chorao Island in Goa provenance, India.

Acknowledgements

Author thanks Dr S R Shetye, Director, National Institute of Oceanography for facilities and encouragement. This work was supported from the grants under OLP0001 from the Council for Scientific and Industrial Research (CSIR), New Delhi. **This is NIO contribution number.**

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Table 1. Differential characteristics of strain NIO-1018^T and its phylogenetically closest relatives of the genus *Agromyces* Strains: 1, NIO-1018^T; 2, *Agromyces soli* KCTC 19549^T; 3, *Agromyces flavus* KCTC 19578^T; 4, *Agromyces aurantiacus* KCTC 9967^T; 5, *Agromyces mediolanus* JCM 346^T; 6, *Agromyces ulmi* KACC 20592^T. All data are from this study except where indicated. All strains are positive for aesculin hydrolysis, D-mannose and D-sucrose utilization, but negative for Gram- stain reaction, H₂S, 5-ketogluconate and indole production. Symbols: +, positive; -, negative; w, weak reaction.

Characteristics	1	2	3	4	5	6
Colony colour	Pale Yellow	Pale yellow	Pale yellow	Orange yellow	Yellow	White
Hyphae	Absent	Absent	Absent	Absent	Absent	Present
Nitrate reduction	-	-	+	-	+	-
Hydrolysis of						
Casein	+	-	+	-	+	-
Gelatin	+	-	+	-	+	-
Starch	+	-	+	+	-	-
Catalase	+	+	+	-	+	-
Growth at 37°C	+	-	+	+	+	+
Acid production						
N-Acetyl-glucosamine	+	+	+	-	-	+
Amygdalin	W	+	-	-	W	+
D-Arabinose	+	+	+	+	W	-
L-Arabinose	-	+	+	+	-	-
Arbutin	+	+	-	-	+	+
D-Fructose	+	+	-	+	-	+
L-Fucose	-	+	+	+	-	-
β-Gentibiose	+	+	-	-	-	+
Gluconate	+	+	+	-	-	-
Glycogen	+	+	+	+	-	+
Inulin	+	-	+	+	-	+
Lactose	W	-	+	-	-	+
D-Lyxose	-	-	+	+	-	+
D-Maltose	W	+	+	-	+	+
D-Mannitol	+	+	+	+	-	-
D-Melezitose	-	+	+	+	-	+
D-Melibiose	+	-	+	+	-	+
Methyl α-D-glucopyranoside	-	+	-	+	-	+
Methyl α-D-mannopyranoside	-	+	-	+	-	+

Raffinose	+	-	W	+	-	+
D-Ribose	+	+	-	-	+	+
Salicin	+	+	-	-	+	+
L-Sorbose	-	-	-	-	-	+
Starch	+	+	+	+	-	-
Trehalose	+	+	+	-	+	+
D-Turanose	-	+	+	W	+	-
Xylitol	-	-	-	-	-	+
D-Xylose	+	+	+	+	-	+
Enzyme Activity						
N-acetyl-β-glucosaminidase	-	+	-	W	+	-
Acid phosphatase	-	+	+	-	+	-
Alkaline phosphatase	+	+	+	-	-	-
Cystine arylamidase	-	+	+	-	+	+
α-Glucosidase	+	-	+	+	+	-
Naphthol-AS-B1-phosphohydrolase	-	+	+	+	+	+
Valine arylamidase	-	+	+	-	+	+
Cell-wall sugars	Rha, Glu, Gal, Xyl	Rha, Gal, Xyl	Rha, Glu, Gal	Rha, Gal, Glu, Man	Rha, Man, Gal	Rha, Fuc, Glu
Menaquinones	MK-12 (-11,-10)	MK-12 (-11, -10)	MK-12, (-11,-13)	MK-12, (-13,-11)	MK-12 (-11,-10)	MK-12 (-11,-10)
G+C content (mol %)	71.8	73.4	70.9	72.8	72.3	72.0
Source of isolation	Marine sediment	Farm soil	Natural soil	Forest soil	Soil	Decayed stump of an elm tree

Data for cell-wall sugars, menaquinones and G+C content (%) were collected from Suzuki et al., 1996; Li et al., 2003; Rivas et al., 2004; Lee et al., 2011; Chen et al., 2011. Gal, galactose; Glu, glucose; Fuc, fucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain NIO-1018^T among other members of the genus *Agromyces*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. *Microbacterium humi* CC-12309^T (GenBank accession number FJ865215) was used as an out group. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the minimum likelihood and maximum-parsimony algorithm. Bar, 0.005 substitutions per nucleotide position.

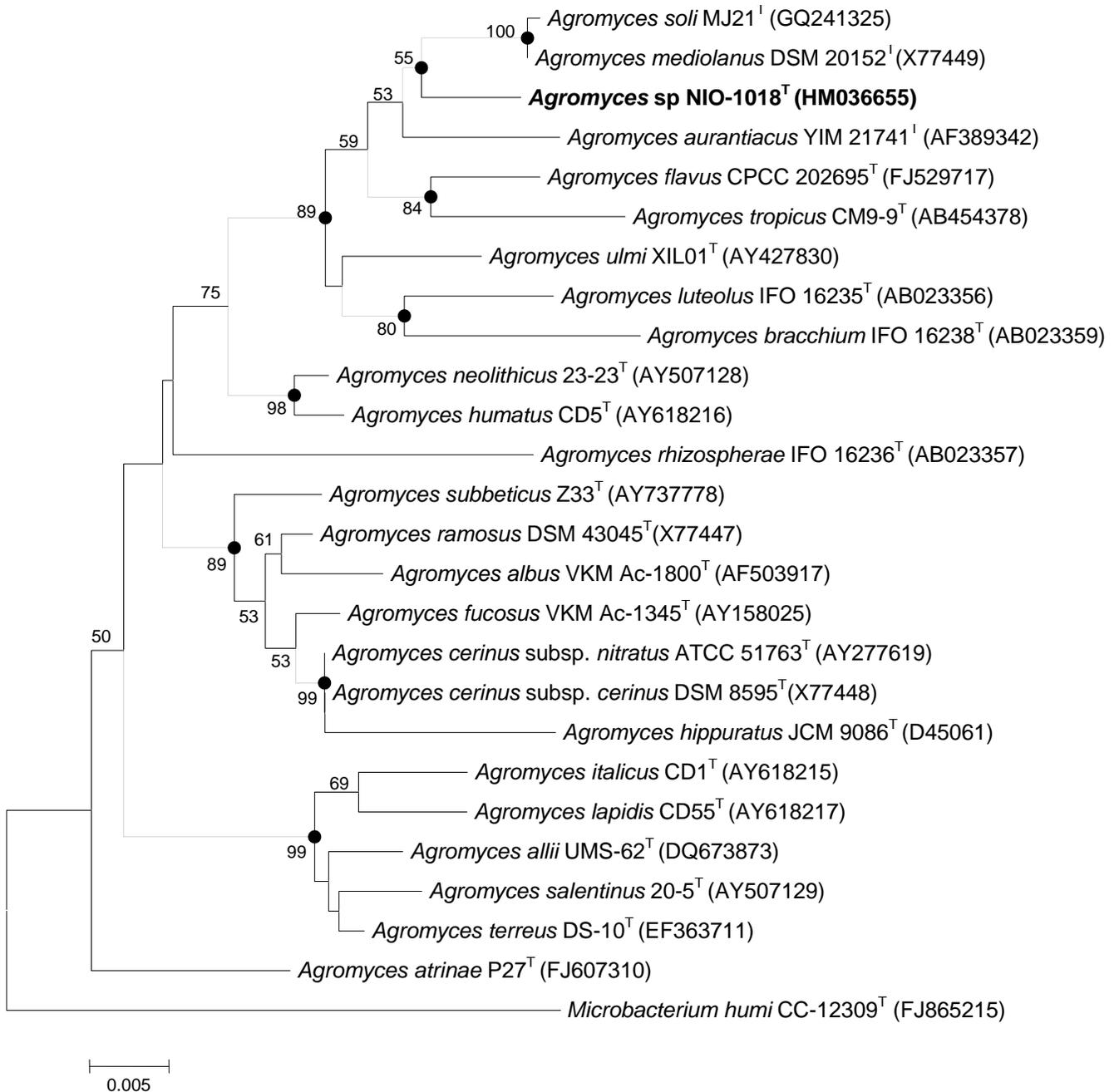
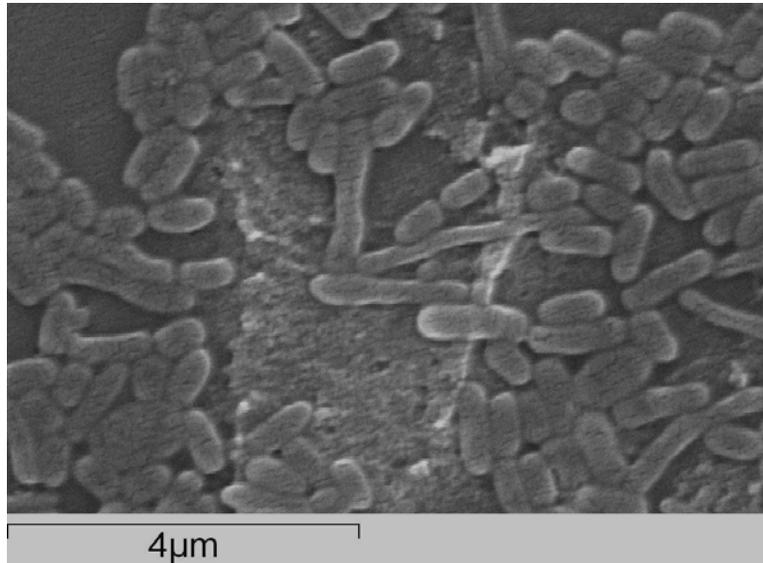


Fig. 2. Scanning Electron micrograph showing morphology of strain NIO-1018^T grown on TSA for 48h.



Supplementary Fig.3. Polar lipid composition of strain NIO-1018^T. I, first dimension of TLC; II, second dimension of TLC. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PL, unknown phospholipids; GL, glycolipid.

