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Nonomuraea monospora sp. nov., an antimicrobial and anticancer compound-producing actinomycete isolated from Thai cave soil and emended description of the genus *Nonomuraea*

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A novel antimicrobial and anticancer compound-producing actinomycete, strain PT708^T, was isolated from cave soil collected in Pha Tup Cave Forest Park, Nan province, Thailand. Chemotaxonomic properties of this strain were consistent with those of members of the genus *Nonomuraea*. The major menaquinone was MK-9(H_4), with minor amounts of MK-9(H_6), MK-9(H_2), MK-10(H_2) and MK-8(H_4). The polar lipid profile contained phosphatidylmonomethylethanolamine, diphosphatidylglycerol, hydroxy-phosphatidylmonomethylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositolmannoside and phosphatidylinositol. The major fatty acids were iso-16:0, 10-methyl 17:0, 16:0 and 17:1 *ω*6*c*. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain PT708^T belongs to the genus *Nonomuraea* and is most closely related to *Nonomuraea rhizophila* YIM 67092^T (98.50%) and *Nonomuraea rosea* GW 12687^T (98.30%). The 16S rRNA gene sequence similarity between strain PT708^T and other members of this genus were lower than 98%. The G+C content of the genomic DNA of strain PT708^T was 73.3 mol%. The distinctive morphology of this strain compared with that of other members in the genus *Nonomuraea* is the formation of single spores at the tips of aerial hyphae. Phenotypic and genotypic differences allowed the distinction of the strain from closely related species. Consequently, strain PT708^T represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea monospora* sp. nov. is proposed, with PT708^T (=TISTR1910^T =JCM16114^T) as the type strain.

The genus *Nonomuria* was described by Zhang *et al.* (1998) and Chiba *et al*. (1999) corrected the spelling to *Nonomuraea*. Species of this genus had been placed in the genera *Actinomadura* (Fischer *et al*., 1983; Athalye *et al*., 1985; Poschner *et al*., 1985) and *Microtetraspora* (Kroppenstedt *et al*., 1990). Because of their spore formation and 16S rRNA gene sequence data, which are distinct from other members of the family *Streptosporangiaceae*, these species were reclassified into a new genus called *Nonomuraea*. At the time of writing, the genus comprises of 27 species and 2 subspecies; *Nonomuraea pusilla* is the type species (Gyobu & Miyadoh, 2001; Stackebrandt *et al.*, 2001; Quintana *et al.*, 2003; Ara *et al.*, 2007 a,b; Le Roes & Meyers, 2008; Kämpfer *et al*., 2010; Li *et al*., 2011; Wang *et al.*, in press; Xi *et al.*, in press; Zhao *et al*., in press). There are diverse natural habitats from which to isolate strains of *Nonomuraea*, including soil, rhizosphere soil, marine and river sediments, caves and plants. Discovery of novel actinomycetes is still valuable to agriculture, medicine and industry. In this report we describe the identification, classification and

nomenclature of a novel antimicrobial and anticancer compound-producing actinomycete, strain PT708^T, isolated from Thai cave soil, which showed a close phylogenetic relationship to the genus *Nonomuraea.*

Soil samples were collected from the Pha Tup Cave Forest Park, Nan province, Northern, Thailand. Soil samples were pretreated with dry heat in a hot air oven at 120°C for 1 hr followed by phenol treatment (Hayakawa *et al.*, 1995) to isolate rare actinomycetes. The soil suspension was spread onto Humic acid-Vitamin (HV) agar (Hayakawa & Nonomura, 1987) containing nystatin and cycloheximide at final concentrations of 50 µg ml^{-1}. The pure isolate was maintained as a working culture on Hickey-Tresner (HT) agar (Hickey & Tresner, 1952) at 4°C and in 20% (v/v) glycerol at -20°C for long term storage.

The capacity of strain PT708^T to produce antibiotics was screened by paper disk diffusion assays after incubation of the strain in AMHU-5 medium and extraction of the cell-free supernatant with ethyl acetate (Nakaew *et al*., 2009). The crude extract was used to determine minimum inhibitory concentrations (MICs) against bacteria: *Bacillus cereus* TISTR 687, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Paenibacillus larvae* LMG 9820, *Staphylococcus aureus* TISTR 517, methicillin-resistant *Staphylococcus aureus* (MRSA) provided by the Department of Associated Medical Science, Chiang Mai University; yeast: *Candida albicans*; and filamentous fungi: *Fusarium oxysporum, Didymella* sp., *Collectotrichum* sp. and *Sclerotium solani* obtained from the Excellent on Sustainable Development of Biodiversity Resources Center, Chiang Mai University, Thailand. The anticancer activity of strain PT708^T against cancer cell lines [human breast cancer (MCF7), human oral cavity cancer (KB), and human small cell lung cancer (NCI-H187)] were determined by the sulphorhodamine B (SRB) assay (Skehan *et al.*, 1990) using the same crude extract as described previously. Doxorubicin and ellipticine were used as positive controls and dimethylsulphoxide (DMSO) as a negative control. The half maximal inhibitory concentration (IC_{50}) was defined as the concentration of crude extract that inhibited 50% of the growth of each cell line.

Morphological and colony characteristics were observed on International *Streptomyces* Project (ISP) media, ISP2; ISP3 and ISP4 (Shirling & Gottlieb, 1966), Czapek's and nutrient agars (Waksman, 1967) at 30°C for 15-30 days. The features of substrate and aerial mycelia and spores were observed by light microscopy (Olympus BH-2) and

scanning electron microscopy (model JSM-5910, JEOL). The colours of colonies and soluble pigments were determined using the NBS/IBCC colour chart (Mundie, 1995). The physiological characteristics, including the ability to grow on a range of sole carbon sources at 1% (w/v) (Pridham & Gottlieb, 1948), degradation of L-tyrosine and casein (Goodfellow, 1971), and utilization of gelatin and starch (Shirling & Gottlieb, 1966), were evaluated.

The biomass for chemotaxonomic studies was obtained after shaking incubation using tryptic soy broth (TSB) at 28°C for 7 days. The isomeric form of diaminopimelic acid and the whole cell sugars were examined according to Hasegawa *et al.* (1983). Menaquinones and polar lipids were extracted and analyzed by 2-dimensional TLC as described by Collins *et al*. (1977) and Minnikin *et al*. (1979), respectively. Cellular fatty acids were also extracted from strain biomass obtained using the protocol of the MIDI system (Microbial ID) version 4.0, the gas chromatograph used is Hewlett Packard HP 5890 Series II GC with an Ultra 2 capillary column (0.2 mm × 25 m). All peaks generated were automatically analyzed by the Microbial Identification software using the ACTINO database (Sasser, 1990) and Kämpfer & Kroppenstedt (1996).

Genomic DNA was extracted from biomass obtained from shaking incubation in ISP2 broth at 28°C for 14 days using the method described by Hopwood *et al.* (1985). The GC content of the DNA was quantified by HPLC according to the protocol of Mesbah *et al*. (1989). The PCR technique was used to amplify the 16S rRNA gene using the universal primers (Lane, 1991) 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'- AAGGAGGTGWTCCARCC-3'). The sequence obtained was compared with all sequences from GenBank using the BLAST program. A multiple sequence alignment was generated and a phylogenetic tree was constructed using the neighbor-joining method of Saitou & Nei (1987) in the Molecular Evolutionary Genetics Analysis (MEGA) program version 4 (Tamura *et al*., 2007). The sequence similarity was computed using the PHYDIT program.

The G+C content in the genomic DNA of strain PT708^T was 73.3 mol%. An almost complete 16S rRNA gene sequence (1453 nucleotides) of strain PT708^T was obtained and compared with representative members of the family *Streptosporangiaceae*. The phylogenetic tree based on the neighbour-joining method showed that strain PT708^T fell within the evolution radiation of the genus *Nonomuraea*. It is evident that strain PT708^T formed a subclade with *Nonomuraea rhizophila* YIM 67092^T (HM755723) and

Nonomuraea rosea GW 12687^T (FN356742) supported by a bootstrap value of 97% (**Fig. 1**). Strain PT708^T shared 16S rRNA gene sequence similarity values of 98.50% and 98.30% with *N. rhizophila* and *N. rosea*, respectively. High similarity values within the range of 98.7–99 % might not be enough to identify strains as novel species (Stackebrandt & Ebers, 2006). Similarity values between 97.1 and 100% have been reported for several members of the genus *Nonomuraea* that showed low DNA:DNA relatedness values (Fischer *et al.*, 1983; Poschner *et al*., 1985; Stackebrandt *et al*., 2001). The type strains of *Nonomuraea kuesteri* and *Nonomuraea turkmeniaca*, for instance, shared a 16S rRNA gene sequence similarity value of 98.9%, but a DNA:DNA relatedness value of 40.5% (Kämpfer *et al*., 2005). Similarly with the study of *Nonomuraea dietziae* and *N. roseola*, which showed 100% 16S rRNA gene sequence similarity value, but only 31% DNA:DNA relatedness (Stackebrandt *et al*., 2001).

Whole-cell hydrolysates of strain PT708^T contained *meso*-DAP, madurose, galactose and arabinose corresponding to cell wall type IIIB (Lechevalier & Lechevalier, 1970). The major menaquinone of strain PT708^T was MK-9(H_4) (73%), with minor amounts of MK-9(H_6) (10%), MK-9(H_2) (9%), MK-10(H_2) (3%) and MK-8(H_4) (3%). This is in good agreement with the menaquinones reported for other members of the genus *Nonomuraea*, where MK-9(H_4) or MK-9(H_6) is the major menaquinone (Kroppenstedt & Goodfellow, 1991; Stackebrandt *et al*., 2001; Quintana *et al*., 2003). Strain PT708^T contained a polar lipid profile of diphosphatidylglycerol (DPG), phosphatidylmonomethylethanolamine (PME), phosphatidylethanolamine (PE), hydroxy-phosphatidylmonomethylethanolamine (OH-PME), phosphatidylglycerol (PG), hydroxy-phosphatidylethanolamine (OH-PE), phosphatidylinositolmannoside (PIM), phosphatidylinositol (PI) and an aminophosphoglycolipid (APGL; possibly an N-acetylglucosamine-containing phospholipid). This polar lipid profile is mostly related to those found for recognized *Nonomuraea* species, however, it differs from *N. rhizophila* as OH-PME and OH-PE were not found in *N. rhizophila* (Zhao *el al*., in press). Strain PT708^T produced a significant amount of OH-PME, but a low amount of OH-PE (**Supplementary Fig. S1**). The major fatty acids were iso-16:0 (19.6%), 10-methyl 17:0 (14.8%), 16:0 (7.6%), 17:1 ω6*c* (6.8%), iso-15:0 (6.1%), iso-16:1 G (6.0%), 10-methyl 16:0 (5.1%), 17:1 ω8*c* (5.0%) and 16:1 ω7*c*/ iso-15:0 2OH (4.8%) and the minor fatty acids were 15:0 (3.6%), 10-methyl 18:0 (3.6%), 14:0 (3.2%), 16:0 2OH (2.8%), 18:0 (1.9%), 17:0 (1.8%), iso-17:0 (1.5%), iso-14:0 (1.4%), 18:1 ω9*c* (1.4%) and anteiso-17:0 (1.3%). The major fatty acids are different with those of *N. rhizophila*, reported as 10-methyl 17:0 (26.66%), iso-16:0 (24.00%), iso-16:1 G (14.11%), 17:1 ω6*c* (5.63%),

iso-15:0 (4.57%), and no 16:1 ω7*c*/ iso-15:0 2OH was found (Zhao *el al*., in press). As 2-hydroxy fatty acids are the precursors for production of OH-PE and OH-PME, the presence of 2-hydroxy fatty acids; 16:1 ω7*c*/ iso-15:0 2OH (4.8%), 16:0 2OH (2.8%) and 15:0 2OH (0.8%) in strain PT708^T is similar to the proportions found in *N. rosea*; 16:1 ω7*c*/ iso-15:0 2OH (4.2%), 16:0 2OH (2.7%) and 15:0 2OH (0.8%) even though the growth medium used was DSMZ medium 65 not TSB (Kämpfer *et al*., 2010). These chemotaxonomic features of strain PT708^T are consistent with membership of the genus *Nonomuraea*.

Staining of the mycelium of strain PT708^T and observation by light microscopy showed that it was Gram-positive with single spores located on the end of each branched hypha (**Fig. 2-A**). The production of single spores is unique to this strain in the genus *Nonomuraea*. Colony morphology, soluble pigment production and amount of growth after cultivation in ISP2, ISP3, ISP4, Czapek's and nutrient agars at 30°C for 15-30 days, compared with *N. rhizophila* are summarized in **Supplementary Table S1** (Zhao *et al*., in press). The cultural characteristics of these strains are distinct. The spore characteristics of strain PT708^T are clearly different from those of its closest phylogenetic relatives after cultivation and observation on ISP3 (**Table 1)**. The features of substrate mycelium, aerial mycelium and single spores of strain PT708^T under scanning electron microscope after cultivation for different periods of time are shown in **Fig. 2**. The diameters of mature single spores (1 month age) varied between 1.5 and 1.7 µm. Biochemical tests of strain PT708^T compared with its closest phylogenetic relatives are summarized in **Table 1** and in the species description. The results show that the strain is clearly different from its phylogenetic relatives. Moreover, the strain was able to produce antimicrobial substances when it was grown in AMHU-5 medium against *B. cereus* TISTR 687, methicillin-resistant *S. aureus* (MRSA) and *P. larvae* LMG 9820 with MIC values of 80, 80 and 175 µg ml^{-1}, respectively. This crude extract also showed the anticancer activity against human small lung cancer cells (NCI-H187) and oral cavity cancer cells (KB) with IC_{50} values of 3.48 and 16.11 µg ml^{-1}, respectively, but no inhibition was observed against breast cancer cells (MCF7) at concentrations up to 50 µg ml^{-1} (Nakaew *et al*., 2009).

According to the chemotaxonomic data together with 16S rRNA gene sequence data, the strain PT708^T should be assigned to the genus *Nonomuraea*. However, the differences in morphological and biochemical characters support the proposal that strain PT708^T represents a novel species of the genus *Nonomuraea*, for which the name

Nonomuraea monospora sp. nov. is proposed.

Description of *Nonomuraea monospora* sp. nov.

Nonomuraea monospora (mo.no.spo´ra. Gr. adj. *monos*-, single; N.L. fem. n. *spora* (from Gr. fem. n. *spora*, seed), spore; N.L. fem. n. *monospora*, single spore)

Gram-positive, the colours of the substrate mycelium vary depending upon the medium used: deep red (ISP2 and HT agar), red (ISP3), vivid yellow pink (ISP4), vivid reddish orange (NA) and brilliant orange yellow (Czapek's agar). White aerial mycelium is observed when cultured on ISP3, ISP4, HT and Czapek's agar. Production of a soluble pigment occurs on ISP2, ISP3 and HT agars. Single spores are observed when cultured on ISP4 for 16 days at 30°C. Sporangia are not found. Mature spore diameters when cultured on ISP4 vary between 1.5 and 1.7 μm. Citrate, L-arabinose, cellobiose, D-fructose, myo-inositol, mannitol, D-mannose, L-rhamnose, sucrose, D-xylose and lactose are utilized as sole carbon sources, but D-raffinose is not utilized. Gelatin, starch, casein and L-tyrosine are decomposed. Antimicrobial substances are produced which are active against *Bacillus cereus* TISTR 687 (MIC, 80 μg ml^{-1}), methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC, 80 μg ml^{-1}) and *Paenibacillus larvae* LMG 9820 (MIC, 175 μg ml^{-1}). Anticancer substances against human small lung cancer cells (NCI-H187) and oral cavity cancer cells (KB) are produced with IC_{50} values of 3.48 and 16.11 μg ml^{-1}, respectively. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Cell hydrolysates contain madurose, galactose and arabinose. The predominant menaquinone is MK-9(H_4) (73%), with minor amounts of MK-9(H_6) (10%), MK-9(H_2) (9%), MK-10(H_2) (3%) and MK-8(H_4) (3%). The polar lipid profile is composed of diphosphatidylglycerol (DPG), phosphatidylmonomethylethanolamine (PME), phosphatidylethanolamine (PE), hydroxy-phosphatidylmonomethylethanolamine (OH-PME), hydroxy-phosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), phosphatidylinositolmannoside (PIM) and phosphatidylinositol (PI). The major fatty acids (>4%) are iso-16:0, 10-methyl 17:0, 16:0, 17:1 *ω*6*c*, iso-15:0, iso-16:1 G, 10-methyl 16:0, 17:1 *ω*8*c* and 16:1 *ω*7*c*/ iso-15:0 2OH, and minor fatty acids are 15:0, 10-methyl 18:0, 14:0, 16:0 2OH, 18:0, 17:0, iso-17:0, iso-14:0, 18:1 *ω*9*c* and ante-iso-17:0. The G+C content of the genomic DNA of the type strain is 73.3 mol%.

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The type strain is PT708^T (=TISTR1910^T =JCM16114^T), which was isolated from a cave soil sample collected from Pha Tup Cave Forest Park, Nan province, Thailand.

Emended description of the genus *Nonomuraea*

The description of the genus is as given by Zhang *et al*. (1998) with the following changes. Aerial hyphae generally bear chains of spores which are hooked, spiral or straight, but single spores may be produced. The G+C range is 64-74 mol%.

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Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses), indicating relationships between *Nonomuraea monospora* sp. nov. PT708^{T} and recognized species of the genus *Nonomuraea*. The out-group used was *Thermopolyspora flexuosa*. Clustering was carried out using the neighbour-joining method, provided by the software package MEGA program, version 4 (Tamura *et al*., 2007), based on 1432 nucleotides (with gaps). Bootstrap values based on 1000 replications are shown as percentages at branching points. Bar, 0.005 K_{nuc}.

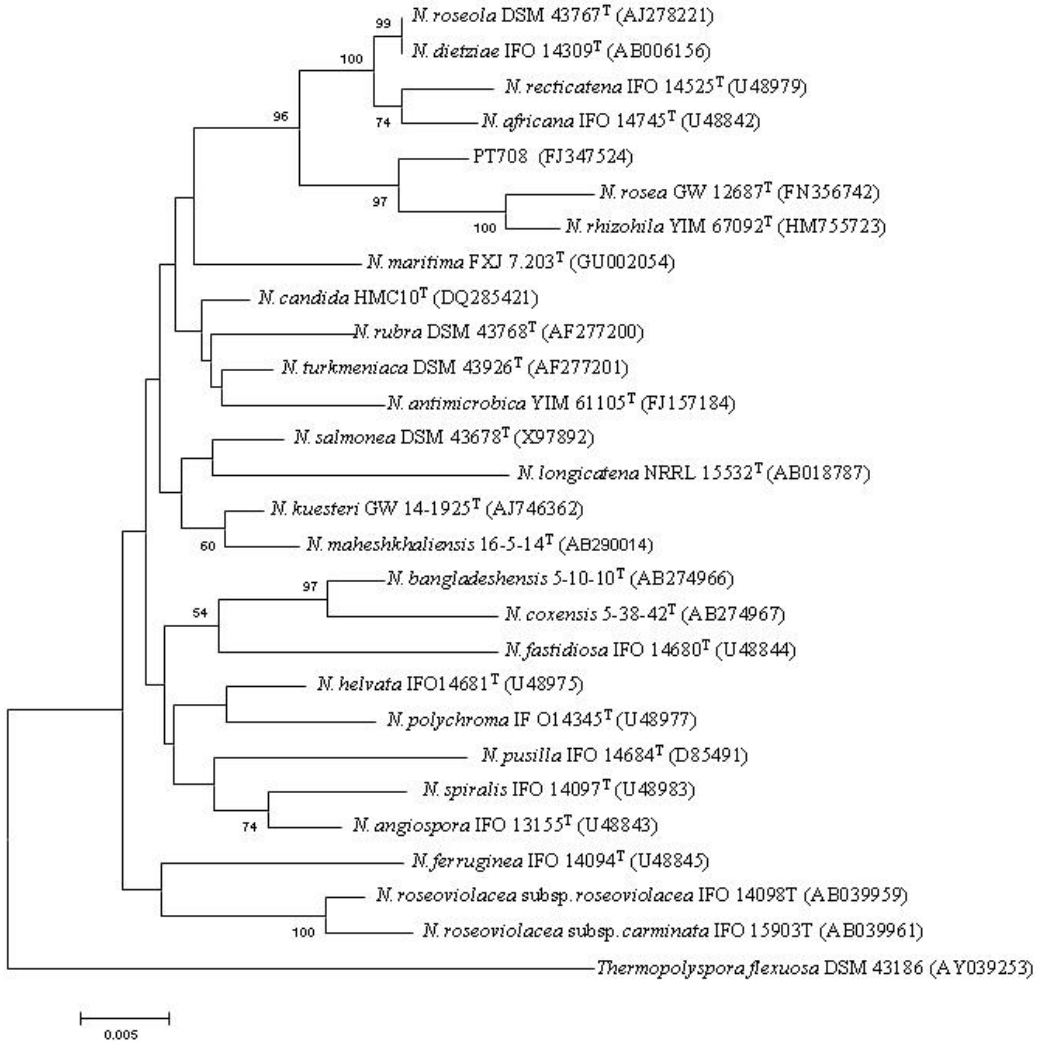

Fig. 2. Light micrograph of strain PT708^{T} showing Gram-positive hyphae and single spores at the hyphal tips after growth on ISP4 agar at 30°C for 16 days; bar 2 μm (**A**). Scanning electron micrographs showing single spores on the tips of branched mycelium after growth on ISP4 agar at 30°C for 16 days; bar 2 μm (**B**) and close-up views of a single spore after growth on ISP4 agar at 30°C for 30 days; bar 1 μm (**C**) and bar 0.5 μm (**D**).

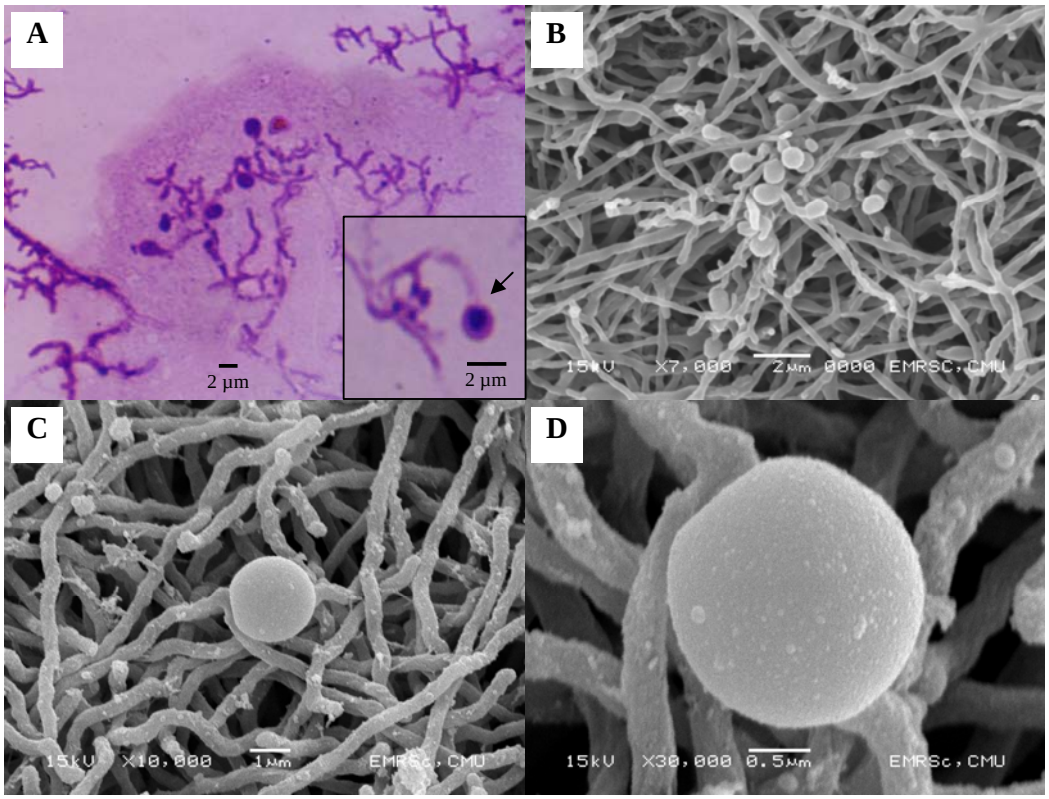

Table 1. Comparison of phenotypic characteristics between strain PT708^T and the closest species *Nonomuraea rhizophila* YIM 67092^T after cultivation at 30ºC for 15 days. Symbols and abbreviations assigned: +, positive; -, negative; ND, not determined.

Characteristic	**Strain PT708^T**	***N. rhizophila* YIM 67092^T**
Spore morphology:		
Spore arrangement	Single spores at the tips of aerial hyphae	Spirals of one or two turns
Spore ornamentation	Smooth	Rough
Number of spore	1	7-10
Growth on ISP3 medium:		
Aerial mycelium	White	White
Substrate mycelium	Vivid red	Brown-yellow
Soluble pigment	Vivid red	None
Biochemical tests:		
Nitrate reductase	+	+
Utilization of:		
L-Arabinose	+	-
Cellobiose	+	+
D-Fructose	+	+
myo-Inositol	+	+
Mannitol	+	+
D-Mannose	+	+
L-Rhamnose	+	+
D-Raffinose	-	+
Sucrose	+	-
D-Xylose	+	-
Lactose	+	+
Degradation of:		
Gelatin	+	-
Starch	+	-
Tyrosine	+	+
Casein	+	+