Amycolatopsis stemonae sp. nov., isolated from a Thai medicinal plant

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A novel actinomycete, strain ST1-08^T, was isolated from the stem of *Stemona* sp. in Thailand. The taxonomic position of this isolate was determined by using a polyphasic approach. Strain ST1-08^T contained *meso*-diaminopimelic acid in the cell-wall peptidoglycan, and arabinose and galactose as diagnostic sugars of the whole-cell hydrolysate, which are typical properties of members of the genus *Amycolatopsis*. Strain ST1-08^T grew at 15–40 °C, pH 6–9 and on 5 % (w/v) NaCl. Gelatin liquefaction, starch hydrolysis and skimmed milk peptonization were positive. The strain utilized L-arabinose, D-glucose, glycerol, *myo*-inositol, D-mannitol and L-rhamnose. The predominant menaquinone was MK-9(H_4) and the major cellular fatty acids were iso-$C_{16:0}$ and iso-$C_{15:0}$.The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, hydroxyl-phosphatidylethanolamine, phosphatidylinositol and phosphatidylglycerol. The 16S rRNA gene sequence analysis revealed that the strain was closely related to *Amycolatopsis pretoriensis* JCM 12673^T (98.99 %) and *Amycolatopsis lexingtonensis* JCM 12672^T (98.87 %). The DNA G+C content of strain ST1-08^T was 71.2 mol%. The DNA–DNA relatedness values among strain ST1-08^T, *A. pretoriensis* JCM 12673^T and *A. lexingtonensis* JCM 12672^T were lower than 70 %, the cut-off level for assigning strains to the same species. On the basis of phenotypic and genotypic characteristics, strain ST1-08^T represents a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis stemonae* is proposed. The type strain is ST1-08^T(=JCM 30050^T=PCU 339^T=TISTR 2278^T).

The genus *Amycolatopsis* belongs to the family *Pseudonocardiaceae* and comprises Gram-stain-positive bacteria that form squarish to rod-shaped fragments on substrate and aerial mycelia. This genus can be separated from others by using morphological and chemotaxonomic characteristics and 16S rRNA gene sequence analysis (Lechevalier *et al.*, 1986; Labeda & Goodfellow, 2012; Tan & Goodfellow, 2012). At the time of writing, the genus *Amycolatopsis* comprises 65 species with validly published names (Euzéby, 2014). Members of the genus *Amycolatopsis* are widely distributed, being isolated from different sources such as equine placenta (Labeda *et al.*, 2003), a human source (Huang *et al.*, 2004), volcanic soil (Ding *et al.*, 2007), rhizospheric soil (Lee, 2009), polluted sediment (Albarracín *et al.*, 2010), arid soil (Zucchi *et al.*, 2012), ocean sediment (Bian *et al.*, 2009) and plants (Duangmal *et al.*, 2011; Miao *et al.*, 2011; Xing *et al.*, 2013). Their secondary metabolites are often utilized as antibiotics (Labeda, 1995) and anti-tumour agents (Kwon *et al.*, 2014). In Thailand, a poly(L-lactic acid)-degrading species, *Amycolatopsis thailandensis*, isolated from soil (Chomchoei *et al.*, 2011) and *Amycolatopsis samaneae* isolated from surface-sterilized roots of *Samanea saman* (Jacq.) Merr. (Duangmal *et al.*, 2011) have been reported. In the course of our investigation on diversity of actinomycetes in medicinal plants, strain ST1-08^T was isolated. In this study, we describe the taxonomic position of strain ST1-08^T as a member of the genus *Amycolatopsis* based on a polyphasic approach.

Strain ST1-08^T was isolated from the stem of *Stemona* sp., a monocotyledon vine in the family *Stemonaceae* (Fig. S1, available in the online Supplementary Material) which was collected from the botanical garden, Faculty of Pharmaceutical

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ST1-08^T is LC011703.

Three supplementary figures and a supplementary table are available with the online Supplementary Material.

Sciences, Chulalongkorn University, Bangkok, Thailand. The leaves and roots of some *Stemona* species contained alkaloids that show insecticidal, larvicidal, toothache-relieving and antitussive activities (Greger, 2006). The surface of the plant sample was washed to remove soil particles and sterilized by soaking in 95 % ethanol for 10 min, followed by 1 % (w/v) sodium hypochlorite for 15 min, and 10 % (w/v) sodium bicarbonate for 10 min before rinsing with sterilized water three times (Indananda *et al.*, 2011). The sample was aseptically cut into small pieces (0.5 × 0.5 cm) and incubated on starch-casein agar (Küster & Williams, 1964) containing nalidixic acid (25 mg l^{-1}) and cycloheximide (50 mg l^{-1}). The colonies of endophytic actinomycetes were picked up to purify on yeast extract-malt extract agar [International *Streptomyces* Project (ISP) 2 medium; Shirling & Gottlieb, 1966] after incubation at 30 °C for 21 days. The purified cultures were preserved as colonies on ISP2 slants for short-term storage and as lyophilized cells in 10 % skimmed milk for long-term storage.

Morphological characteristics and the features of substrate mycelium, aerial mycelium and spores were observed using light and scanning electron microscopy (JSM-5410LV) after incubation on ISP2, ISP3 and ISP4 agar at 30 °C for 14 days. Cultural characteristics of strain ST1-08^T and related type strains, including the ability of growth, the colour of colonies and soluble pigments on various media (Shirling & Gottlieb, 1966), were determined using the NBS/IBCC colour chart (Mundie, 1995) after incubation at 30 °C for 14 days.

Physiological and biochemical characteristics of strain ST1-08^T and related type strains including growth at various pH, temperature and NaCl (%), using ISP2 medium, the utilization of various carbohydrates as sole carbon source on ISP9 medium, gelatin liquefaction, milk peptonization, nitrate reduction and starch hydrolysis were determined as previous reports (Arai, 1975; Williams & Cross, 1971) after incubation at 30 °C for 14 days. Acid production from carbohydrates was determined as described by Gordon *et al.* (1974).

Freeze-dried cells for chemotaxonomic studies were obtained from cultures grown in yeast extract-glucose broth on a rotary shaker (150 r.p.m.) at 30 °C for 4–7 days. The isomers of diaminopimelic acid were determined by TLC following the method of Staneck & Roberts (1974). The whole-cell sugars were analysed using HPLC as described by Mikami & Ishida (1983). Phospholipids were analysed by two-dimensional-TLC as described by Minnikin *et al.* (1984). Cellular fatty acids were analysed using GC according to the protocol of the MIDI Sherlock Microbial Identification System (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The mycolic acid was analysed by TLC as described by Tomiyasu (1982). Menaquinones were extracted according to the method of Collins *et al.* (1977) and analysed by HPLC.

Genomic DNA was prepared as described by Tamaoka (1994). The DNA base composition was measured using HPLC according to the method of Tamaoka & Komagata (1984). The 16S rRNA gene was amplified by PCR as described previously (Yamada *et al.* (2000), and the PCR products were sequenced (Macrogen) using universal primers (Lane, 1991). The 16S rRNA gene sequence of strain ST1-08^T was aligned using BioEdit software and compared with related sequences from the GenBank/EMBL/DDBJ databases with the sequence similarities determined by the EzTaxon-e database BLAST program (Kim *et al.*, 2012). A phylogenetic tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987) in MEGA 5.0 software (Tamura *et al.*, 2011), comparing with maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Felsenstein, 1983) methods. The confidence values of branches of the phylogenetic tree were determined using bootstrap resampling with 1000 replication (Felsenstein, 1985). DNA–DNA relatedness between strain ST1-08^T and closely related type strains were determined using a colorimetric method according to the procedure of Ezaki *et al.* (1989).

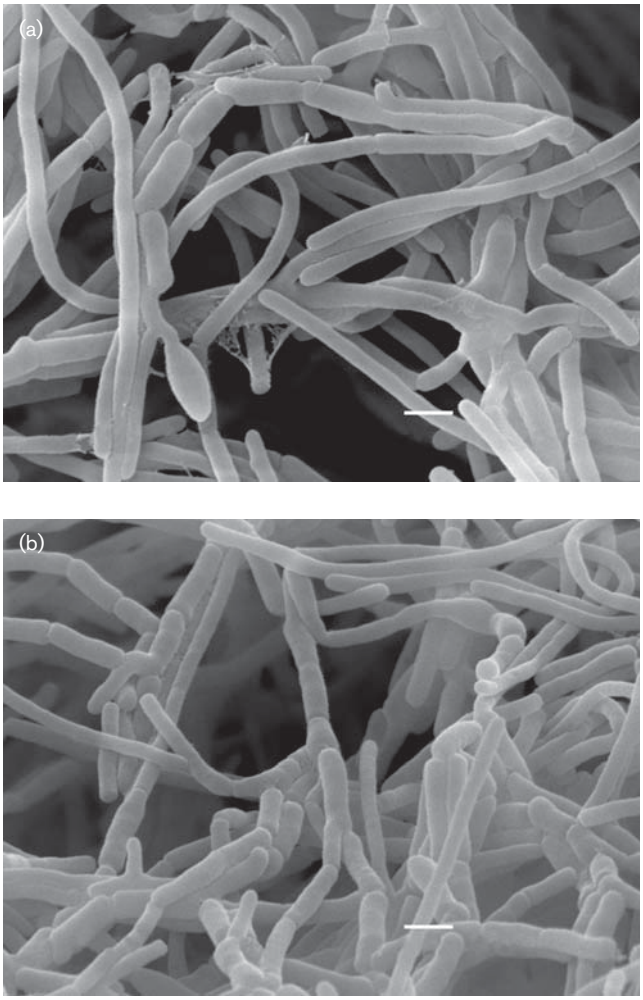

Fig. 1. Scanning electron micrograph of strain ST1-08^T, showing squarish to ovule fragments on substrate mycelium (a) and spore-like chain on aerial mycelium (b) after incubation on ISP2 agar at 30 °C for 14 days. Bar, 1 μm.

Strain ST1-08^T had typical characteristics of the genus *Amycolatopsis*. It showed squarish to ovule fragments on substrate mycelium and spore-like chains on aerial mycelium (Fig. 1). The strain grew well on ISP2 and ISP7 media. Moderate growth was found on ISP3, ISP4, ISP5, Czapek's sucrose, glucose-asparagine and nutrient agar media but poor growth was observed on ISP6 medium. Strain ST1-08^T formed white aerial masses on various media, but did not form soluble pigments. All cultural characteristics on various media are described in Table S1. The strain grew at 15–40 °C (optimum 25–37 °C) and in the presence of 5 % (w/v) NaCl. The pH range for growth was 6.0–9.0 (optimum pH 7.0). This strain was differentiated from related type strains based on gelatin liquefaction, starch hydrolysis, skimmed milk peptonization, nitrate reduction, utilization of L-arabinose, D-glucose, lactose, D-mannitol, raffinose, L-rhamnose and D-sorbitol, and acid production from adonitol, L-arabinose, D-galactose, cellobiose, lactose, maltose, D-mannitol, melibiose, methyl α-D-glucoside, D-sorbitol, sucrose, trehalose and D-xylose. Physiological and biochemical characteristics of strain ST1-08^T are given in the species description and in Table 1. Strain ST1-08^T contained *meso*-diaminopimelic acid in the cell-wall peptidoglycan. Galactose, glucose, arabinose, rhamnose, ribose and mannose were detected in the whole-cell hydrolysate. Arabinose and galactose are the diagnostic sugars as type A of Lechevalier & Lechevalier (1970). Mycolic acid was absent. The polar lipids were phosphatidylinositol, phosphatidylethanolamine, hydroxylphosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, two unknown phospholipids, unknown lipids and unknown glycolipids (Fig. S2). The predominant menaquinone was MK-9(H_4). The major cellular fatty acids of strain ST1-08^T were iso-$C_{16:0}$ and iso-$C_{15:0}$; minor amounts (<10 %) of $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{15:1}\omega6c$, $C_{17:1}\omega6c$, $C_{17:1}\omega8c$, iso-$C_{14:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and 10-methyl $C_{16:0}$ were also detected (Table 2). Strain ST1-08^T showed the same cellular fatty acid profile as *Amycolatopsis pretoriensis* JCM 12673^T and *Amycolatopsis lexingtonensis* JCM 12672^T (Table 2). The DNA G+C content of strain ST1-08^T was 71.2 mol%.

The almost-complete 16S rRNA gene sequence (1462 nt) of strain ST1-08^T was closely related to *A. pretoriensis* JCM 12673^T and *A. lexingtonensis* JCM 12672^T with 16S rRNA gene sequence similarity values of 98.99 and 98.87 %, respectively. The phylogenetic tree analysis using neighbour-joining (Fig. 2), maximum-likelihood and

Table 1. Differential characteristics of strain ST1-08^T and type strains of related species of the genus *Amycolatopsis*

Strains: 1, ST1-08^T; 2, *A. pretoriensis* JCM 12673^T; 3, *A. lexingtonensis* JCM 12672^T. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3
Growth temperature (°C)	15–40	20–45	20–45
Gelatin liquefaction	+	−	−
Nitrate reduction	−	+	+
Skim milk peptonization	+	−	−
Starch hydrolysis	+	−	−
Carbon source utilization			
L-Arabinose	+	W	+
D-Glucose	+	W	+
Lactose	−	W	+
D-Mannitol	+	W	+
Raffinose	−	W	−
L-Rhamnose	+	W	+
D-Sorbitol	−	+	W
Acid production from:			
Adonitol	+	−	−
L-Arabinose	+	W	+
D-Galactose	W	W	+
Cellobiose	−	W	+
Lactose	−	+	+
Maltose	+	W	+
D-Mannitol	+	W	+
Melibiose	+	−	+
Methyl α-D-glucoside	+	W	+
D-Sorbitol	−	+	+
Sucrose	+	W	+
Trehalose	+	W	+
D-Xylose	+	−	+

Table 2. Cellular fatty acids of strain ST1-08^T and type strains of related species of the genus *Amycolatopsis*

Strains: 1, ST1-08^T; 2, *A. pretoriensis* JCM 12673^T; 3, *A. lexingtonensis* JCM 12672^T. Values are percentages of total cellular fatty acids. −, Not detected.

Fatty acid	1	2	3
Straight-chain			
$C_{15:0}$	3.0	−	−
$C_{16:0}$	4.8	7.0	8.6
$C_{17:0}$	2.2	3.4	2.9
Unsaturated			
$C_{15:1}\omega6c$	1.4	0.2	0.4
$C_{17:1}\omega6c$	7.5	8.9	7.6
$C_{17:1}\omega8c$	5.3	1.0	1.7
Branched			
iso-$C_{14:0}$	1.7	1.8	1.7
iso-$C_{15:0}$	17.3	21.4	19.0
iso-$C_{16:0}$	27.8	36.8	34.9
iso-$C_{17:0}$	3.7	4.2	4.2
anteiso-$C_{15:0}$	3.4	2.6	1.8
anteiso-$C_{17:0}$	8.6	5.9	4.3
Summed features*			
3	8.1	4.4	7.3
9	1.3	0.3	1.2

*Summed features are groups of two or more fatty acids that could not be separated using the Microbial Identification System (MIDI). Summed feature 3 contains $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2OH/$C_{16:1}\omega6c$; summed feature 9 contains $C_{16:0}$ 10-methyl/iso-$C_{17:1}\omega9c$.

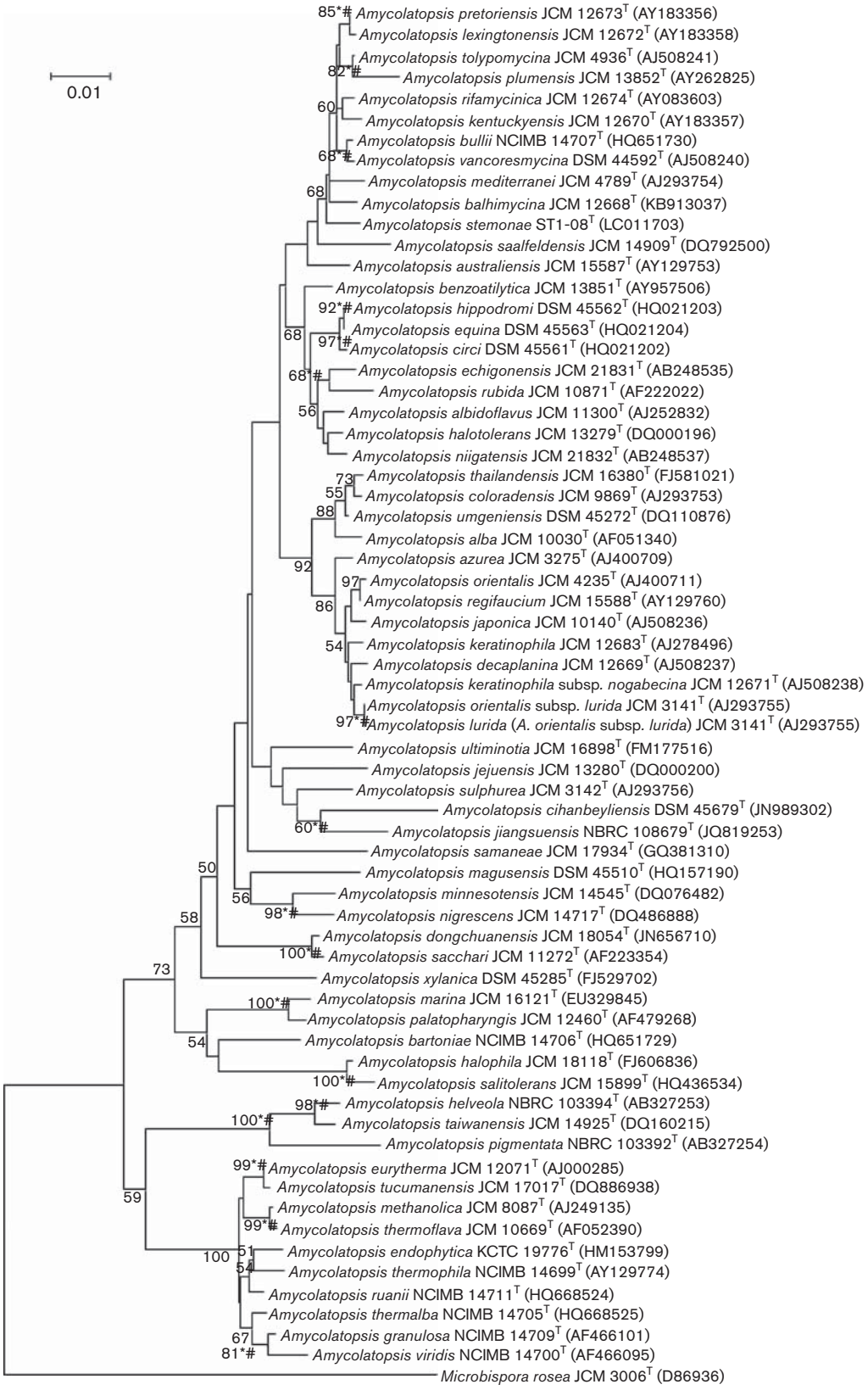

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequence showing the relationship between the strain ST1-08^T and all members of the genus *Amycolatopsis*. Asterisks (*, #) indicate that the corresponding nodes were also found in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Numbers at the nodes are bootstrap percentage values of 1000 replications; only value above 50% are shown. Bar, 0.01 nucleotide substitutions per site.

maximum-parsimony methods showed that strain ST1-08^T was placed the same node as *A. pretoriensis* JCM 12673^T and *A. lexingtonensis* JCM 12672^T (Labeda *et al.*, 2003). The DNA–DNA relatedness values among strain ST1-08^T, *A. pretoriensis* JCM 12673^T (17.4±5.2 % to 57.6±6.4 %) and *A. lexingtonensis* JCM 12672^T (19.9±2.9 % to 42.2±1.8 %) were lower than 70 %, the cut-off level for assigning strains to the same species (Wayne *et al.*, 1987). This result indicates that strain ST1-08^T represents a genomic species distinct from these recognised species of the genus *Amycolatopsis*.

According to the morphological characteristics, chemotaxonomic data and 16S rRNA gene sequence analysis, strain ST1-08^T belongs to the genus *Amycolatopsis* (Lechevalier *et al.*, 1986). However, the differences of physiological and biochemical characteristics and DNA–DNA relatedness values indicate that strain ST1-08^T represents a novel species of the genus *Amycolatopsis*, which the name *Amycolatopsis stemonae* sp. nov. is proposed.

Description of *Amycolatopsis stemonae* sp. nov.

Amycolatopsis stemonae (ste.mo′nae. N.L. gen. n. *stemonae* of the plant genus *Stemona* from which type strain was isolated).

Gram-stain-positive, aerobic, non-motile, filamentous actinomycete that forms abundant white aerial mycelia and yellow to orange vegetative mycelia on yeast extract-malt extract, oatmeal, inorganic salts-starch, glycerol-asparagine, peptone-yeast extract, tyrosine (ISP2–7 media), Czapek's sucrose, glucose-asparagine and nutrient agar, which fragments into substrate mycelium. Grows at 15–40 °C, pH 6–9 and with 5% (w/v) NaCl. Gelatin liquefaction, starch hydrolysis and skimmed milk peptonization are positive. Nitrate reduction and milk coagulation are negative. Utilizes L-arabinose, D-glucose, glycerol, *myo*-inositol, D-mannitol and L-rhamnose, but not lactose, raffinose or D-sorbitol. Acid is produced from adonitol, L-arabinose, D-galactose (weakly), *myo*-inositol, maltose, D-mannitol, melibiose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin, sucrose, trehalose and D-xylose, but not from cellobiose, lactose or D-sorbitol. The cell wall contains *meso*-diaminopimelic acid in the peptidoglycan. Arabinose and galactose are the diagnostic sugars of the whole-cell hydrolysate. The polar lipids are phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol, hydroxylphosphatidylethanolamine, phosphatidylglycerol, unknown phospholipids, unknown lipids and unknown glycolipids. The predominant menaquinone is MK-9(H_4). Major cellular fatty acids are iso-$C_{16:0}$ and iso-$C_{15:0}$.

The type strain, ST1-08^T (=JCM 30050^T=PCU 339^T =TISTR 2278^T), was isolated from the stem of *Stemona* sp. The DNA G+C content of the type strain is 71.2 mol%.

Acknowledgements

This research was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), 2014 and The Faculty of Pharmaceutical Sciences Research Fund, Chulalongkorn University, 2014. We thank Dr Toshiya Iida and Dr Mitsuo Sakamoto, Japan Collection of Microorganisms, Tsukuba, Japan for their technical assistance; Associate Professor Thatree Phadungcharoen, Faculty of Pharmaceutical Sciences, Chulalongkorn University for the plant sample; and Professor Aharon Oren, the Hebrew University of Jerusalem, Jerusalem, Israel for the etymology.

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