Chiang Mai J. Sci. 2012; 39(3) : 373-388
http://it.science.cmu.ac.th/ejournal/
Contributed Paper

Actinomycetes from Tropical Limestone Caves

Nanthavut Niyomvong [a], Wasu Pathom-aree [b], Arinthip Thamchaipenet [c, d] and Kannika Duangmal* [a,d]

[a] Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[b] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
[c] Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[d] Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.
*Author for correspondence; e-mail: fscikkd@ku.ac.th

Received: 4 November 2011
Accepted: 28 May 2012

ABSTRACT

The cultivable actinomycetes from tropical limestone caverns at Khao No-Khao Kaeo karst, Thailand, were investigated. In total, 276 actinomycetes were isolated from 10 soil samples using different selective isolation procedures. The predominant actinomycetes from all samples were members of the genus *Streptomyces* (94.6%) as they contained *LL*-diaminopimelic acid (A_2pm) in their whole cell hydrolysates. The remaining 15 isolates (5.4%), rich in *meso*-A_2pm in whole cell hydrolysates were characterized by 16S rRNA gene sequencing. They were shown to represent strains from 8 different genera namely *Actinomadura*, *Actinoplanes*, *Gordonia*, *Microbispora*, *Micromonospora*, *Nocardia*, *Nonomuraea* and *Saccharopolyspora*. A good agreement was found between the results based on morphology, presence of *meso*-A_2pm and 16S rRNA gene phylogeny. Microwave treatment of soil samples was found to promote the isolation of rare actinomycetes. Successful isolation of members of the genus *Actinomadura* and *Saccharopolyspora* from caves was reported for the first time. This study confirms significant diversity of cultivable actinomycetes from neglected habitats such as limestone caves in Thailand.

Keywords: actinomycetes, limestone cave, selective isolation, 16S rRNA gene

1. INTRODUCTION

Members of the actinomycetes, especially streptomycetes, have long been recognized as prolific producers of useful bioactive compounds [1]. The search for novel antibiotics and other bioactive microbial metabolites is important for the fight against new and emerging pathogens [2, 3, 4]. Isolation of actinomycetes from unique natural habitat is of interest to avoid re-isolation of strains that produce known bioactive metabolites and usually lead to highly diversified actinomycete communities. There is an increasing awareness of the potential value of rare actinomycetes (non-streptomycete actinomycetes) as sources of new metabolites. Neglected habitats are proving to be a particularly good source of novel actinomycetes that produce new

bioactive compounds, as exemplified by studies on actinomycetes isolated from desert soil [5, 6], lichens [7] and marine habitats [8, 9, 10]. Therefore, actinomycetes have been intensively surveyed in different ecological area and underexplored environ ments in various parts of the world [11, 12, 13].

Cave is one of habitat attracting microbiologists' interest during the past decade [14, 15] with a combination of unique conditions including high humidity, relatively low and stable temperature [16]. Many microbiologists believe that microbes collected from pristine sites that were unexplored or rarely visited by humans were likely to be novel taxa or strains which produce unique beneficial chemical compounds. Limestone karsts are sedimentary rocks which consist primarily of calcium carbonate. In Southeast Asia, karsts cover an area of around 400,000 square kilometers, with geological ages ranging from the Cambrian to the Quaternary [17]. The high species diversity on karsts made from a various ecological afforded by complex terrains, fissured cliffs and caves, and variable climatic conditions [18].

In an early work, great diversity of cultivable actinomycetes was found in the Altamira cave, Cantabria, Spain [14, 16], Tito Bustillo, Spain [16, 19] and Grotta dei Cervi, Italy [20]. Many novel species have been discovered from cave environments, such as *Actinocorallia cavernae* [21], *Agromyces subbeticus* [22], *Amycolatopsis halotolerans* [23], *Nocardia altamirensis* [24], *Nocardia speluncae* [25], including novel genera such as *Knoellia* [26] and *Hoyosella* [27]. However, the studies of actinomycetes diversity in caves of Thailand are still rare [28, 29].

The primary aim of this present study was to determine the diversity of cultivable rare actinomycetes from limestone caves in Khao No-Khao Kaeo karst area of Thailand. The efficiency of various selective isolation procedures employed was also discussed.

2. MATERIALS AND METHODS

2.1 Samples Collection

Ten soil samples were collected from 4 unnamed caves located in Khao No-Khao Kaeo karst, Nakhon Sawan province, Thailand (15° 56' N and 99° 52' E) in March 2007. The upper 5 cm layer of cave soils were collected in sterile plastic bags and transported to the laboratory. Soils were air dried at room temperature for 7-10 days before grind with mortar and sieved. The pH of each soil sample was determined following the procedure described by Reed and Cummings [30] using a glass electrode pH meter and expressed as an average of triplicate readings.

2.2 Selective Isolation and Enumeration of Actinomycetes

One gram of dried soil was aseptically diluted in 9 ml of sterile 0.85% NaCl solution. The resultant 10^{-1} soil suspension was agitated on a shaker at 150 rpm at room temperature and subjected to the following pretreatment regimes unless otherwise indicated:

a) Wet heat: Soil suspension was heated in a water bath at 50°C for 6 min and cooled at room temperature [31].

b) Dry heat: One gram of dried soil sample was heated at 120°C in hot air oven for 1 h [32].

c) Dry heat and phenol: One gram of heated soil sample (at 120°C for 1 h) was diluted in 9 ml of sterile 0.85% NaCl solution. The resultant soil suspension (1 ml) was added to 9 ml of 1.5% phenol solution [32]. The mixture was then incubated at room temperature for 30 min.

d) Electromagnetic wave: This pretreatment was adapted from Bulina et al. [33]. Soil suspension was irradiated with the microwave oven at frequency of 2460 MHz and a power setting at 100 watt for 45 sec.

e) No treatment: Soil suspension was diluted without any prior pretreatment.

The pretreated samples were further diluted down to 10^{-4} using 0.85% NaCl solution. One hundred microliters of an appropriate dilution were spread over the surface of starch casein (SC) agar plates [34] for pretreatment regime a-d and Humic acid-Vitamin (HV) agar [35] for regime d and e. All media were supplemented with nalidixic acid (25 µg/ml) and ketoconazole (100 µg/ml) to inhibit the growth of bacteria and fungi, respectively. The pH of all media was adjusted to 7.

Numbers of presumptive actinomycete colonies on each plate were counted after incubation of the inoculated plates at 30°C for 2 weeks and expressed as mean colony forming units (c.f.u.) per gram dry weight soil of three plates. The putative actinomycete colonies were randomly selected and purified by streak plate method on GYE agar [1% glucose (w/v), 1% yeast extract (w/v) and 1.5% agar (w/v)] until pure culture was obtained. Purified isolates were maintained on GYE and oatmeal agar (ISP 3) slopes [36]. Stock cultures were prepared by transferring mycelium and spores of purified isolates into 20% glycerol solution and kept at -20°C for long term preservation [37].

2.3 Colour Grouping and Characterisation of the Isolates

Putative actinomycete isolates were inoculated onto oatmeal agar [36] and peptone-yeast extract-iron agar plates (ISP 7) [36] and incubated at 30°C for 14 and 4 days, respectively. The oatmeal agar plates were examined for aerial spore mass colour, substrate mycelium colour and diffusible pigments using NBS/IBCC color chart [38]. The peptone-yeast extract-iron agar plates were recorded for melanin pigment production.

The presence of isomer of diamino pimelic acid (A_2pm) in whole cell hydrolysates were determined according to the methods described by Becker et al. [39] and Hasegawa et al. [40] using biomass obtained from a culture grown on ISP 2 agar [36]. The spore chain morphology of selected *LL*-A_2pm containing isolates grown on oatmeal agar plates was examined by light microscopy. Spore-chain morphology and spore ornamentation of representative *meso*-A_2pm containing strains were observed using both light microscopy and scanning electron microscopy (JEOL JSM 5600 LV).

2.4 16S rRNA gene Amplification and Analysis

Total genomic DNA from 15 isolates that contained *meso*-A_2pm was extracted using standard method [41]. The 16S rRNA gene was amplified by PCR with the universal bacterial primer 27f (5'AGAGTTTGAT CMTGGCTCAG 3') and 1525r (5' AAGGA GGTGWTCCARCC 3' [42]. Amplification were performed using GeneAmp PCR System 9700, according to the following profile: initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; these cycles were followed by a final extension at 72°C for 5 min. The PCR products were purified and sequenced (Macrogen, Korea) using universal primers [42]. The resultant 16S rRNA gene sequences were aligned against corresponding sequences of the type strains of actinomycete species, retrieved from EMBL/GenBank database, using the

CLUSTAL_X [43] and PHYDIT programs (http://plaza.snu.ac.kr/~jchun/phydit/). An unrooted phylogenetic tree was inferred using the neighbour-joining and maximum-parsimony tree-making algorithms [44]. The tree topology was evaluated by bootstrap analyses of the neighbour-joining data based on 1000 resamplings. All analyses were carried out using TREECON software [45].

3. RESULTS AND DISCUSSION

3.1 Characteristics of Cave and Cave Soils

Khao No-Khao Kaeo karst is an area which formed 440 million years ago, situated 282 m above medium sea level on the west of Banpotpisai district, Nakhon Sawan Province, Thailand. All caves are not tourist attraction sites and free from human activities. Samples were light brown in colour with bat guano in sample A1 and A2; fine soil with stone in sample B1-B3, sandy soil in sample C1-C3 and fine soil with slug shell in sample D1 and D2. The average pH of all soil samples was between 5.5 and 7.6 (Table 1). Most samples were weak acid (5.5 - 6.5), except sample B2, B3, C1 and C3 which were neutral (6.5 - 7.6). The slightly acidic pH may be due to animal secretion such as bat guano. Mineralization processes of such animal inputs resulted in the production of nitric acid and sulphuric acid have been reported [46]. The alkali pH of 8.29 was reported from soil collected from Phanangkoi cave in northern Thailand [28].

3.2 Selective Isolation and Enumeration of Actinomycetes

A total of 276 actinomycete strains were isolated from 10 cave soil samples with the use of various methods. In general, actinomycetes were isolated from all samples. The highest number of isolates was from sample A2 (78 isolates) with the lowest numbers from sample C3 (3 isolates) (Table 1). The total actinomycete counts were ranged from 0 - 8.5×10^4 c.f.u. per gram, a

Table 1. Soil pH and total actinomycetes counts in soil samples from different pretreatment methods (c.f.u./g dry soil).

Sample	pH	a) Wet heat (SC)	b) Dry heat (SC)	c) Dry heat and phenol (SC)	d) Electromagnetic wave		e) No treatment (HV)	Total number of isolates
					(SC)	(HV)		
A1	6.4	1.2×10^3	3.6×10^3	ND	1.2×10^3	6.0×10^3	1.2×10^3	5
A2	6.2	5.8×10^4	1.2×10^3	3.0×10^2	2.3×10^3	1.2×10^4	2.8×10^3	78
B1	5.8	3.0×10^2	ND	ND	4.0×10^2	3.0×10^2	2.1×10^3	4
B2	7.6	7.2×10^4	2.0×10^3	4.1×10^3	8.4×10^3	7.2×10^4	3.5×10^3	76
B3	7.5	8.5×10^4	1.6×10^3	1.3×10^3	5.2×10^4	7.7×10^4	6.0×10^4	62
C1	6.9	1.7×10^4	5.0×10^3	1.6×10^3	6.4×10^4	1.4×10^4	2.0×10^4	34
C2	5.5	4.0×10^2	ND	ND	4.0×10^2	2.0×10^2	2.0×10^3	6
C3	6.8	8.5×10^2	ND	ND	4.0×10^3	3.9×10^4	7.0×10^3	3
D1	5.7	1.4×10^3	1.0×10^2	ND	1.0×10^2	8.0×10^2	1.2×10^3	4
D2	6.5	9.5×10^2	ND	ND	3.4×10^3	6.0×10^3	3.0×10^3	4
								276

N.D. - Not detected, no any colonies on the agar plates which incubated at 30°C for up to 4 weeks.

slightly higher count than the earliest report in Thailand by Laorpaksa et al. [47] (4.4 - 7.0 × 10^3 c.f.u. per gram), which may be a result from the use of selective pretreatments in this study. Several selective isolation procedures were employed in order to isolate actinomycetes from cave soil samples collected from Khao No-Khao Kaeo limestone karst. It has been shown previously that the treatment of samples may lead to growth inhibition of some genera and stimulated germination of other genera [11, 33]. Both moist and dry-heat pretreatment regimens have long been used to select for various actinomycete groups [48]. Moist-heat by heating the soil dilution in a water bath at 45, 50 or 70°C has been used to eliminate the spreading of bacterial colonies on actinomycete isolation plates. They are extremely useful standard procedures for routine isolation [48]. In this experiment, the general selective isolation procedure for actinomycetes by spreading on starch casein agar and humic acid vitamin agar showed the numbers of actinomycete isolates in each cave soil sample ranged from 3.0 × 10^2 to 8.5 × 10^4 c.f.u. per gram dry soil sample (Table 1). Dry heat treatment either at 120°C for 1 hr (procedure b) or dry heat at 120°C for 1 hr and 1.5% phenol (procedure c) showed the most specific and effective in reducing the number of undesirable bacteria. However, these treatments also resulted in a reduction in numbers of viable actinomycetes recovered. For example, there was no colony appeared on the agar plates from sample B1, C2, C3 and D2 when procedure b was used as pretreatment. Similar result was also observed with pretreatment procedure c for sample A1, B1, C2, D1 and D2, even after the plates were incubated at 30°C for up to four weeks.

The efficiency of microwave treatment was evident in this study as most non-streptomycetes actinomycetes were obtained using this regime. Pretreatment of soil samples with 2460 MHz microwaves at 80 W for 30 sec resulted in a significant increase (threefold on average) in the proportion of rare actinomycetes isolated [33]. The analysis of whole cell hydrolysates revealed that most non-streptomycetes (*meso*-A_2pm containing strains) were recovered from isolation associated with procedure d, which used microwave irradiation at 2460 MHz, setting at 100 watt for 45 sec, before spreading on HV agar. Twelve isolates of actinomycetes, which are rich in *meso*-A_2pm, were recovered from this procedure. The remaining 4 non-streptomycete actinomycetes were also isolated from HV agar seeded with un-treated samples. HV agar was reported to support good sporulation of rare actinomycetes which offers an advantage for rapid morphological identification [35, 49] with long-working distance objective lens. This media was designed for isolation and enumeration of soil actinomycetes [35]. It has been used successfully in recovery of various rare and novel actinomycete taxa from environments [28, 29, 50, 51, 52].

3.3 Colour Grouping and Chemotaxonomic Characterization

A total of 276 actinomycetes with different colony types were recovered from cave soil samples with the use of various methods. Chemotaxonomic analysis of whole cell hydrolysates revealed the presence of the *LL*-A_2pm isomer in 261 isolates (94.6%) and *meso*-A_2pm isomer in 15 isolates (5.4%). The *LL*-A_2pm containing isolates formed branched substrate mycelia with aerial mycelia that formed chains of spores. They were assigned to the genus *Streptomyces* [53]. These isolates could be assigned to 10 multi-membered colour groups based on

observation of spore color on oatmeal agar. The largest taxon, dominated by grey spore colour group, encompassed 39.5%, following by white (33.8%) and brown (16.1%) spore colour group. The assignment of these isolates to various colour groups indicated high degree of diversity. Colour grouping has been shown to provide a valuable index of taxonomic diversity of streptomycetes in natural habitats [6, 54]. On the basis of their morphological characteristics and analysis of diaminopimelic acid isomer, all cave soil samples were dominated by representative of the genus *Streptomyces*, a result in good agreement with those from earlier surveys [20, 29, 55, 56, 57].

The 15 remaining *meso*-A_2pm containing actinomycetes, assigned to non-streptomycete actinomycetes or rare actinomycetes were subjected to 16S rRNA gene sequencing. Morphological examined by light microscopy and scanning electron microscopy (Figure 1) was also supported the results received from the detection of diaminopimelic acid type in cell hydrolysates. Cylindrical sporangia were well developed on the substrate mycelium of *Actinoplanes* sp. NN272 (Figure 1a). *Actinomadura* sp. NN242 formed spiral spore chains with smooth spore ornamentation (Figure 1b). *Gordonia* sp. NN234 grew as short rods that occur in short chains (Figure 1c). *Nocardia* sp. NN256 formed substrate mycelium that fragments into rod-shaped (Figure 1d). It is evident that these strains presented morphological properties typical of their assigned genera.

3.4 Molecular Taxonomic of *meso*-A_2pm Containing Actinomycetes

All 16S rRNA gene sequencing studies were focused on non-streptomycetes isolates which were preliminary identified on the basis of morphological characteristics and chemotaxonomic analysis. Fifteen isolates which contained *meso*-A_2pm in whole cell hydrolysates were selected for molecular taxonomical studied for nearly completed 16S rRNA gene sequence analysis. The resulting sequences, 1366 nt to 1428 nt, depending on the isolate (except for strain NN274, 977 nt) were determined and compared with the

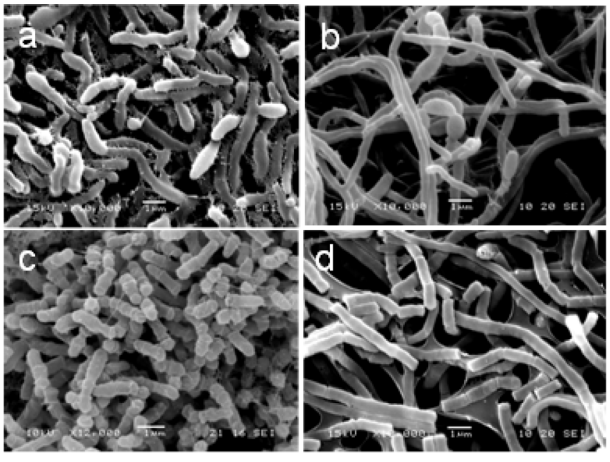

Figure 1. Scanning electron micrographs of spore ornamentation of representative isolates from Khao No-Khao Kaeo limestone karst. (a) *Actinoplanes* sp. NN272 grown on ISP 4 [36] for 21 days; (b) *Actinomadura* sp. NN242 grown on ISP 3 for 21 days; (c) *Gordonia* sp. NN234 grown on GYE agar for 5 days; (d) *Nocardia* sp. NN256 grown on ISP 2 for 7 days. Bar intervals is 1 μm.

sequences available in the GenBank databases using BLASTN. The 16S rRNA gene sequences revealed that they were members of the following 8 genera: *Actinomadura* (2 isolates, family *Thermomonosporaceae*), *Actinoplanes* (2 isolates, family *Micromonosporaceae*), *Gordonia* (2 isolates, family *Nocardiaceae*), *Microbispora* (2 isolates, family *Streptosporangiaceae*), *Micromonospora* (3 isolates, family *Micromonosporaceae*), *Nocardia* (1 isolate, family *Nocardiaceae*), *Nonomuraea* (2 isolates, family *Streptosporangiaceae*) and *Saccharopolyspora* (1 isolate, family *Pseudonocardiaceae*), with 97.7 - 99.8% similarity to their nearest neighboring strains. The percentages of 16S rRNA gene sequence identity of these isolates to the closest type strain are presented in Table 2. Phylogenetic tree constructed based on nearly complete 16S rRNA gene sequences confirmed their affiliation to 5 actinomycete families *Micromonosporaceae*, *Nocardiaceae*, *Pseudonocardiaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* and supported by high bootstrap values (Figure 2).

Two *Actinomadura* strains NN236 and NN242 were isolated from sample B2 and D2, respectively. Both strains were most closely related to *A. napierensis* sharing a 16S rRNA gene similarity of 99.03 and 98.96%, respectively. They clustered with *A. meyerae* in the 16S rRNA gene tree but formed well separated subclade support by 99% bootstrap value (Figure 2). Members of genus *Actinomadura* were widely distributed in soils but have not been reported from the caves before. However, other members of family *Thermomonosporaceae*, genera *Actinocorallia* and *Spirillospora* were reported from caves in Northern Thailand [28, 29]. In addition, a new species of *Actinocorallia cavernae* isolated from cave in Korea has been described [21].

Table 2. Analysis of *meso*-A_2pm containing isolates based on nearly completed sequence of the 16S rRNA gene with the most identity to the closest strain from GenBank database.

Genus	Strain	Source (treatment)	Closest type strain	Similarity (%)
Actinomadura	NN236	B2 (e, HV)	*Actinomadura napierensis*	99.03
	NN242	D2 (e, HV)	*Actinomadura napierensis*	98.96
Actinoplanes	NN265	A2 (d, HV)	*Actinoplanes xinjiangensis*	98.27
	NN272	A2 (e, HV)	*Actinoplanes xinjiangensis*	98.14
Gordonia	NN234	C2 (e, HV)	*Gordonia terrae*	99.80
	NN279	C2 (d, HV)	*Gordonia terrae*	99.79
Microbispora	NN274	A1 (d, HV)	*Microbispora mesophila*	97.74
	NN277	A1 (d, HV)	*Microbispora mesophila*	97.90
Micromonospora	NN275	D2 (d, HV)	*Micromonospora siamensis*	99.0
	NN264	D2 (d, HV)	*Micromonospora siamensis*	98.97
	NN271	A2 (d, HV)	*Micromonospora chalcea*	99.57
Nocardia	NN256	B3 (d, HV)	*Nocardia asteroides*	99.08
Nonomureae	NN273	B2 (d, HV)	*Nonomuraea roseoviolacea*	98.49
	NN278	B2 (d, HV)	*Nonomuraea candida*	99.26
Saccharopolyspora	NN257	B3 (d, HV)	*Saccharopolyspora phatthalungensis*	98.57

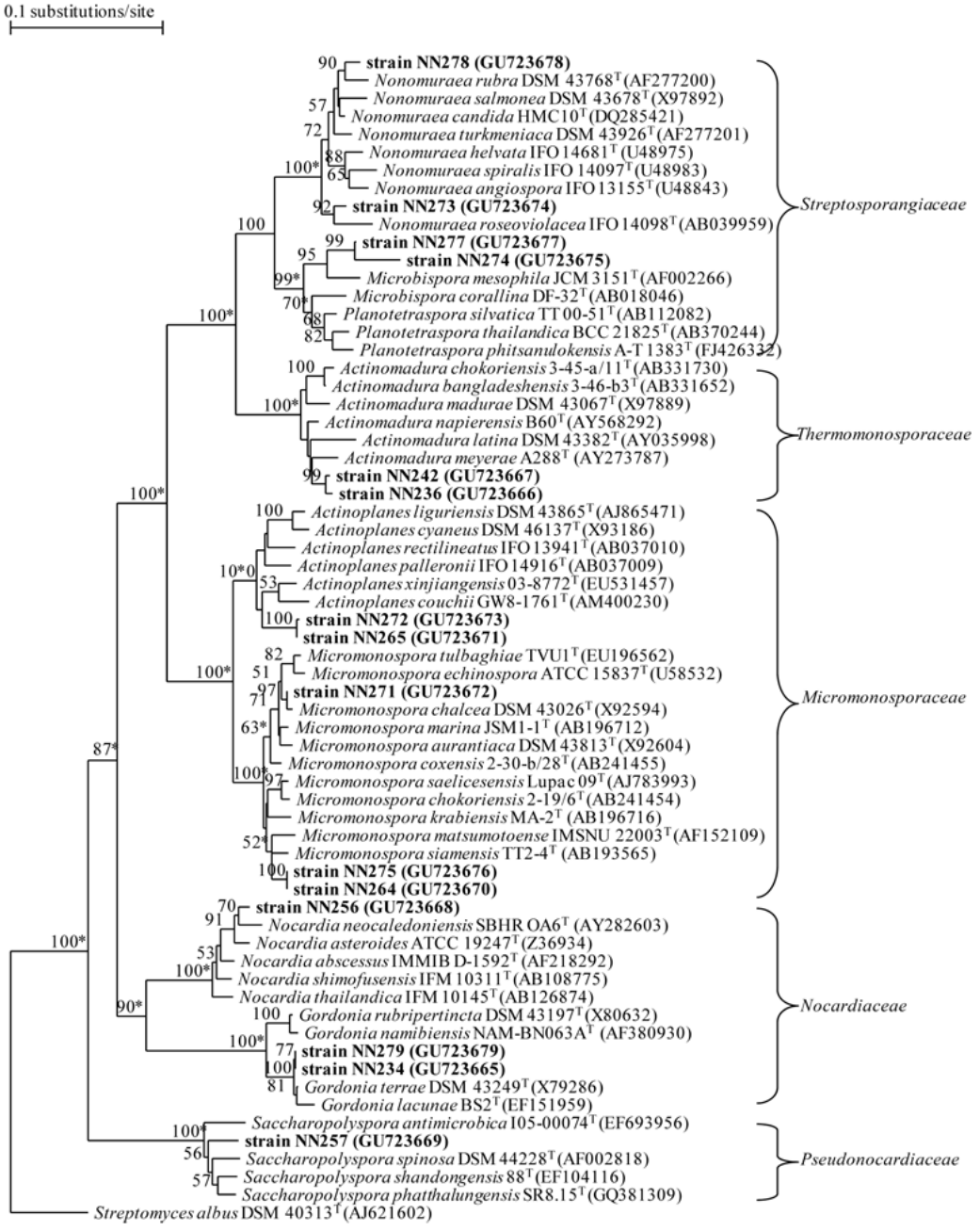

Figure 2. Neighbor-joining tree showing the position of various genera of the selected actinomycetes isolated from the Khao No-Khao Kaeo limestone karst amongst its phylogenetic neighbors based on almost complete 16S rRNA gene sequences. Asterisks indicate branches of the tree that was also found using maximum-parsimony tree making algorithm. Numbers at the nodes show the percentage bootstrap values. The bar represents 0.1 substitutions per nucleotide position.

Strains NN265 and NN272, were found to present only in sample A2. A 16S rRNA gene tree showed that they formed a separate line of descent from their nearest *Actinoplanes* neighbours (Figure 2), the relationship strongly supported by 100% bootstrap value. Sequence similarity calculated based on neighbour joining analysis indicated that they were closest to *A. xinjiangensis*, sharing 98.27 and 98.14% 16S rRNA gene similarity values, respectively. These observations suggested that these 2 strains may represent a new species. Representatives from other genera in family *Micromonosporacea* previously reported from caves were *Dactylosporangium* [55] and *Micromonospora* [29].

The 3 *Micromonospora* strains, another member of family *Micromonosporaceae*, were also isolated from sample A2 (strain NN271) and sample D2 (strains NN264 and NN275). Strain NN271 was closely related to *M. chalcea* with 99.57% similarity. Strains NN264 and NN275, both shared the highest 16S rRNA gene similarity values of 98.97% and 99% with *M. siamensis*, respectively. Both strains were likely to be a *M. siamensis* considering their high 16S rRNA gene similarity values. *M. siamensis* was recently proposed for strain originated from Thai peat swamp forest of Thailand [58]. The finding of this species in cave soil suggested it is widely distributed in terrestrial habitats. The isolation of *Micromonospora* strains were also reported from caves in northern Thailand [29] and Korea [55]. In addition, one new genus in family *Micromonosporaceae* was proposed as *Catelliglobosispora koreensis* for strain isolated from gold mine cave in Korea [59, 60].

Only one *Nocardia* strain NN256 was isolated from sample B3. It was clustered together with *Nocardia asteroides* and *N. neocaledoniensis* with 99.08 and 98.8% level of 16S rRNA gene similarity, respectively. Another member of the family *Nocardiaceae*, is genus *Gordonia* represented by strains NN234 and NN279. Both were isolated from sample C2. They were located adjacent to *G. terrae* in the phylogenetic tree and showed highest level of similarity of 99.8% (Figure 2). Members of the family *Nocardiaceae* are common and widely distributed in aquatic and terrestrial environments; they are probably involved in the turnover of organic matter in natural habitats.

Family *Streptosporangiaceae* was represented by members of the genus *Microbispora* and *Nonomuraea*. Strains NN274 and NN277, isolated from sample A1, fell within the evolutionary radiation occupied by genus *Microbispora* (Figure 2). They are most closely related to *M. mesophila* with relatively low 16S rRNA similarity values of 97.3% and 97.6%, respectively. The highest 16S rDNA similarity between the *Microbispora* type strains was with *M. corallina* NBRC 16416^T and *M. siamensis* DMKUA 245^T. Although these organisms have a relatively high sequence similarity (98.4%) they have a low DNA-DNA relatedness value [61], a value well below the recommended cut-off point for the delineation of bacterial species [62]. Therefore, strains NN274 and NN277 may merit recognition as new validly described *Microbispora* species subjected to polyphasic taxonomic characterization.

Two *Nonomuraea* strains NN273 and NN278 were isolated from sample B2. They were closely related to *N. roseoviolacea* and *N. candida*, respectively. Strain NN273 shared the highest 16S rRNA gene similarity of 98.4% with *N. roseoviolacea* whereas those of strain NN278 and *N. candida* were 99.49%. Members of this genus were also found in a recent study by Nakaew et al. [29] including a hitherto unknown subline. Of note is that genus *Nonomuraea* so far has been reported

only from Thai caves. No previous studies reported the presence of members of this particular genus from other caves either by culture dependent or culture independent approaches. It is still unclear about the nature of its distribution and function in cave habitats.

Strain NN257 was identified as member of the genus *Saccharopolyspora* with *S. phatthalungensis* as its nearest neighbour sharing a 16S rRNA similarity value of 98.57%. It formed a separated subclade with its nearest neighbours in the 16S rRNA gene tree (Figure 2). Members of genus *Saccharopolyspora* were mostly isolated from soil. Recently, new species isolated from lake sediment and marine sponge were also reported. Members of genus *Saccharopolyspora* have previously only been reported from Altamira cave, Spain by culture independent survey using DGGE analysis. This is the first time that cultured representative of genus *Saccharopolyspora* was isolated from caves. Members of this genus are of particular interest as they are known producers of industrially important antibiotics such as the macrolide antibiotic erythromycin produced by *Saccharopolyspora erythraea*. Worldwide annual sales of erythromycin and its derivatives are worth billions of dollars [63]. Other family *Pseudonocardiaceae* members included *Amycolatopsis* [16, 20]; *Pseudonocardia* [29] and *Saccharomonospora* [55] were previously reported from caves. Indeed, 2 new species of *Amycolatopsis*, *A. halotolerans* and *A. jejuensis* [23] and one new species of *Pseudonocardia*, *P. kongjuensis* [64] were also described.

Generally, a 16S rRNA gene similarity of below 97% between a newly isolated strain and validly described species warrant recognition as a new species [65]. It has been shown that a type strain of actinomycetes with almost identical 16S rRNA gene sequences have DNA-DNA relatedness values well below the 70% cut-off point recommended by Wayne et al. [62] for the delineation of strains that belong to the same genomic species. For example, the type strains of *Nonomuraea dietzia* and *N. roseola* have identical 16S rRNA gene sequence but showed less than 31% of DNA-DNA relatedness value [66]. The 16S rRNA sequence data show that several strains isolated from this study belong to putatively new species as they are well separated from their known nearest phylogenetic neighbours (Figure 2). Though detailed polyphasic taxonomic characterization of these strains are required to clarify their status.

Early works on cultivable cave actinomycetes were limited to only caves located in Italy and Spain. Soil samples collected from Altamira [14, 16], Tito Bustillo and Llonín caves in Spain [16, 19] and Grotta dei Cervi, Italy [20] resulted in the isolation of 18 genera in 12 families. *Streptomyces* were dominated in all caves. Rare genera such as *Amycolatopsis*, *Microbacterium*, *Nocardia* and *Rhodococcus* were also widely distributed in these caves. In Asia, there were only reports from China, Korea and Thailand.

A recent study by Nakaew et al. [29] also reported the isolation of several rare actinomycete genera (*Actinocorallia*, *Catellatospora*, *Microbispora*, *Micromonospora*, *Nonomuraea*, *Pseudonocardia*, *Saccharothrix* and *Spirillospora*) from Phatup Cave Forest Park and Phanangkhoi cave in northern Thailand, many of which were also found in the present study. However, Phatup and Phanangkhoi caves are situated in a tourist attraction area which is open to public access. The impact of human introduction may contribute to the diversity of actinomycetes found from the site. Indeed, a correlation between the number of visitors and diversity of bacteria has been reported, the higher

the number of visitors the higher the diversity of isolated strains, as exemplified by the data obtained from Altamira, Tito Bustillo and Llonín caves [16]. Caves with restricted visits in northern Spain (Llonín and La Garma caves) also yielded lower diversity [19]. Nevertheless, the results from Nakaew et al. [29] and our study extended the range of cultivable actinomycetes from Thai caves to 14 genera including some that never been recovered from cave habitats before such as *Nonomuraea* and *Saccharopolyspora*. These findings strongly suggested that actinomycetes are an indigenous population of microbial community inhabited in caves. The notion that is well supported by data from culture independent studies.

In conclusion, large numbers of actino mycetes were isolated from Khao No-Khao Kaeo limestone karst using various established isolation procedures. All samples were dominated by *Streptomyces* species. However, diverse rare genera were also present. HV agar supplemented with nalidixic acid and ketoconazole in combination with microwave irradiation pretreatment of soil samples has been demonstrated to permit the recovery of rare actinomycetes from limestone cave soils compared with other pretreatments. A 16S rRNA gene sequences analysis suggested that some of the isolates may represent novel species.

At the time of writing, 4 new genera and 20 new species of actinomycetes from caves representing 11 families have been described since 1999 (gathered from International Journal of Systematic and Evolutionary Microbiology; [67]). However, the results from previous culture independent studies revealed a greater diversity than conventional cultural based studies [57, 68, 69, 70, 71, 72]. An availability of pure cultures of such diversity is important to better our understanding on the physiology of these bacteria and their functions in cave habitats. The discrepancy of actinobacterial diversity previously reported by culture dependent and culture independent studies including our results highlighted an urgently need for improved selective isolation procedures to assess the true diversity of actinomycetes in caves for bioprospecting. The present findings not only provide further evidence that a variety of actinomycetes are common in caves but show implications of this diversity for biotechnological exploitation as actinomycetes are well known as the most prolific producer of bioactive compounds [1, 3, 73].

ACKNOWLEDGEMENTS

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Kasetsart University Research and Development Institute (KURDI).

REFERENCES

[1] Watve M.G., Tickoo R., Jog M.M. and Bhole B.D., How many antibiotics are produced by the genus *Streptomyces*?, *Arch. Microbiol*., 2001; **176**: 386-390.

[2] Goodfellow M. and O'Donnel A.G., Search and Discovery of Industrially Significant Actinomycetes; in Baumberg S., Hunter I.S., and Rhodes P.M., eds., *Microbial Products: New Approaches*, Cambridge University Press, Cambridge, 1989: 343-383.

[3] Berdy J., Bioactive microbial metabolites, *J. Antibiot.*, 2005; **58**: 1-26.

[4] Busti E., Monciardini P., Cavaletti L., Bamonte R., Lazzarini A., Sosio M. and Donadio S., Antibiotic producing ability by representatives of a newly discovered lineage of actinomycetes, *Microbiol*., 2006; **152**: 675-683.

[5] Takahashi Y., Matsumoto A., Seino A., Iwai Y. and Omura S., Rare actinomycetes isolated from desert soils, *Actinomycetol*., 1996; **10**: 91-97.

[6] Okoro C.K., Brown R., Jones A.L., Andrews B.A., Asenjo J.A., Goodfellow M. and Bull A.T., Diversity of culturable actinomycetes in hyper-arid soils of the Atacama desert, Chile, *Antonie van Leeuwenhoek*, 2009; **95**: 121-133.

[7] Gonzalez I., Ayuso-Sacido A., Anderson A. and Genilloud O., Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences, *FEMS Microbiol. Ecol.*, 2005; **54**: 401-415.

[8] Pathom-aree W., Stach J.E.M., Ward A.C., Horikoshi K., Bull A.T. and Goodfellow M., Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench, *Extremophiles*, 2006; **10**: 181-189.

[9] Schneider K., Keller S., Wolter F.E., Röglin L., Beil W., Seitz O., Nicholson G., Bruntner C., Riedlinger J., Fiedler H.P. and Süssmuth R.D., Proximicins A, B, and C -antitumor furan analogues of netropsin from the marine actinomycete *Verrucosispora* induce upregulation of p53 and the cyclin kinase inhibitor p21, *Angew. Chem. Int. Ed. Engl*., 2008; **47**: 3258-3261.

[10] Maldonado L.A., Fragoso-Yáñez D., Pérez-García A., Rosellón-Druker J. and Quintana E.T., Actinobacterial diversity from marine sediments collected in Mexico, *Antonie van Leeuwenhoek*, 2009; **95**: 111-120.

[11] Bredholdt H., Galatenko O.A., Engel hardt K., Fjærvik E., Terekhova L.P. and Zotchev S.B., Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity, *Environ. Microbiol*., 2007; **9**: 2756-2764.

[12] Cragg G.M. and Newman D.J., Nature: a vital source of leads for anticancer drug development, *Phytochem. Rev*., 2009; **8**: 1568-7767.

[13] Williams P.G., Panning for chemical gold: marine bacteria as a source of new therapeutics, *Trends Biotechnol*., 2009; **27**: 45-52.

[14] Laiz L., Groth I., Gonzalez I. and Saiz-Jimenez C., Microbiological study of the dripping waters in Altamira cave (Santillana del mar, Spain), *J. Microbiol. Meth*., 1999; **36**: 129-138.

[15] Barton A.H., Introduction to cave microbiology: A review for the non-specialist, *J. Cave Karst Stud.*, 2006; **68**: 43-54.

[16] Groth I., Vettermann R., Schuetze B., Schumann P. and Saiz-Jimenez C., Actinomycetes in Karstic caves of northern Spain (Altamira and Tito Bustillo), *J. Microbiol. Meth*., 1999; **36**: 115-122.

[17] Day M.J. and Urich P.B., An assessment of protected karst landscapes in Southeast Asia, *Cave and Karst Sci*., 2000; **27**: 61-70.

[18] Clements R., Sodhi N.S., Schilthuizen M. and Ng P.K.L., Limestone karsts of southeast Asia: imperiled arks of biodiversity, *BioSci*., 2006; **56**: 733-742.

[19] Laiz L., Gonzalez-Delvalle M., Hermosin B., Ortiz-Martinez A. and Saiz-Jimenez C., Isolation of cave bacteria and substrate utilization at different temperatures, *Geomicrobiol. J.*, 2003; **20**: 479-489.

[20] Groth I., Schumann P., Laiz L., Sanchez-Moral S., Canaveras J.C. and Saiz-

Jimenez C., Geomicrobiological study of the Grotta dei Cervi, Porto Badisco, Italy, *Geomicrobiol. J*., 2001; **18**: 241-258.

[21] Lee S.D., *Actinocorallia cavernae* sp. nov., isolated from a natural cave in Jeju, Korea, *Int. J. Syst. Evol. Microbiol*., 2006; **56**: 1085-1088.

[22] Jurado V., Groth I., Gonzalez J.M., Laiz L. and Saiz-Jimenez C., *Agromyces subbeticus* sp. nov., isolated from a cave in southern Spain, *Int. J. Syst. Evol. Microbiol*., 2005; **55**: 1897-901.

[23] Lee S.D., *Amycolatopsis jejuensis* sp. nov. and *Amycolatopsis halotolerans* sp. nov., novel actinomycetes isolated from a natural cave, *Int. J. Syst. Evol. Microbiol*., 2006; **56**: 549-553.

[24] Jurado V., Boiron P., Kroppenstedt R.M., Laurent F., Couble A., Laiz L., Klenk H.-P., González J.M., Saiz-Jimenez C., Mouniée D., Bergeron E. and Rodríguez-Nava V., *Nocardia altamirensis* sp. nov., isolated from Altamira cave, Cantabria, Spain, *Int. J. Syst. Evol. Microbiol.*, 2008; **58**: 2210-2214.

[25] Seo J.P., Yun Y.-W. and Lee S.D., *Nocardia speluncae* sp. nov., isolated from a cave, *Int. J. Syst. Evol. Microbiol*., 2007; **57**: 2932-2935.

[26] Groth I., Schumann P., Schutze B., Augsten K. and Stackebrandt E., *Knoellia sinensis gen*. nov., sp. nov. and *Knoellia subterranea* sp. nov., two novel actinobacteria isolated from a cave, *Int. J. Syst. Evol. Microbiol*., 2002; **52**: 77-84.

[27] Jurado V., Kroppenstedt R.M., Saiz-Jimenez C., Klenk H.-P., Mouniée D., Laiz L., Couble A., Pötter G., Boiron P. and Rodríguez-Nava V., *Hoyosella altamirensis* gen. nov., sp. nov., a new member of the order *Actinomycetales* isolated from a cave biofilm, *Int. J. Syst. Evol. Microbiol*., 2009; **59**: 3105-3110.

[28] Nakaew N., Pathom-aree W. and Lumyong S., First record of the isolation, identification and biological activity of a new strain of *Spirillospora albida* from Thai cave soil, *Actinomycetol*., 2009; **23**: 1-7.

[29] Nakaew N., Pathom-aree W. and Lumyong S., Generic diversity of rare actinomycetes from Thai cave soils and their possible use as new bioactive compounds, *Actinomycetol.*, 2009; **23**: 21-26.

[30] Reed J. and Cummings F.S., Soil reaction-glass electrode and colorimetric methods for determining pH values of soil, *Soil Sci*., 1945; **59**: 97-104.

[31] Duangmal K., Ward A.C. and Good fellow M., Selective isolation of members of the *Streptomyces violaceoruber* clade from soil, *FEMS Microbiol. Lett.*, 2005; **245**: 321-327.

[32] Hayakawa M., Sadakata T., Kajiura T. and Nonomura H., New methods for the highly selective isolation of *Micromonospora* and *Microbispora* from soil, *J. Ferment. Bioeng*., 1991; **72**: 320-326.

[33] Bulina T.I., Alferova I.V. and Terekhova L.P., A novel approach to isolation of actinomycetes involving irradiation of soil samples with microwaves, *Microbiol*., 1997; **66**: 278-282.

[34] Küster E. and Williams S.T., Selection of media for isolation of streptomycetes, *Nature*, 1964; **202**: 928-929.

[35] Hayakawa M. and Nonomura H., Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes, *J. Ferment. Technol*., 1987; **66**: 501-509.

[36] Shirling E.B. and Gottlieb D., Methods for characterization of *Streptomyces* species, *Int. J. Syst. Bacteriol.*, 1966; **16**: 313-340.

[37] Wellington E.M.H. and Williams S.T., Preservation of actinomycete inoculum in frozen glycerol, *Microbiol. Lett*., 1978; **6**: 151-157.

[38] Mundie D.A., The NBS/ISCC Color System/David A. Mundie Pittsburgh, PA:24 Polymath Systems 535.6 dc-20, 1995. (http://www.anthus.com/color/cent.html).

[39] Becker B., Lechevalier M., Gordon R. and Lechevalier H.A., Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates, *Appl. Microbiol*., 1964; **12**: 421-423.

[40] Hasegawa T., Takizawa M. and Tanida S., A rapid analysis for chemical grouping of aerobic actinomycetes, *J. Gen. Appl. Microbiol*., 1983; **29**: 319-322.

[41] Kieser T., Bibb M.J., Buttner M., Chater K.F. and Hopwood D.A., *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, 2000.

[42] Lane D.J., 16S/23S rRNA Sequencing, in: Stackebrandt E., and Goodfellow M., eds., *Nucleic Acid Techniques in Bacterial Systematic*, Wiley, New York, 1991: 115-175.

[43] Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G., The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Res*., 1997; **25:** 4876-4882.

[44] Jukes T.H. and Cantor C.R., Evolution of Protein Molecules; in Munro H.N., ed., *Mammalian Protein Metabolism*, vol 3. Academic Press, New York, 1969; 21-132.

[45] Van de Peer Y. and De Wachter R., TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment, *Comp. Appl. Biosci*., 1994; **10**: 569-570.

[46] Bolter M., Blume H.P., Schneider D. and Beyer L., Soil properties and distributions of invertebrates and bacteria from King George Island (Arctowski Station), maritime Antarctic, *Polar Biol.*, 1997; **18**: 295-304.

[47] Laorpaksa S., Yingyong A., Thoong suwan S. and Pongsopida A., Study on antibiotic producing actinomycetes from cave soil in central region of Thailand, *J. Natl. Res. Council Thailand*, 1987; **19**: 61-79.

[48] Labeda D.P. and Shearer M.C., Isolation of Actinomycetes for Biotechnological Applications; in: Labeda D.P., ed., *Isolation of Biotechnological Organisms from Nature*, McGraw-Hill Publishing Company, Toronto, 1990: 1-20.

[49] Hayakawa M., Studies on the isolation and distribution of rare actinomycetes in soil, *Actinomycetol*., 2008; **22**: 12-19.

[50] Tamura T., Hatano K. and Suzuki K., A new genus of the family *Micromonosporaceae*, *Polymorphospora* gen. nov., with description of *Polymorphospora rubra* sp. nov., *Int. J. Syst. Evol. Microbiol*., 2006; **56:** 1959-1964.

[51] Tseng M., Yang S.F., Li W.J. and Jiang C.L., *Amycolatopsis taiwanensis* sp. nov., from soil, *Int. J. Syst. Evol. Microbiol*., 2006; **56**: 1811-1815.

[52] Ara I., Matsumoto A., Bakir M.A., Kudo T., Ōmura S. and Takahashi Y., *Pseudosporangium ferrugineum* gen. nov., a new member of the family *Micromonosporaceae*, *Int. J. Syst. Evol. Microbiol.*, 2008; **58**: 1644-1652.

[53] Williams S.T., Goodfellow M., and Alderson A., Genus *Streptomyces* Waksman and Henrici 1943, 339AL.; in Williams S.T., Sharpe M.E., and Holt J.G., eds., *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins, Baltimore, 1989: 2452-2492.

[54] Antony-Babu S. and Goodfellow M., Biosystematics of alkaliphilic streptomycetes isolated from several locations across a beach and dune sand system, *Antonie van Leeuwenhoek*, 2008; **94**: 581-591.

[55] Kim B.S., Lee J.Y. and Hwang B.K., Diversity of actinomycetes antagonistic to plant pathogenic fungi in cave and sea-mud soils of Korea, *J. Microbiol*., 1998; **36**: 86-92.

[56] Groth I. and Saiz-Jimenez C., Actinomycetes in hypogean environments, *Geomicrobiol. J*., 1999; **16**: 1-8.

[57] Laiz L., Groth I., Schumann P., Zezza F., Felske A., Hermosin B. and Saiz-Jimenez C., Microbiology of the stalactites from Grotta dei Cervi, Porto Badisco, Italy, *Int. Microbiol*., 2000; **3**: 25-30.

[58] Thawai C., Tanasupawat S., Itoh T., Suwanborirux K. and Kudo T., *Micromonospora siamensis* sp. nov., isolated from Thai peat swamp forest, *J. Gen. Appl. Microbiol*., 2005; 51: 229-234.

[59] Lee S.D., Kang S.O. and Hah Y.C., *Catellatospora koreensis* sp. nov., a novel actinomycete isolated from a gold-mine cave, *Int. J. Syst. Evol. Microbiol*., 2000; **50**: 1103-1111.

[60] Ara I., Bakir M.A. and Kudo T., Transfer of *Catellatospora koreensis* Lee *et al*. 2000 as *Catelliglobosispora koreensis* gen. nov., comb. nov. and *Catellatospora tsunoense* Asano *et al*. 1989 as *Hamadaea tsunoensis* gen. nov., comb. nov., and emended description of the genus *Catellatospora* Asano and Kawamoto 1986 emend. Lee and Hah 2002, *Int. J. Syst. Evol. Microbiol.*, 2008; **58**: 1950-1960.

[61] Boondaeng A., Ishida Y., Tamura T., Tokuyama S. and Kitpreechavanich V., *Microbispora siamensis* sp. nov., a thermotolerant actinomycete isolated from soil, *Int. J. Syst. Evol. Microbiol*., 2009; **59**: 3136-3139.

[62] Wayne L.G., Brenner D.J., Colwell R.R., Grimont P.A.D., Kandler O., Krichevsky M.I., Moore L.H., Moore W.E.C., Murray R.G.E., Stackebrandt E., Starr M.P. and Trüper H.G., International Committee on Systematic Bacteriology, Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematic, *Int. J. Syst. Bacteriol*., 1987; **37**: 463-464.

[63] Chng C., Lum A.M., Vroom J.A. and Kao C.M., A key developmental regulator controls the synthesis of the antibiotic erythromycin in *Saccharopolyspora erythraea*, *Proc. Natl. Acad. Sci*., 2008; **105**: 11346-11351.

[64] Lee S.D., Kim E.S., Min K.L., Lee W.Y., Kang S.O. and Hah Y.C., *Pseudonocardia kongjuensis* sp. nov., isolated from a gold mine cave, *Int. J. Syst. Evol. Microbiol*., 2001; **51**: 1505-1510.

[65] Stackebrandt E. and Goebel B.M., Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology, *Int. J. Syst. Bacteriol*., 1994; **44**: 846-849.

[66] Stackebrandt E., Wink J., Steiner U. and Kroppenstedt R.M., *Nonomuraea dietzii* sp. nov., *Int. J. Syst. Evol. Microbiol*., 2001; **51**: 1437-1441.

[67] Jurado V., Laiz L., Rodriguez-Nava V., Boiron P., Hermosin B., Sanchez-Moral S. and Saiz-Jimenez C., Pathogenic and opportunistic micro-organisms in caves, *Int. J. Speleo.*, 2010; **39**: 15-24.

[68] Holmes A.J., Tujula N.A., Holley M., Contos A., James J.M., Rogers P. and Gillings M.R., Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia, *Environ. Microbiol.*, 2001; **3**: 256-264.

[69] Schabereiter-Gurtner C., Saiz-Jimenez C., Pinar G., Lubitz W. and Rolleke S., Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Palaeolithic paintings, *Environ. Microbiol.*, 2002; **4**: 392-400.

[70] Schabereiter-Gurtner C., Saiz-Jimenez C., Pinar G., Lubitz W. and Rolleke S., Altamira cave paleolithic paintings harbor partly unknown bacterial communities, *FEMS Microbiol. Lett.*, 2002; **211**: 7-11.

[71] Barton A.H., Taylor M.R. and Pace N.R., Molecular phylogenetic analysis of a bacterial community in an oligotrophic cave environment, *Geomicrobiol. J.*, 2004; **21**: 11-20.

[72] Portillo M.C., Gonzalez J.M. and Saiz-Jimenez C., Metabolically active microbial communities of yellow and grey colonizations on the walls of Altamira cave, Spain, *J. Appl. Microbiol.*, 2008; **104**: 681-691.

[73] Lazzarini A., Toppo C.G. and Marinelli F., Rare genera of actinomycetes as potential producers of new antibiotics, *Antonie van Leeuwenhoek*, 2000; **79**: 399-405.