CHAPTER 2

DIVERSITY OF RARE ACTINOMYCETES FROM CAVE SOILS IN NORTHERN THAILAND

2.1 INTRODUCTION

The actinomycetes especially *Streptomyces* are remarkable as antibiotic producers for their unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Ten of thousands of such compounds have been isolated and characterized, many of which have been developed into drugs for treatment of a wide range of human diseases (Bull *et al.*, 1992; Franco and Coutinho, 1991). Main problem in discovery of new drug is the re-isolation of strains producing previously known antibiotics. Less studied organisms such as non-streptomycete species (rare actinomycetes) especially novel strain of them are attractive for exploring new antibiotics (Takahashi and Omura, 2003). Successful approaches for discovery of novel rare actinomycetes need the ecological study for their distribution in diverse habitats and development of appropriate procedures for isolation and cultivation in laboratory. Novel ecosystem that no one investigated yet is attractive place for the finding of novel actinomycetes.

Thailand is located in Southeast Asia, a place well known for its species-rich (Bull *et al.*, 1992; Myers, 1988). The plant and animal species diversity in this area have been reasonably well documented but microbial diversity remains inadequate. Thus, this zone may be valuable for investigation. Furthermore, few genera of rare

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

actinomycetes such as *Micromonospora*, *Actinomycetospora* and *Microbispora* have been reported as new species. Thus, it encourages us to investigate diversity of them in this zone.

Commonly, cave soil was low in nutrient, temperatures and light intensity but high humidity (Schabereiter-Gurtner *et al*., 2002). This factor might prompts encourage the competition, which enhances the production of substance that inhibits growth of other microorganism such as antibiotics and hydrolytic enzymes. Due to several new species of actinomycetes had been isolated from cave such as gold mine cave in Korea (Lee *et al*., 2000a, b, d), Reed Flute cave in China (Groth *et al.*, 1999), Grotta Dei Cervi cave in Italy (Jurado *et al*., 2005a) and cave of bats in Spain (Jurado *et al*., 2005b). Therefore, cave soil will be excellent source to isolate rare actinomycetes.

In this chapter, several selective methods were used to isolation of rare actinomycetes from cave soil in northern Thailand. Three approaches, morphological features, the composition of cell wall diaminopimelic acid and 16S rRNA gene sequence-based phylogenetic analysis, are combined to identify actinomycetes. Briefly, two approaches were used to distinguish into Streptomycetes and rare actinomycetes group and comparism of quantity of them between rhizosphere soil and cave soil was performed. Then all of rare actinomycetes were group based on their spore formation and colony morphology. Last approach was used to identify representative isolate of each group to genus level. By phylogenetic analysis based on 16S rRNA sequence data from selected isolate and known species, comparative of population of each genus, evidence for the presence of novel genera and their ability to produce antibiotics were described.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2 LITERATURE REVIEWS

2.2.1 BIOLOGY OF ACTINOMYCETES

Actinomycetes are defined as Gram-positive bacteria that have high G+C (>55%) content in their DNA (Williams *et al*., 1989). Most of these organisms grow as thin filaments rather than as single cells. Actinomycetes dominate the microbial life in soil where they play a major role in the decay of dead organic matter. Many of them are sources of valuable antibiotics, including streptomycin, erythromycin, and the tetracycline that are highly useful pharmaceutical products. In 2000, three quarters of all known naturally occurring antibiotics were derived from actinomycetes (Hopwood *et al*., 2000).

The morphology and arrangement of spores and chemistry (cell wall and whole cell composition, type of lipid, isoprenoid quinones) are particularly important in actinomycetes taxonomy and are used to divide these bacteria into different groups. The 1st edition of Bergey's Manual divides the actinomycetes into 7 sections, primarily based on cell wall type, conidia arrangement, and the presence or absence of a sporangium (Holt *et al*., 1994). The 2nd edition added the use of 16S rRNA sequences to create a single large phylum Actinobacteria. This phylum contains one class, five subclasses, six orders, 14 suborders and 40 families (Williams *et al*., 1989).

2.2.2 RARE ACTINOMYCETES

Rare actinomycetes (non-*Streptomyces* group) are a group of actinomycete genera other than *Streptomyce*s. Due to their growth normally slower than *Streptomyces* and other microorganism and requires complicated procedure for isolation, preservation and cultivation in some genera (Lazzarini *et al*., 2000). Thus, they were regarded as less exploited microorganism. Presently, rare actinomycetes

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

were mentioned that they are less abundant in the environment or are they just more difficult to isolate and cultivate? Cause of various isolation procedures has been developing for isolate them from their habitat. Currently, some genera such as *Micromonospora* was eliminated from group of rare actinomycetes in some researcher group by reason of their frequency found.

In Thailand, new species of rare actinomycetes are less report due to their complication of isolation and identification. However, only two genus of new species of rare actinomycetes, *Micromonospora* (Thawai *et al.*, 2004, 2005a, b, 2007, 2008; Suchada *et al.*, 2008), *Microbispora* (Nakajima *et al.*, 1999), were reported.

2.2.3 DIVERSITY OF ACTINOMYCETES

The value of microorganism is they are able to recycle nutrients, produce and consume gases that affect global climate, destroy pollutants, treat wastes, and they can be used for biological control of plant and animal pests. Applying processes carried out by them to solve problems in agriculture, food production, human health, environmental quality and industry have been highly successful. The study of microbial diversity is also important to let us know about the distribution of them in each environment, expanding the knowledge about the strategies and limits of their life and diversity knowledge can be used for monitoring and predicting environmental change. Furthermore, the untapped diversity of microorganisms is a key resource for new genes and organisms of value to biotechnology. The prolific resource of new compound is new organism that might be diverse in new ecological system. Identification of novel ecological systems is therefore providing for the discovery of novel rare actinomycetes.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Recent data suggests that perhaps 300,000 to 1 million species of bacteria exist on earth yet only 3,100 bacteria are described in Bergey's Manual systematic of bacteria. A gram of typical soil contains about 1 billion bacteria, but only 1% of those can be cultured. Similarly low fractions of microorganisms have been cultured from fresh water and ocean environments. Hence, most microbes remain to be discovered. Several methods had been developed for isolate unexplored organism from their habitat for achieve correct diversity data. This traditional method which depending on cultivable waste a lot of time to complete program and much effort waste if the genera of interest are not present. Currently, the application of molecular techniques to the study of microbial diversity has change the face of microbial ecology. Microbe can be recognized at the DNA or RNA level without the cultivation of pure cultures on selective media (Gathogo *et al.*, 2004). Although, this method is rapid but it just use as pre-screening material to know how many genera and evidence to present the new species within each environments. The exploration of its ability to produce new compound in biotechnological field need both isolation and cultivation.

Priorities of microbial diversity research are;

1. Addresses gaps in our basic understanding of how microbial diversity originates and where it resides.

2. Focuses on the discovery of the unknown microbes, including the new methods that are needed to culture and rapidly characterize the previously unculturable organisms.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3. Addresses the need of preserving newly discovered, often fastidious organisms, including in situ and consortia preservation as well as more rapid and efficient methods for preservation.

4. Focuses on organizational and infrastructure needs, including improvements in databases, centralized facilities for specialized routine efforts and training of a new generation of microbial diversity and taxonomy experts.

2.2.4 RARE ACTINOMYCETE HABITATS

Although soil is the main habitat of rare actinomycetes, they also can be isolated from sediments, water, plants, stones and animals (Groth and Saiz-Jimenez, 1999). The population and types of rare actinomycetes in each ecosystem are affected by numerous physical, chemical and biological factors.

2.2.4.1 SEDIMENTS

Takizawa *et al.* (1993) isolated actinomycetes from Chesapeake Bay sediments and found that the majority of isolates were actinoplanetes. Bimodal distribution of actinomycetes in near-shore tropical marine environments, with streptomycetes predominating at shallow depths and an increase in actinoplanetes with increasing depth was also reported (Jensen *et al.* (1991).

2.2.4.2 TERRESTRIAL SOILS

Terrestrial soils are the good source of actinomycetes. Wang *et al.* (1999) isolated five thousand actinomycetes from soil samples collected from rainforests in Singapore and the generic identities of these isolates were determined by using a procedure that combined morphological, chemotaxonomic and 16S rDNA sequence-based phylogenetic analyses. The most abundant isolates are members of *Streptomyces*. However, *Micromonospora, Actinoplanes, Actinomadura,*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Nonomuraea, Nocardia and *Streptosporangium* were also found. Consistent with the results of many other studies, *Streptomyces* are the most abundant and *Micromonospora* isolates were the second.

2.2.4.3 WATER

Biodiversity of actinomycetes in water was reported by many researchers who studied from many habitats, such as Lake Baikal, that most of the water isolates belong to the genus *Streptomyces* and most of the sediment isolates belong to the genus *Micromonospora* (Terkina *et al.,* 2002). The shallow water sediments of the Trondheim fjord (Norway) were investigated and found that the predominant genera were clearly *Streptomyces* and *Micromonospora,* representatives of *Actinocorallia*, *Actinomadura*, *Knoellia*, *Glycomyces*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Pseudonocardia*, *Rhodococcus* and *Streptosporangium* were also isolated as well (Bredholdt *et al.,* 2007). Aquatic actinomycetes in 12 lakes of the Middle Plateau of Yunnan were studied from 1983 to 1993. Sixteen genera of actinomycetes were isolated from these samples. *Micromonospora* assumed a notable dominance (from 39 to 89%). *Streptomyces* were the second most abundant organisms (Jiang and Xu, 1996).

2.2.4.4 PLANTS

Actinomycetes are widely distributed in association with plant in natural environments. These plant-associated (endophytic) microorganisms are opportunistic and/or saprophytic but do not show pathogenicity in general. Taechowisan *et al.* (2003) isolate endophytic actinomycetes from the varous tissues of 36 plant species. Most of taxa being *Streptomyces* sp. (n=277); other strains belonged to *Microbispora* sp. (n=14), *Nocardia* sp. (n=8), *Micromonospora* sp. (n=4).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2.4.5 ANIMAL

Olson *et al.* (2007) isolated *Tsukamurella spongiae* sp. nov from a deep-water marine sponge collected off the coast of Curaçao in the Netherlands Antilles.

2.2.4.6 CAVES

Caves are generally constant low temperatures, high relative humidity, low quantities of organic nutrients and high mineral concentrations. Therefore, caves can be considered extreme environments for life, which only highly specialized microorganisms, can growth. Groth *et al.* (1999) studied actinomycetes in Karstic caves of northern Spain. It was found that actinomycetes revealed a great taxonomic diversity with the predominant isolates belonging to the genus *Streptomyces*. Members of the genera *Nocardia*, *Rhodococcus*, *Nocardioides*, *Amycolatopsis*, *Saccharothrix*, *Brevibacterium*, *Microbacterium* and coccoid actinomycetes (family *Micrococcaceae*) were also found. Laiz *et al.* (2000) investigated the microbiology of active stalactites from Grotta dei Cervi, Porto Badisco and southeastern Italy. Genus *Streptomyces* were the most abundant, followed by members of the genus *Bacillus*. Further isolates were assigned to the genera *Amycolatopsis, Arthrobacter, Agromyces, Micrococcus*, *Nocardiopsis* and *Rhodococcus* of the order *Actinomycetales*

Several new species of actinomycetes had been isolated from cave such as *Catellatospora koreensis* sp. nov. and *Saccharothrix violacea* sp. nov. from gold mine cave in Korea (Lee *et al.*, 2000a and Lee *et al.*, 2000b), *Agromyces salentinus* sp. nov. and *Agromyces neolithicus* sp. nov. from Grotta Dei Cervi cave in Italy (Jurado *et al.*, 2005a), *Agromyces subbeticus* sp. nov. from cave of bats in Spain (Jurado *et al.*, 2005b) and *Nocardia speluncae*, *Actinocorallia cavernae*,

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Amycolatopsis jejuensis and *Amycolatopsis halotolerans* from natural cave on Jeju island in Korea (Lee, 2006 a, b; Soe *et al.*, 2007) . Moreover new genus also found such as *Beutenbergiaa cavernae* gen. nov. (Groth *et al.*, 1999) and *Hongia* gen. nov. (Lee *et al.*, 2000b)

2.2.5 ISOLATION OF RARE ACTINOMYCETES FROM SOIL

Rare actinomycte are normally slow growth thus, may be covered by faster growing microorganism such as orther bacteria, fungi and *Streptomyces* although many particular rare actinomycete may exist in the soil samples. Hence, to selectively isolation of rare actinomycete in soil, methods to enhance rare actinomycete (enrichment) and eliminate unwanted microorganisms (pretreatment) must be used.

However, various enrichment and pretreatment methods have been developed for selective isolation of rare actinomycetes (Table 1).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Table 1 Summary of methods developed for the selective isolation of rare actinomycete from soil (1987–2007) (Hayakawa, 2008)

Pretreatment	**Culture media**	**Genera selected**
Physical:		
None	HV agar with or without nalidixic and trimethoprim	*Streptomyces* and other genera
Dry heat at 120℃ for 1 h	HV agar with nalidixic	Many rare actinomycete genera including *Spirilliplanes*
Chemical:		
SDS 0.05% and yeast extract 5%	HV agar with nalidixic	*Streptomyces* and other genera
Phenol 1.5%	HV agar with nalidixic and tunicamycin	*Micromonospora*
Phenol 1.5%	HV agar	*Streptomyces violaceusniger*
Chloramine-T	HV agar with nalidixic	*Herbidospora, Microbispora,Microtetraspora, Nonomuraea, Streptosporangium*
Physical and chemical:		
Dry heat at 110 ℃ for 1 h and phenol 1.0%	HV agar with kanamycin, josamycin, lysozyme and nalidixic acid	*Actinomadura viridis*
Dry heat at 120℃ for 1 h and phenol 1.5%-CG 0.01%	HV a gar with nalidixic acid	*Microbispora*
Dry heat at 120 ℃ for 1 h and BC 0.01% (or 0.03%)	HV a gar with nalidixic acid and leucomycin (or tunicamycin)	*Streptosporangium* or*Dactylosporangium*
Dry heat at 110 ℃ for 1 h and BC 0.05%	LSV-SE agar with kanamycin, nalidixic acid and norfloxacin	*Microtetraspora*
Enrichment:		
Chemotaxis (γ-collidone, vanillin)	HV agar with nalidixic acid	*Actinoplanes, Catenuloplanes,Dactylosporangium* and *Virgosporangium*
Enrichment and physical:		
Pollen-baiting and drying	HV agar with nalidixic acid	*Actinoplanes*
Rehydration (30℃, 90 min) and centrifugation (1,500 × g, 20 min)	HV agar with nalidixic acid and trimethoprim	*Actinoplanes, Actinokineospora,Actinosynnema,Catenuloplanes, Cryptosporangium, Dactylosporangium, Geodermatophilus,Kineosporia,Sporichthya*
$CaCO_3$, rehydration and centrifugation	HV agar with fradiomycin, kanamycin, nalidixic and trimethoprim	*Actinokineospora*
Sucrose-gradient centrifugation (240× g, 30 min)	HV agar with nalidixic and chlortetracycline	*Nocardia*
Moist incubation and drying	HV agar with nalidixic acid and trimethoprim	*Streptomyces* and other genera

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2.5.1 SELECTIVE MEDIA

Due to rare actinomycetes was competitive disadvantage when culture on agar media with other fast growing microorganism. Thus, the media for isolating them was designed to reduce growth of competitive microbe and simultaneously not affecting growth of them (Williams and Wellington, 1982). In 1987, Hayakawa and nonomura formulated humic acid-vitamin (HV) agar, a medium containing humic acid as the sole carbon and nitrogen source. Humic acid was extremely heterogeneous cross-linked polymers (Kumada, 1975) and resistant to biological decomposition. However, actinomycetes can utilize it (Yanze and Blondeau, 1991) and use it to supported sporulation. While it restricting growth of non-filamentous bacteria colonies.

Solidifying agents cause effect on spore formation. Gellan gum plus calcium chloride significantly stimulated the formation of spores and aerial mycelium of *Actinobispora* (Suzuki *et al.,* 1998).

Hair hydrolysate vitamin agar (HHVA) was suitable medium for isolation of rare actinomycetes especially the following genus, *Micromonospora, Microbispora, Actinoplanes* and *Streptosporangium* (Seong *et al.*, 2001).

2.2.5.2 PHYSICAL BARIER

During selective isolation of rare actinomycetes, reducing the number of nonfilamentous bacteria on the isolation medium is important. Hirsch and Christensen (1983) employed a physical barrier for selective isolation of filamentous actinomycetes. Soil particles were placed on a 0.22 µm pore-size membrane filter located on the surface of a humus-based growth medium. Actinomycetes mycelia are thin enough to penetrate the pores and grow into the agar below, while fungi and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

other bacteria are confined to the membrane surface. Removing the filter after several days of incubation left only the actinomycetes behind.

2.2.5.3 HEAT-TREATMENT TECHNIQUE

Heat treatment of soil is another method for inhibiting bacterial growth. *Streptosporangium* and *Microbispora* species were more resistant to dry heat than the spore of Streptomycetes species (Nonomura and Ohara, 1969). This method has been used for the isolation of certain actinomycetes genera such as *Micromonospora, Microbispora*, *Streptosporangium, Dactylosporangium* and *Actinomadura* (Hayakawa *et al.*, 1991a, b; 1995).

2.2.5.4 SELECTIVE ANTIBITOTIC

The use of antibiotics to select desired microorganisms or against unwanted group has become one of the most important selective techniques for the isolation of different actinomycete genera. The starch-casein containing novobiocin 1-50 µg/ml was used to isolate *Actinoplanaceae* (Palleroni, 1980). Kanamycin promoted growth of *Actinomadura* and *Nocardia*. Rubomycin promoted growth of *Actinomadura*. Tavromycetin favored isolation of *Actinomadura* and *Nocardia*. Roseofungin favoured isolation of *Actinomadura* (Vetlugina *et al.*, 1990).

2.2.5.5 CHEMOTACTIC METHOD

Some members of the *Actinoplanaceae* form flagellated motile spores. These organisms can be isolated by baiting aqueous sources with insoluble substrates, such as keratin or pollen granules (Couch, 1950). Makkar and Cross (1982) used the spore motility of actinomycetes include strains of *Actinoplanes*, *Ampullariella*, *Amorphosporangium*, *Catenuloplanes*, *Dactylosporagium* and *Geodermatophilus* to isolated them from their habitat. Selective isolation of them, they were desiccated and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

rehydrated for release their motile spore. An alternative method for baiting techniques for isolation *Actinoplanaceae* is based on the attraction of zoospore to chloride ions and consist of flooding soil sample with water, immersing capillaries filled with phosphate buffer containing KCl in the liquid phase and collecting zoospores in the capillaries (Palleroni, 1980). Isolation method by using γ-collodine as the chemoattractant for isolation of *Actinoplanes* and *Dactylosporangium* from soil was successfully used (Hayakawa *et al.*, 1991a).

2.2.5.6 DIFFERENTIAL CENTRIFUGATION

Sucrose-gradient centrifugation was used in combination with a selective medium (HV agar) supplemented with chlortetracycline successfully and efficiently isolated soil *Nocardia* populations that are suppressed by conventional dilution plating approaches (Yamamura *et al.*, 2003).

2.2.5.7 USE OF BACTERIOPHAGE FOR THE SELECTIVE ISOLATION OF THERMOPHILIC ACTINOMYCETES

A method was developed to reduce the numbers of thermophilic bacteria on isolation plates, which in turn facilitated the detection and isolation of thermophilic actinomycetes. The phage susceptibility of thermophilic bacteria provided a selective means of reducing their numbers on isolation plates and hence increased the numbers of *Thermomonospora*, *Saccharopolyspora rectivirgula*, and thermophilic *Streptomyces* spp. on these media in comparison with the numbers recorded from control plates (Kurtboke *et al.*, 1993).

2.2.6 CHARACTERIZATION OF RARE ACTINOMYCETES

Characterization of actinomycetes is important step in any antibiotic screening program. Rapid identification of isolates, at least to the genus level, can provide

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

valuable information for later stages in development, such as fermentation scale-up. Identification to species level will often give the isolation biochemist a clue as to whether or not a metabolite is novel.

Originally, identification of actinomycetes was based on morphological observations and it is still important for describing the taxa, but it not sufficient to distinguish between many genera. Thus, hemotaxonomic data are need for identification of actinomycetes into genera level. Flow chart for identification of actinomycetes isolates to the genus level is presented in Figure 1.

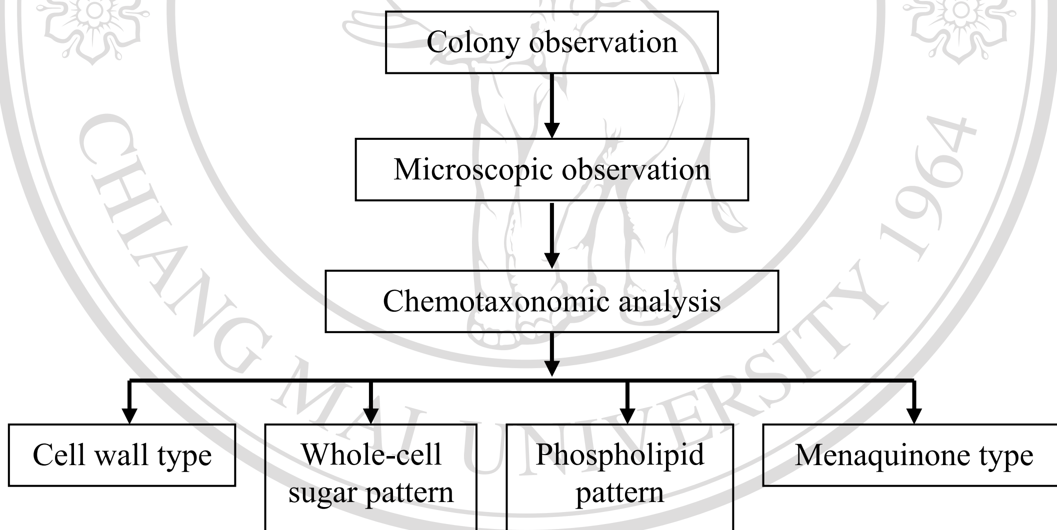


Figure 1 Guide to identification of actinomycetes

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.2.6.1 MORPHOLOGICAL CHARACTERIZATION

Several cultivation media suggested for the international *Streptomyces* project (Shirling and Gottlieb, 1966) and by Pridham *et al.*, (1957) are helpful to characterization. Some actinomycete genera such as *Actinomyces* (Anaerobic) or *Frankia* are requiring very special growth media and incubation conditions for

growth. Sporoactinomycetes require special media to allow differentiation and the development of characteristic spore and pigment such as media which contain colloidal chitin, soil extract, or decoctions of plant materials. Some species grow thinly but sporulate profusely on tap water agar.

Correlation between colony morphology and kind of mycelium production are shown in Table 2.

Table 2 Correlation between colony morphology and kind of mycelium production

Typical of mycelium	Description	Colony morphology
Substrate mycelium	A spore or fragments of mycelium develop into hyphae that penetrate the agar and hyphae that branch repeatedly and become cemented together on the surface of agar.	Tough, leathery
Aerial mycelium.	Free, erect hyphae surrounded by a hydrophobic sheath that grow into the air away from the colony.	Powdery, velvety
Fragmentation mycelium	the hyphae break up into rods and cocci	Soft, friable

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Bergey's Manual of Determinative Bacteriology 9 edition divide actinomycetes in to 8 groups (Group 22-29) based on morphology. Morphological feathers include the following:

1. Mycelium
2. Conidia
    a. Single conidia

b. Pairs of conidia

c. Short chains of conidia

d. Long chains of conidia

e. The conidia-bearing hyphae

3. Sporangia

4. Other structure such as the spore bearing synemata and sclerotia

Morphological characters are still widely used for characterizing genera, for example, the presence or absence of spores on the substrate mycelium or the formation of zoospores in specialized spore vesicles or sporangia although using it alone is insufficient.

2.2.6.2 CHEMOTAXONOMIC ANALYSIS

Chemotaxonomic criteria are need for differentiation of many actinomycetes genera, because of some genera are similar morphologically, but differ with their chemical composition. Cell-wall type and whole cell sugar pattern are the primary chemotaxonomic data that need to be determined. In some taxa, only chemotaxonomic data is sufficient to identify to the genus level. Most actinomycetes can be keyed to the appropriate genus after micromorphological observations have been made and cell-wall type-whole cell sugar pattern have been determined.

CELL-WALL ANALYSIS

DIAMINOPIMELIC ACID (DAP) ISOMERS

The classification and identification of actinobacteria depends on several chemotaxonomic characters (Goodfellow and O'Donnell, 1994). Especially identifying the third (diamino) acid of the glycan tetrapeptide, i.e. lysine or diaminopemilic acid (DAP) which is holds significant systematic information of

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

actinomycetes (Shleifer and Kandler, 1972). The differentiation between lysine and DAP is readily achieved by thin layer chromatography (Hancock, 1994). However, the identification of the DAP diastereomer (DD, LL or meso) is considerably more difficult and many methods have been reported in the literature. These include paper chromatography (Rhuland *et al.*, 1955; Becker *et al.*, 1964), and TLC (Staneck and Roberts, 1974; Hasegawa *et al.*, 1983; Tajima *et al.*, 2001), which is probably the most popular currently used method (Friedman *et al.*, 1998). Recently, Schön and Groth (2006) introduced pyridine-free solvent system for TLC on cellulose sheets which efficiently separate the DAP sterioisomers. However, both suffer from poor sensitivity and long analyses times, and the LL and DD isomers are not resolved. More recently HPLC and supercritical fluid chromatographic methods, based either on diastereomeric derivatives of DAP (El-Waziry *et al.*, 1996; Kudo *et al.*, 1998) or chiral mobile (Wiseman and Nichols, 1984) or chiral stationary phases (Nagasawa *et al.*, 1993; Medvedovici *et al.*, 1996) have been used. Not all of these methods have been applied to bacterial cultures. Some are unsuitable for complex amino acid mixtures (Nagasawa *et al.*, 1993), or need sample preparation that are tedious and time consuming (Kudo *et al.*, 1998).

LL-DAP is found in the cells of strain of the genera streptomycetes and *Nocardioides* etc. *Kitasatospor*a is the first bacterium recognized as having both *meso*-and LL-DAPs in the cell-wall (Omura *et al.*, 1982). In fact, *meso*-DAP is found in the cells of vegetative and filamentous mycelia, while LL-DAP is found in the submerged and aerial spores (Takahashi *et al.*, 1984). In such a case, fractionation is required for cell harvest.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3-Hydroxy-DAP (DD-DAP) is found in some actinomycetes, for example *Actinoplanes* and *Ampullariella*. In most cases, it is found together with *meso*-DAP. Amino acids other than DAP is need to determined when none of the DAP isomers are detected.

Lechevalier and Lechevalier (1980) defined 9 chemotypes, I-IX, based on the cell-wall amino acids (Table 3).

Table 3 Cell-wall chemotype of the actinomycetes

Major constituent	**Chemotype**								
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX***
LL-DAP	+								
Meso-DAP		+	+	+					+
DAB							+		
Aspartic acid						V			
Glycine	+	+					+		
Lysine					+		V		
Ornithine					+			+	
Arabinose				+					
Galactose				+		V			

V, Variable amount; DAP, 2,6 Diaminopimelic acid; DAB, 2,6 Diaminobutyric acid
* This type has numerous amino acids
(Lechevalier and Lecheralier, 1981)

WHOLE-CELL SUGARS

The sugar composition presents valuable information on the classification and identification of actinomycetes as same as DAP isomers. Paper chromatography gives good separation, although development takes more than one day. The solvent sytem used is n-butanol-water-pyridine-toluene (5:3:3:4). The whole-cell sugar pattern divides the actinomycetes containing *meso*-DAP in the peptidoglycan into five types

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

(Lechevalier and Lechevalier, 1981) (Table 4). The presence of madurose (3-O-methyl-D-galactose) is an important marker for some genera of actinomycetes, for example *Actinomadura*, *Streptosporangium*, *Spirillospora* and *Microbispor*a.

Table 4 The whole-cell sugar pattern of the actinomycetes containing *meso*-DAP

Pattern	**Sugar**				
	Arabinose	**Fructose**	**Galactose**	**Madurose**	**Xylose**
A	+		+		
B				+	
C			No diagnostic sugars		
D	+				+
E		+			

PHOSPHOLIPID COMPOSITION

The five phospholipids patterns observed in the *Actinomycetales* are:

PI no nitrogenous phospholipids present

PII phosphatidylethanolamine is the only nitrogen-containing phospholipids present

PIII phosphatidylcholine present

PIV phosphatidylethanolamine and glucosamine-containing phospholipid present

PV glucosamine-containing phospholipids are the only nitrogenous phospholipids present

The phospholipids pattern is important, however, to distinguish between some genera, such as *Amycolata* and *Amycolatopsis,* which have the same general morphology and cell-wall type but differing phospholipids pattern.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

MENAQUINONES PATTERN

Isoprenoid quinines are constitutents of bacterial plasma membrane and important in the functioning of the electron transport system in respiration. Various kinds of quinines are found in bacterial cells (Figure 2), with most aerobic bacteria processing isoprenoid menaquinone and/or isoprenoid ubiquinone (Komagata and Suzuki, 1987). The inherent structural variation exhibited by isoprenoid quinones might be of value in microbial systematics (Lester and Crane, 1959; Page *et al.*, 1960). Menaquinone patterns appear to be somewhat genus specific and may be used to confirm the assignment of a strain to a given genus.

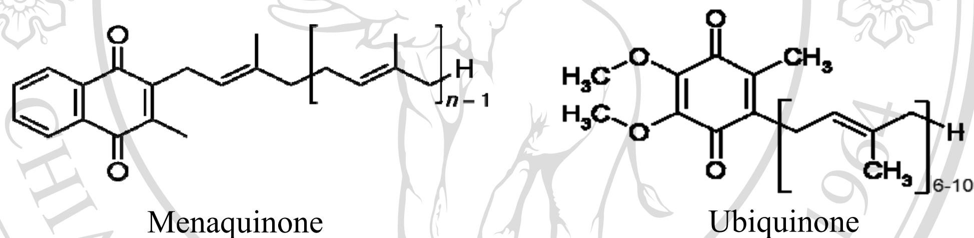


Figure 2 Isoprenoid menaquinone and/or isoprenoid ubiquinone

2.2.6.3 16S rRNA GENE SEQUENCE ANALYSIS

Among the genera of actinomycetes, their morphology, physiology and biochemical were diverse. On the basis of these characteristics various keys for the identification of species have been constructed (Dietz, 1986), but specialists working in this field could not agree for a long time on how many species should be distinguished. It has been suggested that reliable suprageneric classifications of bacteria are not possible using traditional approaches based upon a priori weighting of a few morphological and physiological features, since Gram-positive bacteria, and among them actinomycetes, form one of about 10 major suprageneric groups

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

(Strackebrandt *et al.*, 1983; Ludwig *et al.*, 1992). Avery and Blank (1954) suggested the use of chemical procedures in order to correlate the composition of the cell wall of the Actinomycetales with their systematic position. Later, in the past three decades, approaches to solve this problem have been made by means of chemical taxonomy (Lechevalier and Lechevalier, 1970a,b; Goodfellow and Minnikin, 1985; Hamid *et al.*, 1993). More recently, new approaches to solve the problem involved molecular methods as comparative sequencing and reassociation of DNA (Schleifer *et al.*, 1992; Schleifer *et al.*, 1993; Park *et al.*, 1993) had been occur. A most useful method for establishing the relatedness of higher taxa is the comparative analysis of the ribonuclease-resistant oligonucleotides of the 16S ribosomal RNA (rRNA) (Stackerbrandt *et al.*, 1985; Stackebrandt and Ludwig, 1989; Rainey *et al.*, 1994; Stackebrandt and Goebel, 1994). Nevertheless, it is not yet possible to compile a comprehensive suprageneric classification of actinomycetes from the results of partial sequencing of the 16S rRNA (Ludwig *et al.*, 1980; Stackebrandt *et al.*, 1981; Stackebrandt *et al.*, 1997). All this information shows that there is a big gap between traditional approaches and modern methods for the classification and identification of actinomycetes. Thus, their classification is still a crux not only for taxonomists, but also for researchers working with this genus, who try to apply valid criteria on their own isolates. Since the discrimination of distinct cultures among morphologically similar *Streptomyces* isolates and the detection of specific biosynthetic pathways in these strains are important steps in the selection of microorganisms to be included in a natural products library, it is important to establish definite criteria for working with species not reported and/or not already studied. Taking into account the mentioned considerations, the majority of researchers finish in building their own collections and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

classification system in order to work with new isolates avoiding repetitions among them.

2.3 METHODS

2.3.1 ISOLATION OF RARE ACTINOMYCETES FROM SOILS

2.3.1.1 SAMPLE COLLECTION

Eight cave soil samples were taken from three sites at Phanangkoi cave in Phrae province and five sites at Pha Tup Cave Forest Park in Nan province, Thailand (Table 5). Four rhizosphere soil samples were collected from medicinal plants (ginger, aloe vera, tumeric and garanga) grown in Nongbualamphu Province. All of them were used to compare diversity of rare actinomycetes between cave soil and rhizosphere soil.

Table 5 Location of soil sampling sites

Sample	Site	Province
1	Jedi Kaew Cave, Pha Tup Cave Forest Park	Nan
2	Khon cave, Pha Tup Cave Forest Park	Nan
3	Bor Nam Thip Cave, Pha Tup Cave Forest Park	Nan
4	Phra cave, Pha Tup Cave Forest Park	Nan
5	Takhian cave, Pha Tup Cave Forest Park	Nan
6	Pha nang khoi cave site 1	Phrae
7	Pha nang khoi cave site 2	Phrae
8	Pha nang khoi cave site 3	Phrae
9	Ginger rhizosphere soil	Nongbualamphu
10	Aloe vera rhizosphere soil	Nongbualamphu
11	Turmeric rhizosphere soil	Nongbualamphu
12	Garanga rhizosphere soil	Nongbualamphu

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.3.1.2 SOIL PH

One gram of each soil sample was suspended in 2.5 ml of distilled water and mixed well. After the soil solution was left for 30 min, the supernatant was gently shaken for 2-3 second and measured pH value by pH meter.

2.3.1.3 SOIL TREATMENT

Soil samples were air-dried at room temperature for 7-10 days and heating in hot air oven at 120 °C for 1 hour (Nonomura and Ohara, 1969).

2.3.1.4 SELECTIVE ISOLATION

A 100 mg of dried soil was suspended in 9 ml of 1.5% phenol solution for 30 minutes to kill fast growing bacteria. The 0.1 ml solution was spread onto Humic acid-Vitamin agar (HVA) (Hayakawa and Nonomura, 1987), Humic acid-vitamin-gellan gum medium (HVG) which was based on HVA but use gellan gum 7% instead of agar and starch casein agar (STC) (Okazaki *et al.*, 1983) which adjusted to different pH (5, 7 and 11). All of media containing cycloheximide and nystatin at the final concentrations of 10 and 25µg/ml, respectively. The incubation for isolation was performed at 28 °C for 20 days. All of isolates were maintained on Hickey-Tresner (HT) agar media (Hickey and Tresner, 1952). Strains obtained were deposited in the Excellent on Sustainable Development of Biodiversity Resources Center laboratory stock, Chiang Mai University, Thailand.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.3.2 TAXONOMIC CHARACTERIZATION OF RARE ACTINOMYCETES

2.3.2.1 CULTURAL AND MORPHOLOGICAL CHARACTERIZATION

All of isolates were cultured on inorganic salts starch agar (ISP-4 medium) or HT agar at 30 °C for 20 days. Color of substrate mycelium, aerial mycelium and

soluble pigment were observed on ISP-2, ISP-4 and HT agar using color chart of the 267 Color Centroids of the NBS/IBCC Color System (Mundie, 1995).

The morphological features of substrate mycelium, aerial mycelium and spore were observed by inserting sterile cover slips in the agar medium at an angle of 45° (Figure 3). The cover slips were stained and observed under light microscopy (Olympus BH-2).

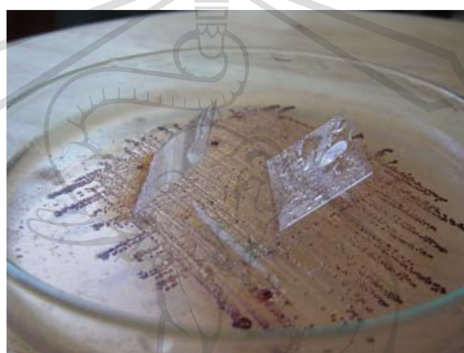


Figure 3 Slide culture technique

Samples for scanning electron microscopy were prepared by cutting a block from an agar plate. It was fixed in 2% glutaraldehyde vapor at room temperature for 3 hours. Then dehydrated through a series of ethanol solution (50, 60, 70, 80 and 95%, 15 min each; twice with 100% ethanol, 30 min/time). Ethanol was substituted with acetone and subject to critical-point dryer (CPD7510, Critical Point Drying Apparatus, Polaron, Rang) and then sputter coating with gold using a Gold Sputter (SPI-Module^{TM} Sputter Coater, SPI Supplies, Division of Structure Probe Inc., USA) and observed by scanning electron microscope (JEOL, JSM-5910, Japan).

To observe the spore formation, actinomycetes is streaked in a cross-hatched pattern on the surface of the agar (Figure 4). The angle of the streaks was subjected to looking for mature hyphae with spore.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

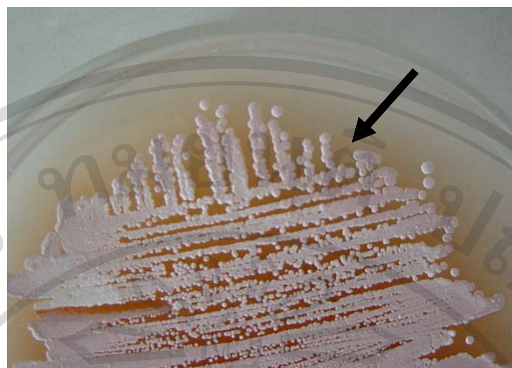


Figure 4 Cross-hatched streak pattern

2.3.2.2 CHEMOTAXONOMIC CHARACTERIZATION

Diaminopimelic acid (DAP) analysis

Dried cells (3 mg) were hydrolyzed with 1 ml of 6 N HCl at 121 °C for 1 h by autoclave. Each sample was applied as 3 µl on Cellulose TLC plate (20×20cm) (e.g. Merck No. 5716) or cellulose whatman No.1 paper. As DAP standard, 1µl of 0.01 M DL-DAP (Sigma Chemical Co., St. Louis, Mo., USA), which contains both *meso*- and LL-DAP isomer was used. Chromatography was developed with MeOH:H_2O:6 N HCl:Pyridine (80:26:4:10, v/v). Sample development required approximately 3 hours or more. Spots were visualized by spraying the chromatogram with 0.2% ninhydrin in water-saturated n-butanol follow by heating at 100 ºC for 5 min. Standard DAP spot was seen with the LL-isomer moving ahead of *meso*- isomer (Figure 5). DAP isomers appear as dark-green spots with R_f 0.29 (LL-isomer) and 0.24 (*meso*-and DD-isomer). DD-DAP appears lower than the *meso*-isomer (R_f approximately 0.20). Spots of other

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

amino acids run faster than DAP (R_f =0.37-0.80). Spots will gradually disappear in a few hours.

Cellulose high-performance TLC (HPTLC) plates (Merck No.5787) are effective for shortening development times (approximately 30 min) with the solvent system mentioned above. Furthermore, the amount of sample to be applied can be decreased to a half to one third. A normal cellulose TLC analysis presents a better separation than an HPTLC. However, it is quite sufficient to determine the three kinds of DAP isomers.

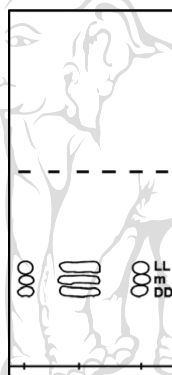


Figure 5 Separation of DAP by TLC (origin at bottom)

Whole-cell sugar

Dried cells (50 mg) were hydrolyzed with 1 ml of 1 N H_2SO_4 at 121°C for 1 h by autoclave. The sample (1 µl) was spotted on cellulose TLC plate or whatman No.1 paper. Solution of mixed standard sugar was spotted. Chromatography was performed with the solvent system *n*-butanol-water-pyridine-toluene (10:6:6:1, v/v). Sample development required less than 4 hours. Spots were visualized by spraying the chromatogram with acid aniline phthalate and heating at 100 ºC for 4 min. After heating, hexose spots (glucose, mannose, rhamnose, galactose and madurose) were

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

yellowish-brown and pentose spots (ribose, arabinose and xylose) were maroon. The stand solution of sugar (0.1% w/v for each sugar) migrated in the following sequence from the origin (slowest to fastest component): galactose, glucose, arabinose, mannose, xylose, ribose and rhamnose (Figure 6). Madurose (3-O-methyl-D-galactose), if present in a hydrolysate, migrated the same distance as xylose but distinguished by its yellowish-brown, while that xylose is maroon. In this system, 1μl spot of a 0.1% solution of any standard carbohydrate could easily be visualized.

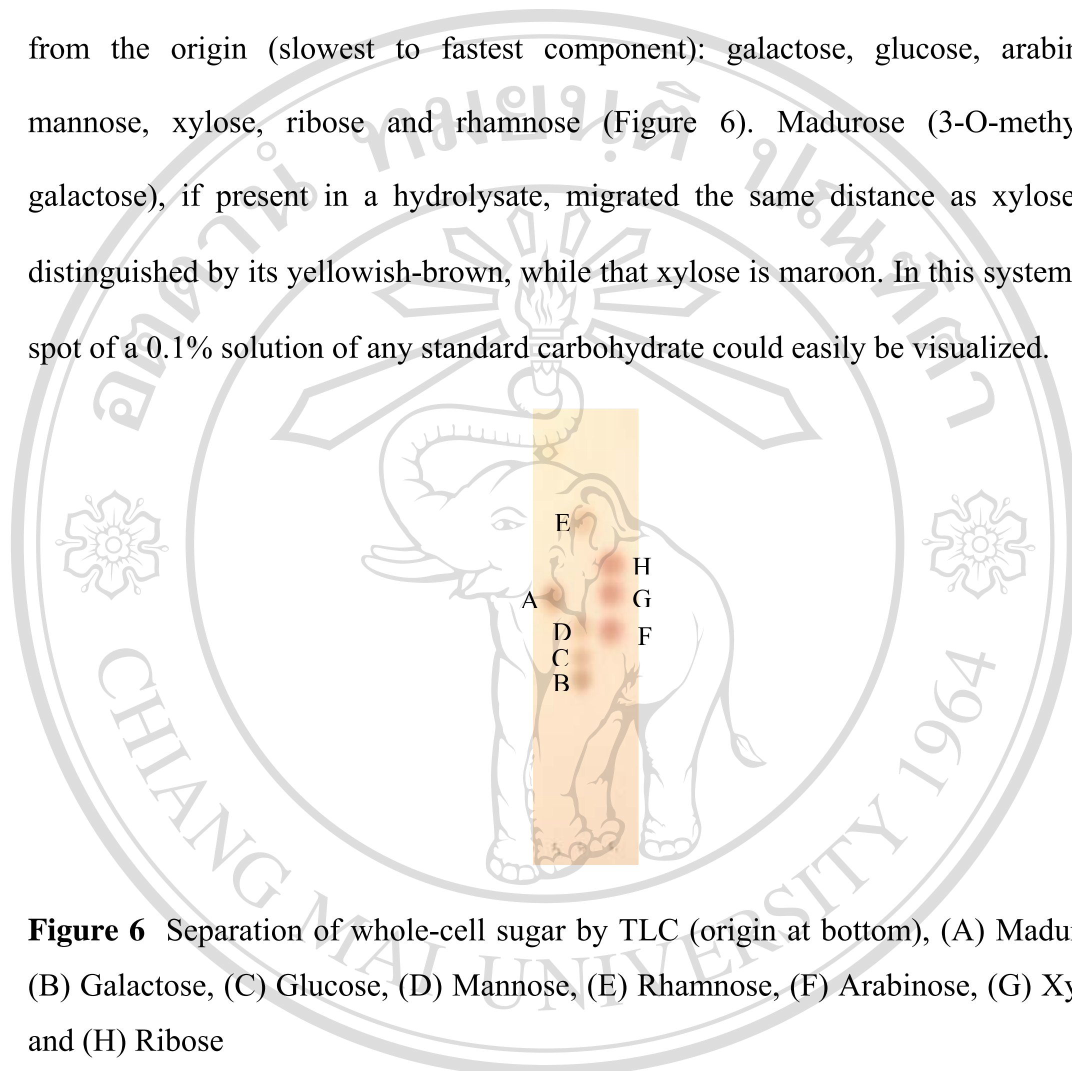


Figure 6 Separation of whole-cell sugar by TLC (origin at bottom), (A) Madurose, (B) Galactose, (C) Glucose, (D) Mannose, (E) Rhamnose, (F) Arabinose, (G) Xylose and (H) Ribose

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.3.2.3 MOLECULAR SYSTEMATIC

DNA extraction

Actinomycete was cultured in ISP-2 broth and incubated in the shaker incubator at 30 ºC for 10 days. The cells were harvested by centrifugation. Cell pellets were washed twice with double-distilled water and suspended in TE buffer pH 8.0 at ratio of 1:1 (V/V). Grind the cell by adding white quartz sand to the solution at

a ratio of 1:3 (v/v) to allow access to nuclear material. CTAB buffer was added for removing unwanted carbohydrate from DNA preparations. The mixture of CTAB and actinomycetes cells was incubated at 60 °C for 1 hour in water bath. After incubation, spin the CTAB/cell extract mixture at 13,000 rpm for 30 min to spin down cell debris. Transfer the supernatant to clean microcentrifuge tube. The tube was added Chloroform: Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 30 min. Transfer the upper aqueous phase only (contains the DNA) to a clean microcentrifuge tube. The tube was added 1ml of ice-cold absolute ethanol. The tube was inverted slowly several times and keeps it at -20°C overnight to precipitate the DNA. After precipitation, the precipitate can be isolated by centrifuged (13,000 rpm) at 4 °C for 15 min. The precipitate was washed by adding 1 ml of ice-cold 70% ethanol, spinning the tube at 13,000 rpm for 15 min and removes the supernatant. Repeat it three times. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Resuspend the DNA in sterile TE buffer pH 7.0 plus RNase A 50 µl.

DNA quality confirmation

Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 ml of 1x TAE buffer in a microwave. Pour it to a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface. Load the DNA plus loading buffer to well. Run the gel at 100 V. Expose the gel to UV light and photograph (demonstration). Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.

16S rRNA gene amplification by PCR

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

16S rRNA gene was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). The 50 µl PCR reaction mixture contained 5 µl of DNA template, 2 µl of 25 mM deoxyribonucleotide triphosphate (dNTPs), 2 µl of each 10 µM primer, 0.4 µl of Taq DNA polymerase, 5 µl of 10X PCR buffer and 33.6 µl of sterilization distilled water. All reactions were preheated at 95 °C for 5 min then 30 cycles of PCR amplification were performed using 95 °C 1 min denaturing, 50 °C 1 min annealing, and 72 °C 1 min extension. The reaction was kept at 72 °C 10 min for completed final extension. PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

Sequence analysis and phylogenetic tree construction

The purified PCR products were sequenced by Macrogen Inc. (Seoul, Korea). All sequences were compared with all accessible sequences in database using BLAST software. Multiple sequence alignment and computation of sequences similarity were conducted using ClustalW within the Bioedit program. Phylogenetic tree was constructed using the neighbour-joining method of Saitou and Nei (1987) in the Molecular Evolutionary Genetics Analysis (MEGA) program version 4 (Tamura *et al.*, 2007).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2.4. RESULTS AND DISCUSSION

2.4.1 COMPARATIVE STUDY OF RARE ACTINOMYCETE DIVERSITY BETWEEN RHIZOSPHERE AND CAVE SOILS

Four rhizosphere soil samples were collected from medicinal plant in Nongbualamphu province. The soil pH was ranging from 5.24-5.90 (Table 6). Cave

soil samples were collected from caves in Phrae and Nan province. The pH of each soil sample was ranging from 7.40-8.29 except sample from Pha nang khoi cave site 1 which was slightly acidic 5.03. The number of isolate between *Streptomyces* and rare actinomycetes group in each sampling site was shown in Table 6.

Table 6 The number of isolate between Streptomycetes and rare actinomycetes group in each sampling site.

Soil sample	Soil pH	No. of isolate		Total
		Streptomyces	**Rare actinomycetes**	
1. Medicinal rhizosphere soil, Nongbualamphu province				
► Ginger	5.62	82(65%)	45(35%)	127
► Aloe vera	5.61	43(66%)	22(34%)	65
► Turmeric	5.24	48(62%)	30(38%)	78
► Garanga	5.90	38(97%)	1(3%)	39
Total		211(68%)	98(32%)	309
2. Pha tup cave forest park, Nan province				
► Jedi kaew cave	7.73	17(28%)	43(72%)	60
► Khon cave	7.40	24(80%)	6(20%)	30
► Bor nam thip cave	7.79	29(60%)	19(40%)	48
► Phra cave	7.40	60(82%)	14(18%)	74
► Takhian cave	7.64	25(38%)	41(62%)	66
Total		155(56%)	123(44%)	278
4. Pha nang khoi cave, Phrae province				
► Site 1	5.03	6(67%)	3(33%)	9
► Site 2	8.28	7(54%)	6(44%)	13
► Site 3	8.29	41(53%)	36(47%)	77
Total		54(55%)	45(45%)	99

Table 6 showed a total of 692 actinomycetes isolated from that soil samples were subjected to identify up to two groups (*Streptomyces* and rare actinomycetes). 420 isolates were belonged to genus *Streptomyces*, based on the formation of spore chain which contained more than 20 spores and cell wall amino acid were contained LL-DAP. 272 isolates belong to rare actinomycete group, based on different in spore formation to *Streptomyces* and cell wall hydrolysates were not contained LL-DAP.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Similar to other studies, *Streptomyces* are the most abundant in both rhizosphere and cave soil (Hu *et al.*, 1995; Jiang and Xu, 1996; Ward-Rainey *et al.*, 1996; Groth *et al.*, 1999). The isolation rate of rare actinomycete between cave soil and rhizosphere soil does not exhibit differences. In addition, it was no correlation between soil pH and rare actinomycete population. Hence, it could be concludes that actinomycetes are ubiquitous.

The distribution of actinomycete has been studied in various types of soils and environments such as rice paddy, lake mud and water, deciduous forest, tropical forest, wasteland and natural caves (Hayakawa *et al.*, 1998; Jiang and Xu, 1996; Lim *et al.*, 1996; Shomura, 1993; Suzuki *et al.*, 1994; Xu *et al.*, 1996; Kim *et al.*, 1998). All of these report found that *Streptomyces* was the most abundant. It might because of its easier and faster grow in isolation media than another genus. Although many particular microorganisms may exist in the soil samples, but the isolation method and media are limited, thus there are only a small number of genus was detected (Iwai and Takahashi, 1992). In addition to the amount of them in soil, their ability to grow on culture media was prerequisite for isolate them from soil habitat.

Morphological features together with the composition of cell wall diaminopimelic acids (DAPs) and whole cell sugars are sufficient for accurate identification of many actinomycete genera (Holt *et al.*, 1994). These properties can be determined quickly by using simple techniques such as light microscopy and thin-layer-chromatography. However, these approaches were not adequately distinctive in several genera that exhibit similar morphological and chemotaxonomic properties, such as members of *Nonomuraea*, *Microbispora*, *Microtetraspora* and *Actinomadura* (Koch *et al.*, 1996; Wang *et al.*, 1996; Zhang *et al.*, 1998).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Furthermore, it was very difficult to distinguish *Streptomyces* from the genus *Kitasatospora*. Because of *Kitasatospora* produced long chain spore and has a cell wall that contains both *meso*-and LL-DAP similar to *Stretomyces* (Willaims *et al.*, 1989). *Meso*-DAP is found in the cells of vegetative and filamentous mycelia, while LL-DAP is found in the submerged and aerial spores (Takahashi *et al.*, 1984). In such a case, non-sporulation medium and fractionation is required for cell harvest.

16S rRNA gene sequence-based phylogenetic analysis has been widely used to determine taxonomic positions of many organisms in virtually all taxonomic ranks (Embley and Stackebrandt, 1994; Olsen *et al.*, 1986; Stackebrandt *et al.*, 1997; Woese *et al.*, 1990). Nowadays, many bacteria, which cannot be cultured in the laboratory, are identified solely by analyzing their 16S rRNA gene sequences (Amann *et al.*, 1995). Assisted by the PCR technique and the ability to directly sequence PCR products, the 16S rDNA sequence of an unknown organism can be quickly obtained and immediately compared with thousands of sequences in public databases. However, DNA sequence analysis is rather expensive when all of sequences need to be determined. Thus, all of isolate were grouping for reduced the numbers to a manageable level for 16S rRNA gene sequence analysis.

2.4.2 TAXONOMIC CHARACTERIZATION OF RARE ACTINOMYCETES STRAIN ISOLATED FROM CAVE SOILS

A total of 168 rare actinomycetes isolated from cave soils were subjected to identify to genus level. based on the formation of single spore on sporophore 50 isolates were belonged to genus *Micromonospora*. One isolate was belonged to genus *Microbispora*, based on the formation of paired spores on aerial mycelia. Representative of these two genera and nine isolates of the other genus, which were

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

distinct in colony morphology, were randomly selected for further taxonomic characterization by chemotaxonomic and molecular systemic based on 16S rRNA gene sequences data. All obtained sequences were submitted to GenBank and compared with all accessible sequences in NCBI database using BLAST software (Table 7).

Table 7 Percent similarities of the 16S rRNA gene sequences between selected rare actinomycetes isolates obtained and the most closely related strain

Group	selected isolate	The most closely related strain	GenBank accession No.	% similarity
2	PNK404	*Micromonospora chersinia* X92628	FJ756552	99%
3	PNK464	*Micromonospora echinaurantiaca* FJ481619	FJ756553	98%
4	PT707	*Nonomuraea maheshkhaliensis* AB290014	FJ756557	99%
5	PT708	*Nonomuraea roseola* EU841591	FJ347524	98%
6	PNK225	*Microbispora rosea* D86936	FJ756549	98%
7	PNK384	*Catellatospora citrea* subsp. citrea AF152106	FJ756550	97%
8	PNK393	*Pseudonocardia halophobica* AJ252827	FJ756551	98%
9	PT725	*Actinocorallia aurantiaca* AF134066	FJ756555	98%
10	PT702	*Nonomuraea roseola* EU841591	FJ756554	99%
11	PT749	*Saccharothrix texasensis* AF114815	FJ756556	97%
12	PNK470	*Spirillospora albida* D85498	EU399546	98%

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Table 7 showed that selected isolate were belonged to 8 genera in 5 families; *Actinosynnemataceae*, *Micromonosporaceae*, *Pseudonocardiaceae*, *Streptosporangiaceae* and *Thermomonosporanceae*.

Family *Actinosynnemataceae*

PT749 was belonged to genus *Saccharothrix* in the family *Actinosynnemataceae*. Family *Actinosynnemataceae* was proposed by Labeda and Kroppenstedt (2000). It comprised of 6 genera; *Actinokineospora*, *Actinosynema*, *Lechevalievia*, *Asiosporangium*, *Lentzea* and *Saccharothrix*. Table 8 showed chemotaxonomic properties that discriminate each genus from another.

Table 8 Chemotaxonomic characteristics of genera of the family *Actinosynnemataceae* (Data taken from Zitouni *et al.*, 2004)

Characteristic	*Actinokineospora*	*Actinosynnema*	*Asiosporangium*	*Lechevalieria*	*Lentzea*	*Saccharothrix*
Whole-cell sugar pattern	Galactose, mannose, rhamnose	Galactose, mannose	Galactose, mannose, ribose	Galactose, mannose, rhamnose	Galactose, mannose, ribose	Galactose, rhamnose, mannose (trace)
Phospholipid type	PII	PII	PII	PII	PII	PII, PIV
Phospholipids	PE, OH-PE	PE, OH-PE, PI, PIMs, DPG	PE	PE	PE, DPG, PG, PI	PE, OH-PE, PI, PIMs, DPG, PG
Predominant menaquinones	MK-9(H_4)	MK-9(H_4), MK-9(H_6)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)	MK-10(H_4), MK-9(H_4)

DPG, diphosphatidyl glycerol ; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol ; PI, phosphatidyl inositol ; PIMs, phosphatidyl inositol mannosides; PME, phosphatidyl methylethanolamine

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Genus *Saccharothrix*

According to blast search result for 16S rRNA gene sequence in Table 7 and its spore formation in Figure 7, it was evident that PT749 belonged to the genus *Saccharothrix* and was closely related to *Saccharothrix texaensis*. The 16S rRNA

gene sequence of this strain was submitted to GenBank under an accession number FJ756556.

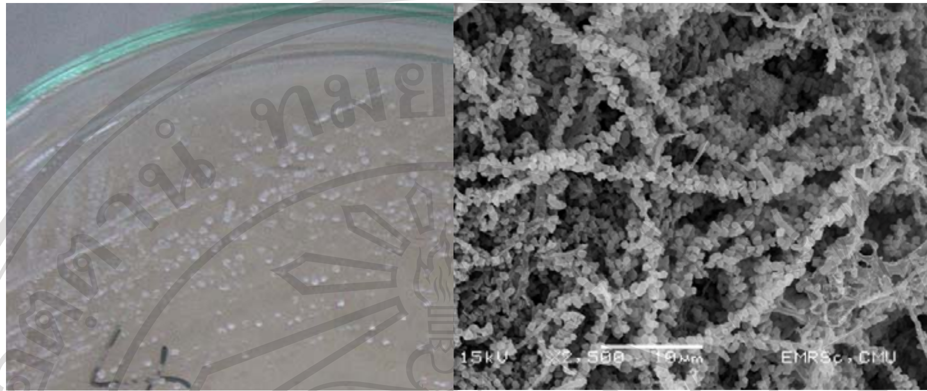


Figure 7 Colony morphology and scanning electron micrograph of a 15-day-old of PT749 grown on HT agar at 30ºC

The genus *Saccharothrix* (Labeda *et al.*, 1984) has undergone several amendments based on accumulated chemotaxonomic and phylogenetic data (Labeda and Kroppenstedt, 2000; Labeda *et al.*, 2001). This genus includes the following recognized species: *Saccharothrix australiensis*, *Saccharothrix coeruleofusca*, *Saccharothrix espanaensis*, *Saccharothrix longispora*, *Saccharothrix mutabilis*, *Saccharothrix mutabilis* subsp. *capreolus*, *Saccharothrix syringae*, *Saccharothrix texasensis* (Labeda *et al.*, 1984; Labeda, 1986, 1989; Labeda and Lechevalier, 1989; Grund and Kroppenstedt, 1989), *Saccharothrix albidocapillata*, *Saccharothrix violacea* (Lee *et al.*, 2000b), *Saccharothrix algeriensis* (Zitouni *et al.*, 2004) and *Saccharothrix xinjiangensis* (Hu *et al.*, 2004).

The genus *Saccharothrix* is phylogenetically heterogeneous on the basis of analysis of almost complete 16S rDNA sequences. An evaluation of chemotaxonomic, morphological and physiological properties in the light of the molecular phylogeny data revealed that several species are misclassified.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Saccharothrix aerocolonigenes and *Saccharothrix flava* constitute a lineage distinct from *Saccharothrix* and separate from *Lentzea*. The genus *Lechevalieria* gen. nov. is proposed for these species. *Lechevalieria aerocolonigenes* comb. nov. is the type species and *S. flava* is transferred as *Lechevalieria flava* comb. nov. Although *Lentzea albidocapillata*, the type species of the genus *Lentzea*, was transferred recently to the genus *Saccharothrix* (Lee *et al.*, 2000d), the revival of *Lentzea* is clearly supported by molecular phylogenetic and chemotaxonomic data. The description of the revived genus is emended to include galactose, mannose and traces of ribose as diagnostic whole-cell sugars and MK-9(H4) as the principal menaquinone and elimination of tuberculostearic acid as a diagnostic component in the fatty acid profile. *S. waywayandensis*, *S. aerocolonigenes* and *Asiosporangium albidum* are members of the amended genus *Lentzea* on the basis of phylogenetic and chemotaxonomic properties. *S. waywayandensis* was transferred to *Lentzea* as *Lentzea waywayandensis* comb. nov., while the new species *Lentzea californiensis* sp. nov. and *Lentzea albida* sp. nov. was described for *S. aerocolonigenes* and *A. albidum*, respectively.

Recently, new antibiotics, mutactimycin PR and saccharomicins, were derived from *Saccharothrix* sp. SA 103 and *Saccharothrix espanaensis* respectively (Zitouni *et al.*, 2004; Singh *et al.*, 2000).

Family *Micromonosporaceae*

Isolate PNK404, PNK464 and PNK384 were belonged to Family *Micromonosporaceae*. The family *Micromonosporaceae* was described by Krasil'nikov (1983). At present, this family comprises 17 genera: *Micromonospora, Actinoplanes, Pilimelia, Dactylosporangium, Catellatospora, Cateuloplanes,*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Couchioplanes, Spirilliplanes, Verrucosispora, Virgisporangium, Asanoa, Longispora, Salinispora, Actinocatenispora, Polymorphospora, Krasilnikovia and *Planosporangium*. Each genus has distinctive morphological and/or chemotaxonomic characteristics. *Micromonospora* and *Salinispora* species form single spores on short or long sporophores. *Actinoplanes*, *Dactylosporangium*, *Pilimelia* and *Virgisporangium* species form sporangia. *Catellatospora*, *Asanoa*, *Catenuloplanes*, *Couchioplanes*, *Longispora*, *Spirilliplanes*, *Actinocatenispora* and *Polymorphospora* species form spore chains. *Verrucosispora* species do not form aerial mycelia or sporangia. *Actinoplanes*, *Dactylosporangium*, *Pilimelia*, *Catenuloplanes*, *Couchioplanes* and *Spirilliplanes* spores show motility. *Krasilnikovia* species form pseudosporangia on short sporangiophores directly above the surface of substrate mycelium contained non-motile spore inside. *Planosporangium* species form two types of spore, motile spores, which were formed in sporangia and single globose spores. *Catenuloplanes* and *Couchioplanes* species have lysine instead of mesodiaminopimelic acid as the diamino acid of the peptidoglycan.

Genus *Catellatospora*

According to blast search result for 16S rRNA gene sequence in Table 7 and consistent with their spore formation (Figure 8), it was evident that isolate PNK384 was belonged to genus *Catellatospora* and was closely related to *Catellatospora citrea* subsp. *citrea*. The 16S rRNA gene sequence of this strain was submitted to GenBank under an accession number FJ756550.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

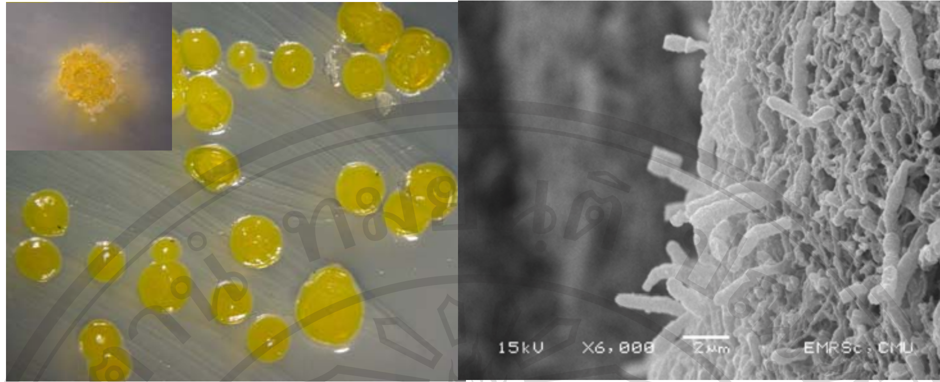


Figure 8 Colony morphology and scanning electron micrograph of a 30-day-old culture of PNK384 grown on HT agar at 30ºC

The genus *Catellatospora* was first described by Asano and Kawamoto (1986) for actinomycete strains that produce short chains of non-motile spores borne directly on the substrate mycelium without the formation of aerial mycelium. This genus originally contained two species, *C. citrea* and *C. ferruginea* which were isolated from woodland soil samples collected in Itsukaichi-shi Tokyo and Yamanashi, Japan, respectively. Subsequently *C. citrea* subsp. *methionotrophica* (Asano and Kawamoto, 1988), *C. matsumotoense*, *C. tsunoense* (Asano *et al.*, 1989) and *C. koreensis* (Lee *et al.*, 2000a) were added. Later, *C. matsumotoense* has been transferred to the genus *Micromonospora* as *Micromonospora matsumotoense*, based on 16S rRNA gene sequence analysis and phenotypic characteristics (Lee *et al.*, 1999). In 2002 *C. ferruginea* was transferred to the genus *Asanoa* as *Asanoa ferruginea* by Lee and Hah. Recently, three isolates of this genus were found in soil samples collected from rainforests in Singapore (Wang *et al.*, 1999). *C. chokoriensis*, *C. coxensis* and *C. bangladeshensis* that were isolated from sandy soil collected at a forest-side waterfall in Chokoria, Cox's Bazar, Bangladesh were proposed by Ara and Kudo (2006).

Genus *Micromonospora*

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

According to blast search result for 16S rRNA gene sequence in Table 7 and consistent with their single spore on aerial mycelium formation (Figure 9), it was evident that isolate PNK404 and PNK464 were belonged to genus *Catellatospora* and were closely related to *Micromonospora chersinia* and *Microbispora echinaurantica* respectively. The 16S rRNA gene sequences of strain PNK404 and PNK464 were submitted to GenBank under an accession number FJ756552 and FJ756553 respectively.

The genus *Micromonospora* was first described by Ørskov (1923) for an actinomycete that produces non-motile spores borne singly on sporophores branched from substrate hyphae. This genus is well established according to its morphological and chemotaxonomic characteristics (Lechevalier and Lechevalier, 1970; Kroppenstedt, 1985) as well as through 16S rDNA-based phylogenetic analyses (Stackebrandt *et al.*, 1997).

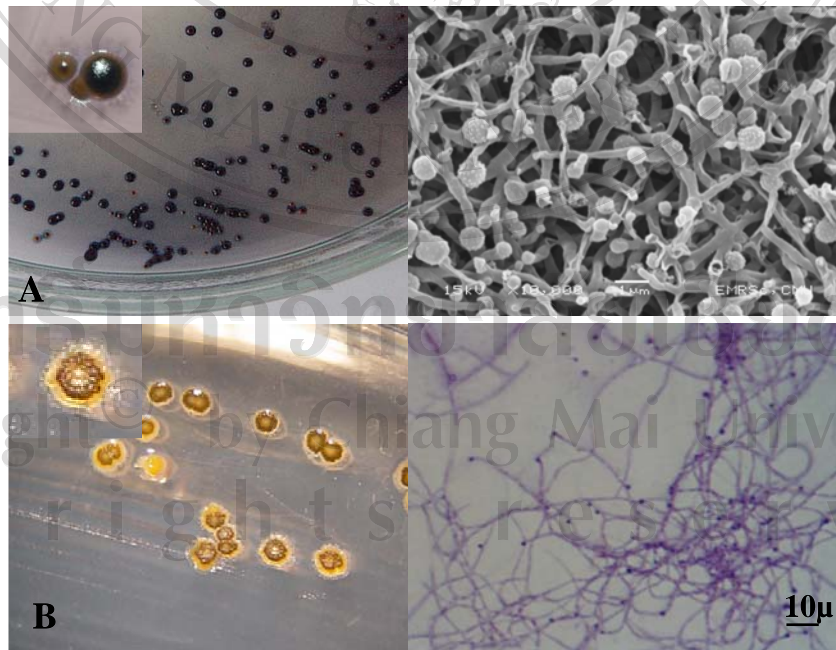


Figure 9 Colony morphology and spore formation of a 10-day-old culture of PNK404 (A) and PNK464 (B) grown on HT agar at 30ºC

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Recently, several new species of this genus from Thailand were reported, *M. narathiwatensis*, *M. siamensis*, *M. aurantionigra* and *M. eburnean* isolated from peat swamp forest soils and *M. pattaloongensis* from mangrove forest soil, *M. chaiyaphumensis* from soil in chaiyaphum province and *M. krabiensis* isolated from marine soil in Krabie province (Thaiwai *et al.*, 2004, 2005a, b, 2007, 2008; Jongrungruangchok *et al.*, 2008a, b).

Various species of this genus are sources of aminoglycoside antibacterial such as vancomycin, gentamycin and streptomycin.

Family *Pseudonocardiaceae*

PNK393 was belonged to genus *Pseudonocardia* in the family *Pseudonocardiaceae.* This family was proposed by Embly *et al.* (1988) based on the basis of 16S rRNA gene sequences and chemotaxonomic features for the mycolate-less wall chemotype IV actinomycetes. Recently, *Actinomycetospora* was proposed by Jiang *et al.* (2008) as a new genus of this family and species *Actinomycetospora chiangmaiensis* isolated from soil of a tropical rainforest in Chiang Mai, Thailand was proposed as the type strain of this genus.

Genus *Pseudonocardia*

According to blast search result for 16S rRNA gene sequence in Table 7 and its zig-zag shaped hyphae formation in Figure 10, it was evident that PNK393 belonged to the genus *Pseudonocardia* and was closely related to *Pseudonocardia halophobica*. The 16S rRNA gene sequence of this strain was submitted to GenBank under an accession number FJ756551.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Figure 10 Colony morphology and light micrograph of a 15-day-old of PNK393 grown on HT agar at 30ºC

The genus *Pseudonocardia* was proposed by Henssen (1957) for mycolate-less, nocardioform actinomycetes that contained *meso*-diaminopimelic acid, arabinose and galactose in the peptidoglycan . The genus *Pseudonocardia* is morphologically characterized by the formation of well-developed vegetative and aerial mycelium, from which spore chains are produced by acropetal budding or fragmentation. It also has *meso*-diaminopimelic acid, arabinose and galactose in the cell wall (a type IV cell wall composition; Lechevalier and Lechevalier, 1970), tetrahydrogenated menaquinone with eight isoprene units [MK-8(H%)] as a major menaquinone, a DNA G-C content of 68-79 mol%, no mycolic acids, and a phospholipid type PII (phosphatidylethanolamine or its derivatives as a diagnostic phospholipid) or PIII (phosphatidylcholine) pattern, according to the species (Lechevalier *et al.*, 1981). Phylogenetically, the genus *Pseudonocardia* is intermixed on the basis the 16S rDNA sequences (Lee *et al.*, 2000a, c).

Members of the genus *Pseudonocardia* are divided into two groups according to their phospholipid patterns. One group is characterized by the presence of phosphatidylcholine (a type PIII pattern) and contains the isolate and eight other

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

species (*P. alni*, *P. autotrophica*, *P. compacta*, *P. hydrocarbonoxydans*, *P. kongjuensis*, *P. petroleophila*, *P. saturnea* and *P. thermophila*), whereas the other group possesses nitrogenous phospholipids (a type PII pattern) in the polar lipid profiles and is composed of *P. saccharolytica*, *P. halophobica* and *P. sulfidoxydans* (Embley, 1992; Reichert *et al.*, 1998; Lee *et al.*, 2001; Lee *et al.*, 2002).

Family *Streptosporangiaceae*

Isolate PT702, PT708, PT707and PNK225 were belonged to Family *Streptosporangiaceae*. The family *Streptosporangiaceae* was described for the redefined maduromycete group. At present, this family comprises of 10 genera: *Streptosporangium*, *Planomonospora*, *Microtetraspora,* *Planobispora*, *Planotetraspora*, *Herbidospora*, *Microbispora*, *Nonomuraea*, *Sphaerosporangium* and *Acrocarpospora*. Each genus is distinguished by morphological feather including the formation of sporangia and the number of spores per sporangia or chain. Members of the family have cell wall type III, whole-cell sugar pattern B or C, fatty acid pattern, major menaquinone MK-9(III, VIII-H_4, H_6, H_2, H_0) and phospholipid type PIV.

Genus *Microbispora*

According to blast search result for 16S rRNA gene sequence in Table 7 and consistent with its pair spore on aerial mycelium formation (Figure 11), it was evident that PNK225 belonged to the genus *Microbispora* and was closely related to *Microbispora rosea*. The 16S rRNA gene sequence of this strain was submitted to GenBank under an accession number FJ756549.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

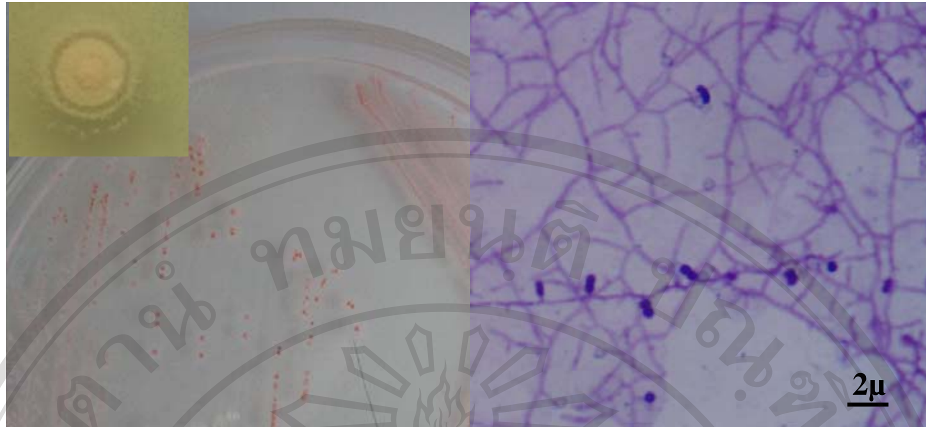


Figure 11 Colony morphology and light micrograph of 15-day-old of PNK225 grown on HT agar at 30ºC

The genus *Microbispora* was proposed by Nonomura and Ohara (1957) for an actinomycete strain producing longitudinally paired spores on aerial mycelia. In the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), 10 species were cited as members of the genus *Microbispora*; later, three others, *M. viridis* (Miyadoh *et al.*, 1985), *M. karnatakensis* and *M. indica* (Rao *et al.*, 1987), were validly proposed as new species. Subsequently, this genus was well defined from a chemotaxonomic point of view, and Kroppenstedt *et al.* (1990) and Miyadoh *et al.* (1990) transferred *Microbispora echinospora* and *M. viridis* to the genus *Actinomadura* as *Actinomadura echinospora* and *Actinomadura rugatobispora*, respectively. On the basis of DNA-DNA hybridization experiments, Miyadoh *et al.* (1990) also proposed that 10 of the remaining species (with the exception of *Microbispora bispora*) should be combined into the type species *M. rosea* with two subspecies, *M. rosea* subsp. *rosea* and *M. rosea* subsp. *aerata*. Analysis of 16S rDNA sequences by Wang *et al.* (1996) indicated that *M. bispora* was phylogenetically distant from the *M. rosea* cluster and other members of the family *Streptosporangiaceae* and this species was transferred to the new genus *Thermobispora* (as *Thermobispora bispora*). Recently,

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Zhang *et al.* (1998) reclassified the genus *Thermomonospora* and proposed the transfer of *Thermomonospora mesophila*, which produces spores singly borne on aerial hyphae, to the amended genus *Microbispora* (as *Microbispora mesophila*).

Genus *Nonomuraea*

According to blast search result for 16S rRNA gene sequence in Table 7, it was evident that strain PT702, PT707 and PT708 were belonged to genus *Nonomuraea* although their cultural characters were diverse, isolate PT707 and PT708 formed spore chain inside sheath on entire sporophore while PT702 formed short chains spore (Figure 12). PT707 was closely related to *Nonomuraea maheshkhaliensis* and it was submitted to GenBank under an accession number FJ756557. PT702 and PT708 were closely related to *Nonomuraea roseola*. The 16S rRNA gene sequences of them were submitted to GenBank under an accession number FJ756554 and FJ347524 respectively.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

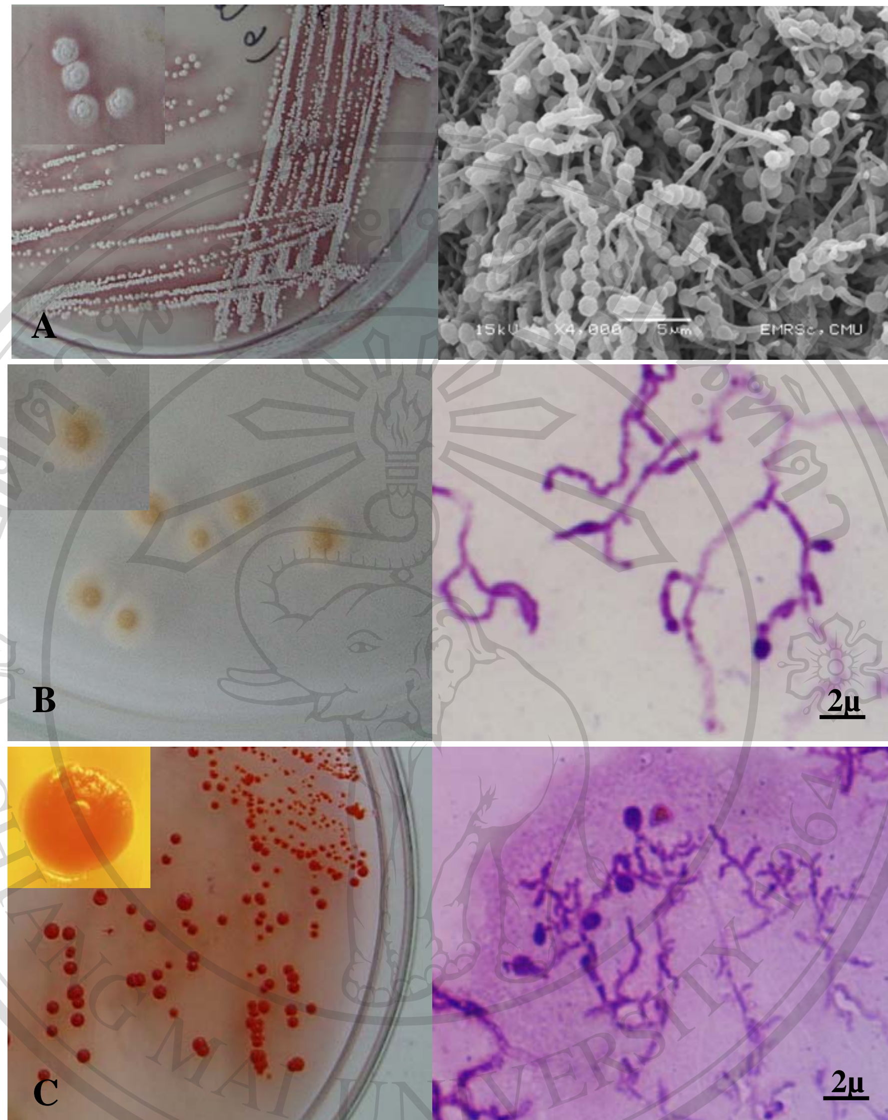


Figure 12 Colony morphology and spore formation of a 15-day-old culture PT702 (A), PT707 (B) and PT708 (C) on HT agar at 30ºC

For the diverse in spore formation and cultural characteristic of this genus, it may simply reflect to mis-identify of them that may affect the diversity population of this genus in this research. Due to members of the genus *Nonomuraea* presently can only be identified by 16S rDNA sequence analysis. No record of this genus in some diversity report in the past might be due to no 16S rDNA sequence analysis available for identification (Hu *et al.*, 1995; Jiang and Xu, 1996; Ward-Rainey *et al.*, 1996).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

The genus *Nonomuria* was proposed by Zhang *et al.*, (1998) prior Chiba *et al.* (1999) proposed to change to the name *Nonomuraea* as the correct spelling of the genus (Rule 57a, Appendix 9; hexahydrogenated menaquinones with nine isoprene). This genus had been placed in genus *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 distinct from other member of the family *Streptosporangiaceae* result in it was separated to a new genus. This genus comprises of 19 validly published species and 2 subspecies; *Nonomuraea pusilla* is the type species (Rose and Meyer, 2007).

Family *Thermomonosporaceae*

PNK470 and PT725 were belonged to Family *Thermomonosporaceae*. The family *Thermomonosporaceae* was emended by Zhang *et al.* (2001). At present, this family comprises 4 genera (Lee and Jeong, 2006): *Thermomonospora, Actinomadura, Spirillospora and Actinocorallia*. On the basis of 16S rDNA sequences analysis of the members of this family, it was observed that *Thermomonospora curvata* and *Spirillospora albida* were intermixed with *Actinomadura* species in a clade distantly separated from their phylogenetic neighbours, the member of the families *Streptosporangiaceae and Nocardiopsiaceae*.

For *Thermomonospora curvata*, though its position in the tree was intermixed with *Actinomadura* species but it does not contain the diagnostic sugar madurose in its whole cell hydrolysated which distinguishes it from *Actinomadura* species (Zhang *et al.*, 1998). Further investigations of its relationship are needed.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Spirillospora albda was closely related to some *Acinomadura* species than *Spirillospora rubra*. However, it formed complex sporangia containing motile spore, a property not found in any *Actinomadura* species (Zhang *et al.*, 2001).

Genus *Actinocorallia*

According to blast search result for 16S rRNA gene sequence in Table 7 and its spore formation in Figure 13, it was evident that PT725 belonged to the genus *Actinocorallia* and was closely related to *Actinocorallia aurantiaca*. The 16S rRNA gene sequence of this strain was submitted to GenBank under an accession number FJ756555.

Figure 13 Colony morphology and scanning electron micrograph of a 15-day-old of PT725 grown on HT agar at 30ºC

The genus *Actinocorallia*, which was originally proposed by Iinuma *et al.* (1994), was recently emended on the basis of a combination of phylogenetic analyses and major chemotaxonomic properties (Zhang *et al.*, 2001). At present, this family comprises 6 species: *A. aurantiaca*, *A. glomerata*, *A. herbida*, *A. libanotica, A. cavernae* and *A. longicatena*. All of the *Actinocorallia* species with validly published names, with the exception of the type species, *A. herbida*, was transferred from the genus *Actinomadura* on the basis of 16S rRNA gene sequence studies and

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

chemotaxonomic characterization (Kroppenstedt *et al.*, 1990; Itoh *et al.*, 1995; Zhang *et al.*, 1998, 2001). Recently, *Actinocorallia cavernae* isolated from a natural cave in Jeju, Korea (Lee, 2006a).

Genus *Spirillospora*

According to blast search result for 16S rRNA gene sequence in Table 7 and its sporangium formation in Figure 14, it was evident that PNK470 belonged to the genus *Spirillospora* and was closely related to *Spirillospora albida*. The 16S rRNA gene sequence of this strain was submitted to GenBank under an accession number EU399546.

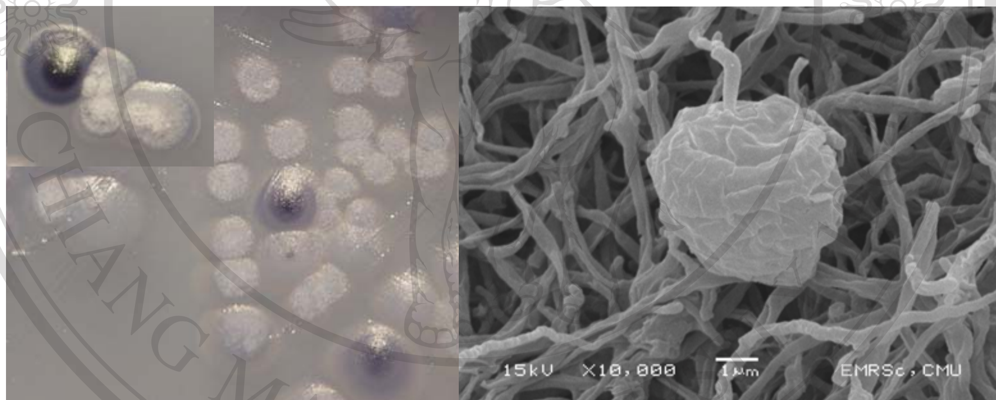


Figure 14 Colony morphology and scanning electron micrograph showing sporangium of 30-day-old of PNK470 grown on HT agar at 30ºC

The genus *Spirillospora* was first proposed by Couch in 1963 for sporangia forming actinomycetes with motile spore and placed in the family *Thermomonosporaceae* on the basis of 16S rRNA gene sequences and chemotaxonomic data (Zhang *et al.*, 1998). It comprises only two species, *Spirillospora albida* and *Spirillospora rubra*.

Spirillospora albida was very difficult and hard to cultivate due to their slow growth. Hence, there is very few published information describing their distribution,

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

isolation and bioactive compounds production since its proposal. Spirillomycin was an only known antibiotics produced by this genus which was reported in 1968 (Domnas, 1968). Until today, there are only three publishes of only one strain of this specie were reported (Hacène *et al.*, 1994; Hacène and Lefebvre, 1995, 1996). *Spirillospora* strain 719 from soil sample in the Algerian Sahara was reported to produce several antibiotics including a new deep red pigment-like antibacterial HP17, which could inhibit the growth of coccoidal Gram-positive bacteria, a new polyene antifungal HM17 and a broad spectrum antibiotics, AH17. There was no report on anticancer compounds produced from members of the genus *Spirillospora*. This is the first report on isolation of *Spirillospora* from cave soil.

2.4.3 PHYLOGENETIC RELATIONSHIP BETWEEN SELECTED STRIAN AND THEIR RELATIVE SPECIES AND EVIDENCE FOR THE PRESENCE OF NOVEL SPECIES

Phylogenetic analyses of the 16S rRNA gene sequences of each selected isolates were performed to evaluate the phylogenetic relationship between selected strain and their relative species were showed in Figure 15.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

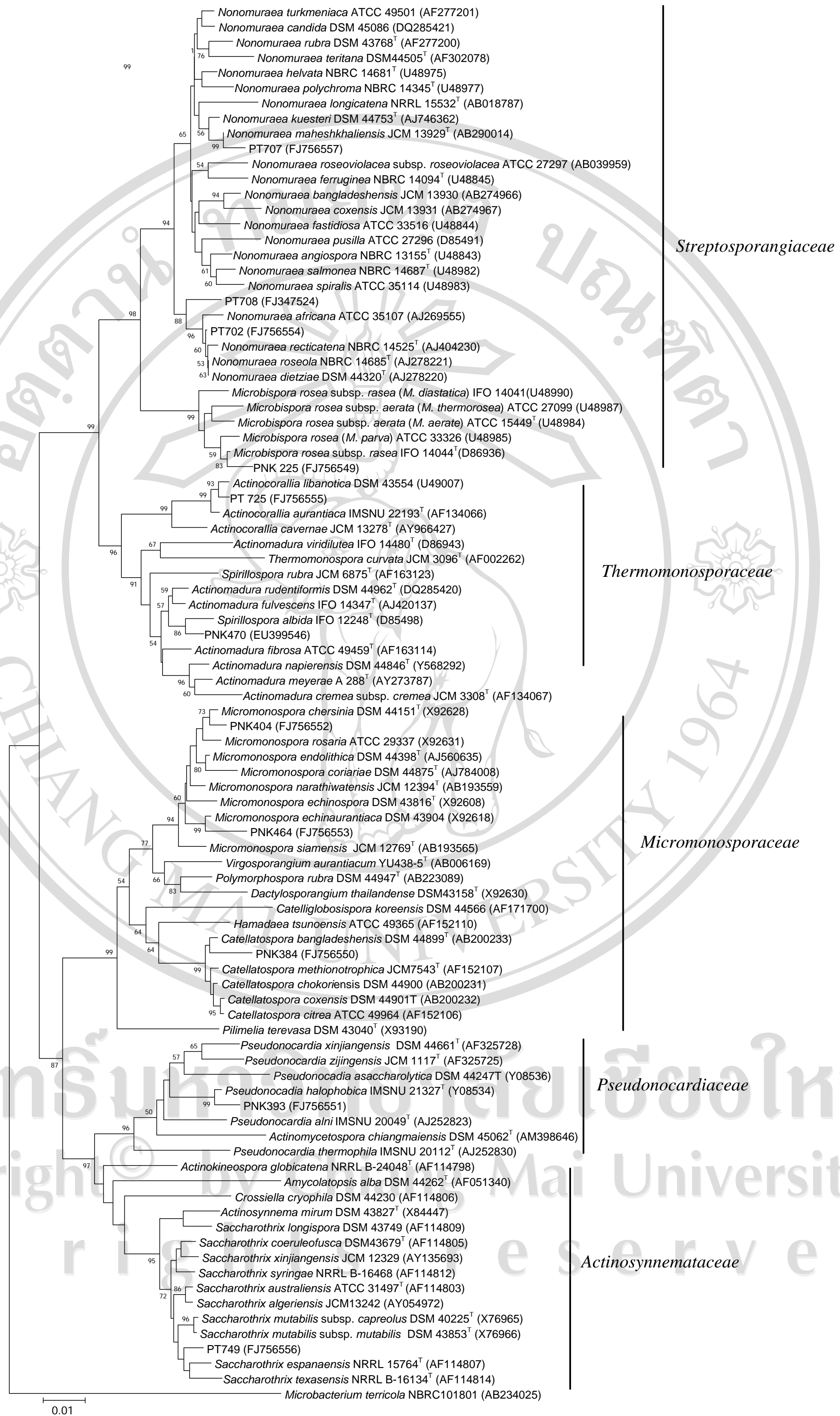


Figure 15 Phylogenetic tree derived from 16S rRNA gene sequences of 11 selected strain and their closely related genera. Numbers at nodes indicate the level (%) of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets. Only value>50% are shown. Bar, 0.01 nucleotide substitution per nucleotides position.

Copyright by Chiang Mai University
All rights reserved

Phylogenetic tree indicated that they should be classified in 8 genera that belong to 5 families; *Micromonosporaceae*, *Streptosporangiaceae*, *Pseudonocardiaceae*, *Actinosynnemataceae* and *Thermomonosporaceae*. This result was consisting with their morphology and chemotaxonomic data in Table 2. Isolate PNK384 was located in the same clade of genus *Catellatospora* and mostly related to *Catellatospora bangladeshensis* DSM 44899 (AB 200233). Two isolates, PNK404 and PNK464 were located in the same clade of genus *Micromonopora* and most closely related to *Micromonospora chersinia* DSM44151^T (X92628) and *Micromonospora echinaurantiaca* DSM 43816^T (X92618) respectively. Isolate PNK470 was located in the same clade of genus *Spirillospora* and mostly related to *Spirillospora albida* IFO 12248^T (D85498). Three isolates, PT702, PT707 and PT708, were located in the same clade of genus *Nonomuraea*. Isolate PT707 was most closely related to *Nonomuraea maheshkhaliensis* JCM 13929^T (AB290014) while PT702 and PT708 was mostly related to *Nonomuraea roseola* NBRC 14685^T (AJ278221). Isolate PT725 was located in the same clade of genus *Actinocorallia* and mostly related to *Actinocorallia libanotica* IFO14095^T (U49007). Isolate PNK393 was located in the same clade of genus *Pseudonocardia* and mostly related to *Pseudonocardia halophobica* IMSNU 21327^T (Y08534). Isolate PT749 was located in the same clade of genus *Saccharothrix* and mostly related to *Saccharothrix espanaensis* NRRL 15764^T (AF114807).

The phylogenetic tree (Figure 15) revealed that 3 of 11 selected isolates (PT749, PT702 and PT708) represent new species in genus *Saccharothrix* and *Nonomuraea* respectively. Because of they form a new phyletic line from each of other species in their genera. Consistent with their low 16S rDNA similarity value

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

that isolate PT749 exhibited (97.75%) to *Saccharothrix espanaensis* NRRL 15764^{T} (AF114807) and (97.35%) *Saccharothrix texasensis* NRRL B-16134 (AF114814) (Table 9).

Table 9 16S rRNA gene sequence percentage similarity between strain PT749 and related taxa

Upper-right triangle contains [NT] Different/Total nucleotides.

	1	2	3	4	5	6	7	8	9	10
1	---	4/142	18/1427	29/1428	35/1428	19/1391	35/1427	43/1427	52/1404	38/1390
2	99.72	---	15/1432	26/1433	32/1433	16/1396	32/1431	40/1432	49/1408	35/1394
3	98.74	98.95	---	26/1509	27/1509	19/1413	28/1507	36/1456	50/1429	33/1468
4	97.97	98.19	98.28	---	17/1510	19/1413	36/1508	34/1455	41/1430	38/1469
5	97.55	97.77	98.21	98.87	---	18/1413	36/1508	32/1455	43/1430	39/1469
6	98.63	98.85	98.66	98.66	98.73	---	33/1411	28/1413	25/1389	28/1373
7	97.55	97.76	98.14	97.61	97.61	97.66	---	41/1453	54/1428	39/1469
8	96.99	97.21	97.53	97.66	97.8	98.02	97.18	---	50/1428	45/1415
9	96.3	96.52	96.5	97.13	96.99	98.2	96.22	96.5	---	48/1411
10	97.27	97.49	97.75	97.41	97.35	97.96	97.35	96.82	96.6	---

Strain: 1, *S. mutabilis* subsp. *capreolus* X76965; 2, *S. mutabilis* subsp. *mutabilis* X76966; 3, *S. espanaensis* AF114807; 4, *S. syringae* AF114812; 5, *S. coeruleofusca* AF114805; 6, *S. australiensis* AF114803; 7, *S. texasensis* AF114814; 8, *S. longispora* AF114809; 9, *S. xinjiangensis* AY135693; 10, strain PT749

Lower-left triangle contains [NT] Similarity.

Although PT702 and PT708 showed high 16S rDNA similarity values to *Nonomuraea roseola* NBRC 14685^{T} (AJ278221) (98.86% and 98.29% respectively) (Table 10) but they remain seem to be new species because a higher 16S rDNA similarity value was found between representatives of validly described members of *Nonomuraea*. Such as *Nonomuraea dietziae* and *Nonomuraea recticatena* which shared 99.4% similarity. However, other taxonomic data are needed to be further investigated for support the phylogenetic results.

Due to morphology and chemotaxonomy characters of each genus in family *Streptosporangiaceae* were almost the same (Ara & Kudo, 2007) thus, members of

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

the genus *Nonomuraea* are not easy to identify to genus level by only using these characters. For ensure identification is correct, it should be used 16S rRNA gene sequencing data to solve that problem. This method was not used in previous actinomycetes diversity studies until 1999. Therefore there was no record of this genus except Wang *et al.*, (1999) that they evaluated the species diversity in the rainforest in Singapore and found 390 isolates of *Nonomuraea* from total 1796 rare actinomycetes (21.71%).

Not only 3 less explored genera *Catellatospora*, *Nonomuraea* and *Spirillospora* that they were absent from other Asian regions, including cave soils (Hopwood *et al.*, 1985; Jurado *et al.*, 2005a; Xu *et al.*, 1996) were found. But also possibly 3 new species of *Nonomuraea* and *Saccharothrix* were also present. Furthermore this is the first record on the isolation of *Nonomuraea* and *Spirillospora* from a cave. It could be concluded that Phatup Cave Forest Park and Phanangkhoi cave in northern Thailand are possibility to use as a source for the isolation of new rare actinomycetes and investigation of their diversity.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Table 10 16S rRNA sequence percentage similarity between isolated *strain* and related taxa

Lower-left triangle contains [NT] Similarity.Upper-right triangle contains [NT] Different/Total nucleotides.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	---	36/1432	31/1447	36/1432	46/1447	52/1396	41/1429	24/1406	24/1406	53/1446	44/1397	35/1404	57/1424	45/1392	55/1393	31/1447	25/1353	47/1445	81/1396	46/1415	59/1450	25/1379	49/1394
2	97.49	---	27/1439	0/1448	44/1437	39/1412	22/1444	28/1422	28/1422	45/1442	27/1412	33/1419	44/1438	42/1406	39/1407	27/1439	9/1368	27/1441	67/1409	43/1429	43/1446	31/1392	33/1408
3	97.86	98.12	---	27/1439	39/1451	46/1403	35/1436	35/1414	35/1414	62/1450	40/1404	44/1411	53/1432	40/1400	51/1401	0/1457	18/1360	41/1449	75/1403	39/1423	55/1455	38/1387	46/1401
4	97.49	100	98.12	---	44/1437	39/1412	22/1444	28/1422	28/1422	45/1442	27/1412	33/1419	44/1438	42/1406	39/1407	27/1439	9/1368	27/1441	67/1409	43/1429	43/1446	31/1392	33/1408
5	96.82	96.94	97.31	96.94	---	54/1401	45/1434	47/1411	47/1411	76/1451	44/1402	55/1408	58/1429	47/1396	58/1397	39/1451	38/1358	49/1451	84/1400	59/1419	62/1456	48/1384	50/1399
6	96.28	97.24	96.72	97.24	96.15	---	32/1410	44/1411	44/1411	48/1408	28/1409	48/1408	45/1403	30/1407	33/1408	46/1403	32/1366	37/1407	55/1383	50/1410	53/1410	46/1390	34/1405
7	97.13	98.48	97.56	98.48	96.86	97.73	---	33/1444	33/1444	50/1462	16/1463	41/1440	48/1463	41/1404	37/1405	35/1436	18/1405	30/1488	68/1457	35/1449	53/1473	41/1403	16/1424
8	98.29	98.03	97.52	98.03	96.67	96.88	97.71	---	0/1447	5/1443	34/1434	16/1443	48/1438	44/1407	49/1407	35/1414	25/1392	40/1441	68/1417	43/1444	55/1444	16/1406	42/1421
9	98.29	98.03	97.52	98.03	96.67	96.88	97.71	100	---	5/1443	34/1434	16/1443	48/1438	44/1407	49/1407	35/1414	25/1392	40/1441	68/1417	43/1444	55/1444	16/1406	42/1421
10	96.33	96.88	95.72	96.88	94.76	96.59	96.58	99.65	99.65	---	39/1430	14/1443	65/1459	48/1404	52/1404	62/1450	30/1388	69/1480	83/1428	53/1448	83/1484	21/1402	47/1417
11	96.85	98.09	97.15	98.09	96.86	98.01	98.91	97.63	97.63	97.27	---	42/1430	43/1431	36/1403	29/1404	40/1404	24/1406	36/1457	48/1434	39/1432	57/1441	42/1403	8/1424
12	97.51	97.67	96.88	97.67	96.09	96.59	97.15	98.89	98.89	99.03	97.06	---	50/1435	48/1404	49/1404	44/1411	29/1389	46/1437	74/1413	54/1441	59/1441	22/1402	50/1417
13	96	96.94	96.3	96.94	95.94	96.79	96.72	96.66	96.66	95.54	97	96.52	---	42/1400	51/1401	53/1432	39/1392	50/1462	83/1432	61/1445	68/1466	52/1399	51/1413
14	96.77	97.01	97.14	97.01	96.63	97.87	97.08	96.87	96.87	96.58	97.43	96.58	97	---	44/1406	40/1400	32/1362	39/1401	52/1379	35/1407	52/1405	45/1387	42/1399
15	96.05	97.23	96.36	97.23	95.85	97.66	97.37	96.52	96.52	96.3	97.93	96.51	96.36	96.87	---	51/1401	37/1363	33/1404	46/1383	58/1408	59/1406	51/1387	35/1400
16	97.86	98.12	100	98.12	97.31	96.72	97.56	97.52	97.52	95.72	97.15	96.88	96.3	97.14	96.36	---	18/1360	41/1449	75/1403	39/1423	55/1455	38/1387	46/1401
17	98.15	99.34	98.68	99.34	97.2	97.66	98.72	98.2	98.2	97.84	98.29	97.91	97.2	97.65	97.29	98.68	---	26/1404	42/1388	31/1390	43/1394	26/1379	31/1377
18	96.75	98.13	97.17	98.13	96.62	97.37	97.98	97.22	97.22	95.34	97.53	96.8	96.58	97.22	97.65	97.17	98.15	---	41/1457	43/1446	64/1488	48/1400	37/1418
19	94.2	95.24	94.65	95.24	94	96.02	95.33	95.2	95.2	94.19	96.65	94.76	94.2	96.23	96.67	94.65	96.97	97.19	---	71/1424	99/1436	67/1381	48/1395
20	96.75	96.99	97.26	96.99	95.84	96.45	97.58	97.02	97.02	96.34	97.28	96.25	95.78	97.51	95.88	97.26	97.77	97.03	95.01	---	60/1451	52/1404	47/1419
21	95.93	97.03	96.22	97.03	95.74	96.24	96.4	96.19	96.19	94.41	96.04	95.91	95.36	96.3	95.8	96.22	96.92	95.7	93.11	95.86	---	53/1403	55/1423
22	98.19	97.77	97.26	97.77	96.53	96.69	97.08	98.86	98.86	98.5	97.01	98.43	96.28	96.76	96.32	97.26	98.11	96.57	95.15	96.3	96.22	---	47/1397
23	96.48	97.66	96.72	97.66	96.43	97.58	98.88	97.04	97.04	96.68	99.44	96.47	96.39	97	97.5	96.72	97.75	97.39	96.56	96.69	96.13	96.64	---

Strain: 1, strain PT708 *;* 2, *N. turkmeniaca* AF277201; 3**,** *N. rubra* AF277200; 4, *N. turkmeniaca* AF277201; 5, *N. teritana* AF30207; 6, *N. salmonea* U48982; 7, *N. kuesteri* AJ746362; 8, *N. roseola* AJ278221; 9, *N. dietziae* AJ278220; 10, *N. recticatena* AJ404230; 11, *N. maheshkhaliensis* AB290014; 12, *N. Africana* AJ269555; 13, *N. pusilla* D85491;14, *N. spiralis* U48983; 15, *N. fastidiosa* U48844; 16, *N. rubra* AF277200; 17, *N. candida* DQ285421; 18, *N. bangladeshensis* AB274966; 19, *N. coxensis* AB274967; 20, *N. roseoviolacea* subsp. *roseoviolacea* AB039959; 21, *N. longicatena* AB018787; 22, Strain PT702; 23, Strain PT707

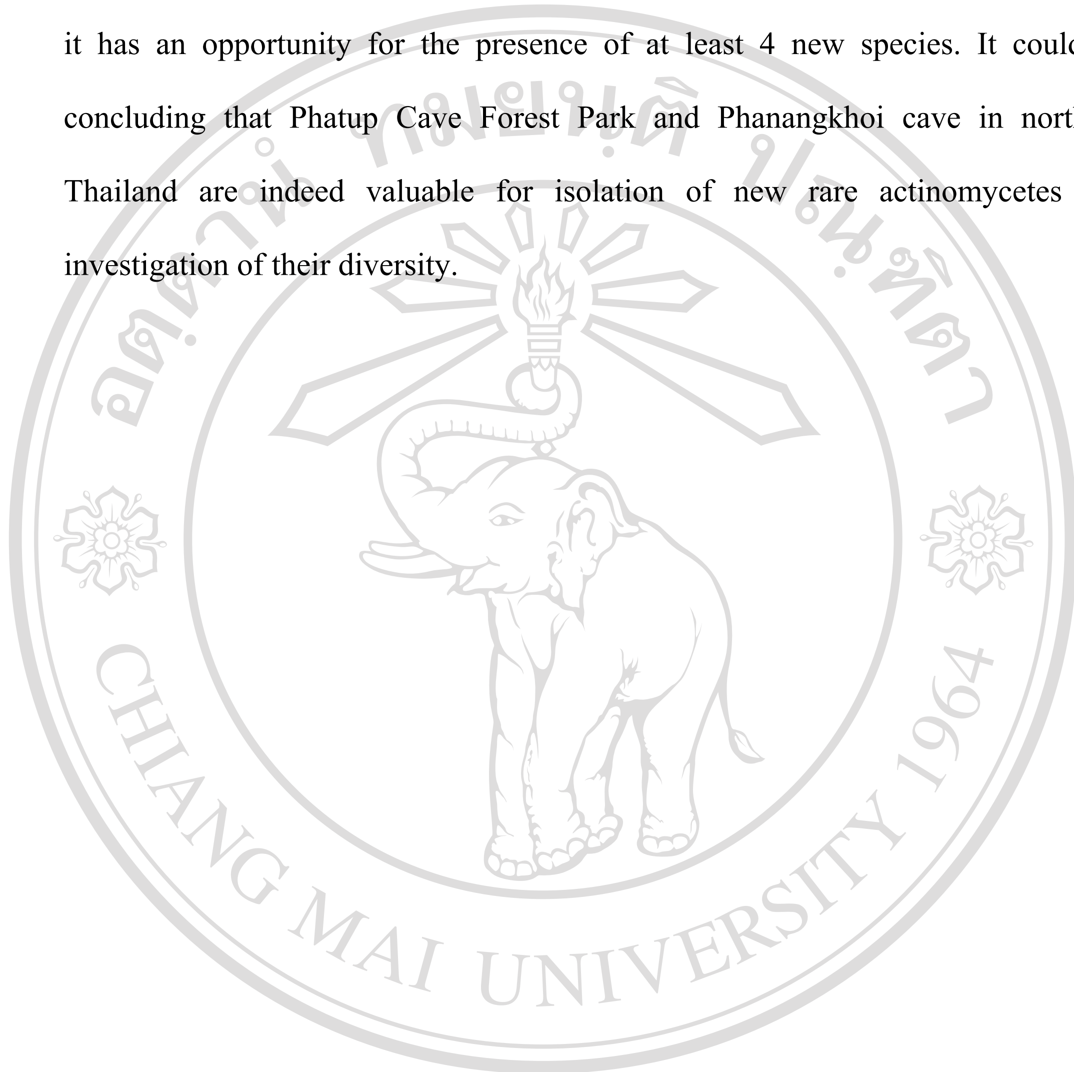
2.4.4 EVALUATON OF GENERIC DIVERSITY

Among the genera found, *Nonomuraea* was complicated to identifying to genus level by their phenotypic and chemotaxonomic data. It can only be identified by 16S rRNA gene sequence analysis. This approach was not used in previous diversity studies. Thus, there was no record of this genus except as reported by Wang *et al.*, (1999). They evaluated the species diversity in the rainforest in Singapore by using 16S rRNA gene sequencing data and found 390 isolates of *Nonomuraea* from total 1796 rare actinomycetes (21.71%). The two genera, *Catellatospora* and *Spirillospora* were also present, however, they were absent from other Asian regions, including cave soils (Hopwood *et al.*, 1985; Jurado *et al.*, 2005a; Xu *et al.*, 1996).

In this report, 8 genera were found in these caves in northern Thailand that covers 0.93 km^2. Thus, approximately one genus was found every 0.12 km^2. Our results suggest that, in these caves, the generic diversity of rare actinomycete is very high when compared to reports from other geographic regions such as tropical rain forest soil in Singapore and soil from various vegetation and climatic zones that approximately found one genus in every 17.8 and 15,041 km^2 (Wang *et al.*, 1999; Xu *et al.*, 1996). Although, this does not exclude the possibility that the results may simply reflect the different procedure and strategies used for isolation and identification in the different studies. Amann *et al.*, (1995) reported that majority of microorganisms in nature have not been cultured in laboratories and remain uncharacterized. Additionally, the number of actinomycetes genera may affect by human biases in selecting colonies in isolation step.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

In addition to the high generic diversity found in these caves, this is the first record on the isolation of *Nonomuraea* and *Spirillospora* from cave. Furthermore, it has an opportunity for the presence of at least 4 new species. It could be concluding that Phatup Cave Forest Park and Phanangkhoi cave in northern Thailand are indeed valuable for isolation of new rare actinomycetes and investigation of their diversity.



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved