BIODIVERSITY OF UNCULTURED *Streptomyces* INVOLVED IN BIOACTIVE SUBSTANCE PRODUCTION FROM SOIL IN THAILAND

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ความหลากหลายทางชีวภาพของสเตรปโตมัยซีสที่เกี่ยวข้องกับการผลิตสารออกฤทธิ์ทางชีวภาพจากดินในประเทศไทยโดยไม่อาศัยการเพาะเลี้ยงเชื้อ

นางสาวสิริณี ยอดเมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	BIODIVERSITY OF UNCULTURED *Streptomyces* INVOLVED IN BIOACTIVE SUBSTANCE PRODUCTION FROM SOIL IN THAILAND
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สิริณี ยอดเมือง : ความหลากหลายทางชีวภาพของสเตรปโตมัยซีสที่เกี่ยวข้องกับการผลิตสารออกฤทธิ์ทางชีวภาพ จากดินในประเทศไทยโดยไม่อาศัยการเพาะเลี้ยงเชื้อ. (BIODIVERSITY OF UNCULTURED *Streptomyces* INVOLVED IN BIOACTIVE SUBSTANCE PRODUCTION FROM SOIL IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.สุชาติ ชะนะมา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.มณี ชะนะมา, 181 หน้า.

สเตรปโตมัยซีส (*Streptomyces*) เป็นแบคทีเรียแกรมบวกที่มีเส้นใย มีนิวคลีโอไทด์ G-C มาก มีความสามารถในการสร้างสารออกฤทธิ์ทางชีวภาพหลายชนิด และพบมากในดิน การศึกษาในครั้งนี้ได้มีจุดมุ่งหมายเพื่อตรวจหาความหลากหลายทางพันธุกรรมของสเตรปโตมัยซีสจากตัวอย่างดินในประเทศไทยโดยใช้เทคนิคแบบไม่อาศัยการเลี้ยงเชื้อ จากการสร้างแบบจำลองแบบแผนการตัดลำดับเบสของ 16S rDNA ด้วยเอ็นไซม์ตัดจำเพาะ 33 ชนิด ด้วยโปรแกรม NEBcutter [สเตรปโตมัยซีส (70 ชนิด), แอคติโนมัยซีส (6 ชนิด) และแบคทีเรียอื่น ๆ (3 ชนิด)] จากฐานข้อมูลสาธารณะ Ribosomal Database Project (RDP) และ National Center for biotechnology Information (NCBI) เพื่อทำให้เกิดแบบจำลองแบบแผนข้อมูล Restriction Fragment Length Polymorphism (RFLP) จากนั้นทำการวิเคราะห์แบบจำลอง RFLP โดยการคำนวณ restriction distance ด้วยวิธีของ Nei-Li และการสร้างแผนภูมิต้นไม้ด้วยวิธี neighbor-joining โดยใช้โปรแกรม PAUP พบว่าจำนวนของแบบแผน RFLP OTUs ที่ได้จากเอ็นไซม์ *Msp*I มีค่าเฉลี่ยจำนวนตำแหน่งที่ถูกตัดใน 16S rDNA ของแบคทีเรียอยู่ในระดับสูง (10.50) และมีความสอดคล้องกันระหว่างวงศ์วานวิวัฒนาการและการสร้างสารออกฤทธิ์ทางชีวภาพ ดังนั้นจึงได้เลือกเอ็นไซม์ *Msp*I สำหรับการวิเคราะห์ RFLP ของ 16S rDNA ของสเตรปโตมัยซีสจากดินเพื่อตรวจหาความหลากหลายของสเตรปโตมัยซีสดังกล่าว จากการสกัดดีเอ็นเอจากดินตัวอย่าง ทำการเพิ่มจำนวน 16S rDNA ด้วยวิธี nested-Polymerase Chain Reaction (nested-PCR) โดยใช้ไพรเมอร์ชนิด universal primers และไพรเมอร์ที่มีความจำเพาะต่อสเตรปโตมัยซีส ในปฏิกิริยา PCR รอบแรกและรอบที่สองตามลำดับ ทำให้ได้ชิ้นดีเอ็นเอของ 16S rDNA ที่มีขนาดประมาณ 1,000 เบส และได้ทำการโคลนเข้าไปใน T/A cloning vecter เพื่อสร้าง clone library หลังจากนั้นก็นำไปทำการวิเคราะห์ความหลากหลายโดยวิธี Restriction Fragment Length Polymorphism (RFLP) ผลการทดลองพบว่าชิ้นดีเอ็นเอของ 16S rDNA ที่ได้จาก 100 โคลนของสเตรปโตมัยซีสในดินในพื้นที่ต่าง ๆ ของประเทศไทย [ภูเขา (MT), ป่าชายเลน (MG), และทุ่งนา (PD)] แสดงแบบแผน RFLP จำนวน 16 OTUs ซึ่งเมื่อนำมาเปรียบเทียบกับแบบจำลองแบบแผน RFLP ที่สร้างขึ้นมาดังกล่าวข้างต้นพบว่าแบบแผน RFLP หรือ OTUs ที่พบมากที่สุดคือชนิด ‘a’ (77%) ซึ่งมีแบบแผน RFLP ตรงกับ OTU ของ *Streptomyces* ที่สร้างสารออกฤทธิ์ทางชีวภาพ ได้แก่ *S. venezuelae* และ *S. fridae* ซึ่งสร้างสารปฏิชีวนะ และ 3 OTUs [i, m, และ f] ซึ่งให้แบบแผนตรงกับ *S. lavendulae* (growth promotant) , *Micromonospora olivasterospora* (antibacterial), และ *Thermomonospora chromogena* ตามลำดับ

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต.....

สาขาวิชา ชีวเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2551 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4872509923 : MAJOR BIOCHEMISTRY

KEYWORDS : *Streptomyces* / 16S rDNA / uncultured soil *Streptomyces*/ biodiverstiy / PCR-RFLP

SIRINEE YODMUANG : BIODIVERSITY OF UNCULTURED *Streptomyces* INVOLVED IN BIOACTIVE SUBSTANCE PRODUCTION FROM SOIL IN THAILAND. ADVISOR : ASST. PROF. SUCHART CHANAMA, Ph. D.; CO-ADVISOR : ASST. PROF. MANEE CHANAMA, Ph. D., 181 pp.

Streptomyces, gram-positive filamentous bacteria and G-C rich nucleotide, have capability of producing several bioactive compounds and predominantly exhibit in soil. This study is aimed to investigate the genetic diversity of *Streptomyces* in soil samples in Thailand by using culture-independent method with total genomic DNA extraction and purification from soil. The 16S rDNA was then amplified by nested Polymerase Chain Reaction (nested-PCR) technique using universal primers and *Streptomyces* specific primers for the first and second PCR reactions respectively. The PCR product about 1 kb was obtained and cloned into T/A cloning vector to make clone library. The biodiversity of 16S rRNA genes was determined by Restriction Fragment Length Polymorphism (RFLP). Based on *in silico* restriction endonuclease digestion of seventy-nine 16S rDNA sequence data [Streptomyces (70 species), Actinomyces (6 species), and outgroup bacteria (3 species)] derived from public sequences databases (RDP and NCBI) using NEBcutter program with 33 restriction endonucleases and calculation of restriction distance using Nei-Li method and construct of Neighbor-joining tree using PAUP program various tpes of RFLP patterns were elucidated. It was found that a number of OTUs of RFLP patterns derived from *Msp*I (isoschizomer of *Hpa*II) yielded high level of average restriction site per species (10.50), and good correlation between the phylogenetic distribution and the production of bioactive compounds. The *Msp*I enzyme was, therefore, selected for the analysis of 16S rDNA gene isolate and amplified from soil in different locations of Thailand (mountain, mangrove forest, and paddy field) in order to investigate the biodiversity of such *Streptomyces* as describe previously. The results showed that 100 clones of 16S rDNA of such soils revealed RFLP patterns of 16 OTUs. Comparison of the RFLP patterns from soils to simulated RFLP patterns (digested with *Msp*I) showed that dominant OTU type was ‘a’ type. The ‘a’ type of RFLP pattern contains several important bioactive producing *Strepomyces* such as *S. venezuelae* and *S. fridae* which are antibiotic producers. Three OTUs (i, m, and f type) were found to match *S. lavendulae* (growth promotant), *Micromonospora olivasterospora* (antibacterial) , and *Thermomonospora chromogena*, respectively.

Department : Biochemistry Student’s Signature .....

Field of Study : Biochemistry Advisor’s Signature .....

Academic Year : 2008 Co-Advisor’s Signature .....

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LIST OF ABBREVIATIONS

16S rDNA	16S ribosomal RNA gene
µg	microgram
µl	microliter
µM	micromolar
bp	base pair
cm	centimeter
dNTPs	deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
kb	kilobase pair
min	minute
ml	milliliter
ng	nanogram
OD260	optical density at 260 nanometer
OTU	Operational taxonomic unit
PCR	polymerase chain reaction
RDP	Ribosomal Database Project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SSU	small subunit
TAE	tris acetate EDTA
tRNA	transfer ribonucleic acid
Tris	This (hydroxymethyl) aminomethane

CHAPTER I

INTRODUCTION

Members of genus *Streptomyces* are Gram-positive filamentas bacteria in the class Actinobacteria (Anzai *et al*., 2008). They are ubiquitous in nature and important members of soil microbial community (Inbar *et al*., 2005). They are known to be producers of many secondary metabolites, which have different biological activities such as antibacterial, antifungal, antiparasitic, antitumor, and immunosuppressive actions (Anderson and Wellington, 2001). Currently, about 10,000 antibiotics have been discovered from microorganisms. It has been estimated that approximately two thirds of these naturally occurring antibiotics were isolated form actinomycetes mainly from the genus *Streptomyces* (Kieser, 2000; Anzai et al., 2008). It has been reported that *Streptomyces* species such as *Streptomyces hygroscopicus, S. griseus, S. fridae,* and *S. lavendulae* produce many biologically active secondary metabolites (Kieser et al., 2000; Strol, 1997). There are also many *Streptomyces* species that have not yet been shown to produce bioactive compounds (Buchanan et al., 1974). However, it has been estimated that only 0.001-15 % of the environmental microbial population can be cultured by standard techniques because culture techniques fail to reproduce in artificial media the niches of many microorganisms found in high-diversity environments such as soil (Gich et al., 2000; Rintala et al., 2001). Thus, the recent development of molecular biology techniques, which do not rely on cultivation methods, allows microbial ecologists to reveal inhabitants of natural microbial communities which have not yet been cultured (Trevors and Elsas, 1995; Paul, 2007). This approach involves examining variations in 16S rRNA or 16S rRNA-encoding DNA (rDNA) within a naturally occurring prokaryotic community (Malo et al., 1991; Moyer et al., 1994; Urakawa et al., 1999). PCR-based methods are culture-independent and potentially more sensitive than culturing, and thus, can provide better tools for exposure assessment (Jurgens, 2002).

As a result, these techniques are now widely applied to characterize microbial community in different environments (Moyer et al., 1994; Moran et al., 1995; Atalan, 2001; Rintala et al., 2002; Inbar et al., 2005). One of these techniques, sequencing, allow us to determine which microorganisms are present in the community, but they are time-consuming. Hybridization and probing are faster, but require a sufficient knowledge of the community to choose the appropriate target sequences. In this study, another molecular biology technique, the restriction fragment length polymorphism (RFLP) based on 16S rRNA gene, is applied to soil sample. Even faster than hybridization and probing, PCR-RFLP of 16S rRNA gene has been used in the analysis of bacterial diversity in environmental community (Moyer et al. 1994; Jurgen, 2000). The 16S rRNA gene contains information which makes it an excellent biomarker of microorganisms. For example, each 16S rRNA gene contains both highly conserved regions found in all living microorganisms and variable regions that are unique to particular microorganisms or closely related group of microorganisms. Analysis of variable regions leads to a specific RFLP pattern, which in turn can be used to define an operational taxonomic unit (OTUs). Although RFLP gives little or no information about the type of microorganisms present in the sample, it can be used for a quick assessment of genotypic comparison from different environment condition.

Since little known about the information on the relationship between the microbial taxonomy, e.g., RFLP data based on 16S rDNA and the bioactive compounds produced. This would be useful for the utilization of industrial microorganisms. Thus, objective of this study was to investigate biodiversity of uncultured bioactive producing soil Streptomyces in Thailand by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR- RFLP). A computer-simulated fragments pattern of seventy-nine16S rDNA sequences was initially used to predict the pattern of digested fragments and useful for selection of enzymes used in vivo experiment.

CHAPTER II

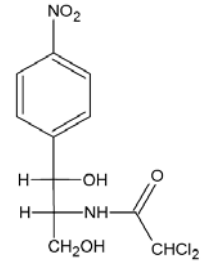
LITERATURE REVIEW

2.1 General Information of *Streptomyces*

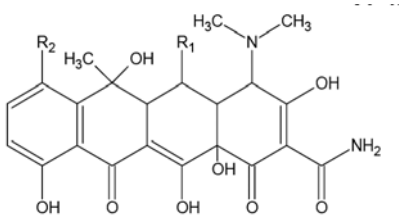
Members of genus *Streptomyces* are Gram-positive, filamentous bacteria that undergo morphological differentiation during their life cycle. They normally occur as spores, but in the presence of sufficient moisture and nutrients, the spores can germinate and form vegetative mycelium. In response to environmental signals, such as a shortage of nutrients or water, the process of differentiation is set in motion, and spores resistant to desiccation and starvation are formed again. At the same time, the production of pigments, antibiotics and other secondary metabolites is initiated (Kieser, 2000). It is known that many antibiotics and biologically active secondary metabolites were produced by many members of in this genus. Table 1 describes a range of useful *Streptomyces* antibiotics. The example of various bioactive substances from *Streptomyces* shown in Figure 1 indicated high diversity of structures.

Table 1 Secondary metabolites from genus *Streptomyces* (Kieser et al., 2000)

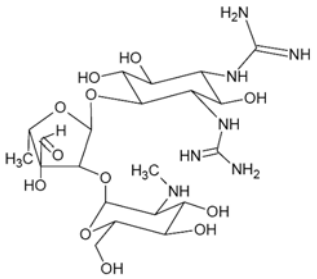
Species	Antibiotic name	Bioactivity
S. albidoflavus	3-phenylpropionic acid	Antifungal
S. alboniger	puromycin	Antibacterial
S. ambofaciens	Spiramycin	Antibacterial
S. avermitilis	Avermectin	Antiparasitic
S. bambergiensis	Bambermycins	Growth promotant
S. coelicolor	Actinorhodin	Antibacterial
S. graminofaciens	Streptogramins	Antibacterial
S. halstedii	Vicenistatin	Antitumor
S. hygroscopicus	Bialophos	Herbisidal
S. hygroscopicus	FK506	Immuno suppressant
S. hygroscopicus	Rapamycin	Antihelminthic
S. kanamyceticus	Kanamycin	Antibacterial
S. *lavendulae*	StreptotricinA-F,X	Antibacterial
S. lividans	Actinorhodin	Antibacterial
S. narbonensis	Josamycin	Antibacterial
S. *natalensis*	Natamycin	Antifungal
S. peucetius	Daunorubicin	Antitumor
S. peucetius subsp. caesius	Doxorubicin	Antitumor
S. rimosus	Oxytetracycline,rimocidin	Antibacterial
S. spectabilis	Spectinomycin	Antibacterial
S. tendae	Nikkomycin	Antifungal,insecticidal
S. tsukubaensis	Tacrolimus (FK506)	Immunosuppressant
S. venezuelae	chloramphenical	Antibacterial
S. viridochromogenes	Phosphinothricin	Herbiside



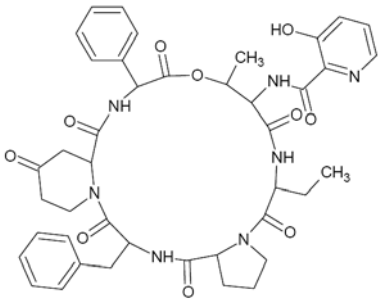
Chloramphenical (nitrogroup containing)
antibacterial from *S. venezuelae*



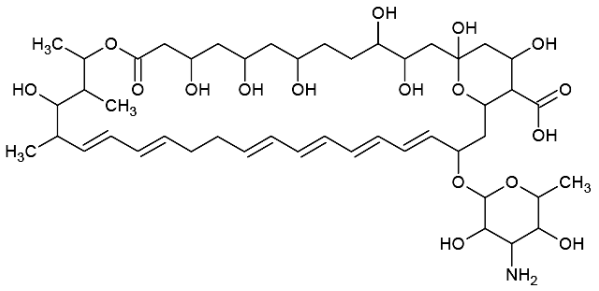
Tetracycline (tetracycline)
antibacterial from *S. aureofaciens*



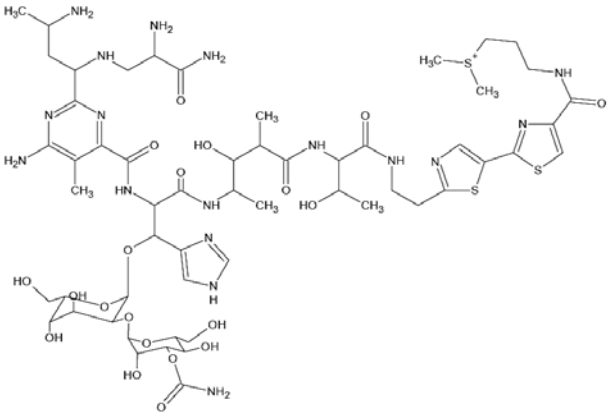
Streptomycin (aminoglycoside)
antibacterial from *S. griseus*



Virginiamycin (depsipeptide)
growth promotant from *S. virginiae*



Nystatin (Polyene)
antifungal from *S. noursei*



Bleomycin A (bleomycin)
antitumor from *S. verticillus*

Figure 1 Example of bioactive substances from *Streptomyces* (Behal, 2002)

2.2 Biology and Ecology of Streptomycetes

2.2.1 Taxonomy

The genus *Streptomyces* was proposed by Waksman & Henrici (1943) (Kampfer, 2006) and classified in the family Streptomycetaceae on the basis of morphology and subsequently cell wall chemotype. A nontaxonomic name for the genus was streptomycetes.

The taxonomy of Streptomyces species are as follows (Buchanan, 1974; Anderson and Wellington, 2001)

Kingdom: Monera

Domain: Eubacteria

Phylum: Actinobacteria

Class: Actinobacteria

Subclass: Actinobacteridae

Order: Actinomycetales

Family: Streptomycetaceae

Genus: *Streptomyces*

The development of numerical taxonomic systems, which utilized phenotypic traits helped to resolve the intergeneric relationships within the family Streptomycetaceae and resulted in the reclassification of six additional genera (*Actinopycnidium*, *Actinosporangium*, *Chainia, Elytrosporangium, Kitasatoa* and *Microellobosporia*) to the *Streptomyces* genus. These early numerical systems utilized phenotypic characters, which were fundamentally changed by the incorporation of molecular biological characteristics into classification systems and thus enabled considerable advances for genus delimitation within the Actinobacteria. Prior to this, the genera *Streptomyces* and *Streptoverticillium* were two distinct genera; both have cell-wall type 1 are lysed by the same phages and are phylogenetically closely related. Immunodiffusion studies linked members of the genus *Streptoverticillium* closely to the *Streptomyces lavendulae* species group and also found similarities using physiological tests. Observed differences in DNA ± RNA pairing; this and the morphological trait of

producing whorls were the only detectable differences between the two genera. concluded from 16S and 23S rRNA comparisons that the genus Streptoverticillium should be regarded as a synonym of *Streptomyces*. *Kitasatosporia* was also included in the genus *Streptomyces*, despite having differences in cell wall composition, on the basis of 16S rRNA similarities (Wellington et al., 1992). This was revoked by Zhang et al. (1997), who demonstrated that members of the genus *Kitasatosporia* always formed a stable monophyletic clade away from streptomycetes when sequences from the entire 16S rRNA genes were compared (Kampfer, 2006). *Kineosporia* and *Sporichthya* are both rare and share many chemotaxonomic similarities with members of the genus *Streptomyces*, which led to their incorporation into the genus. The *Kineosporia* and *Sporichthya* have since been reinstated as independent genera on the bases of ribosomal sequencing: *Sporichthya* is a genus of the family Sporichthyaceae of the suborder Frankineae and the Kineosporia are grouped with the Kineococcus. These changes resulted in the genus *Streptomyces* being the sole member of the family Streptomycetaceae.

On the basis of 16S rRNA/DNA sequence comparisons, members of the genus *Streptomyces* form a separate line of descent, and (Stackebrandt et al., 1993) proposed the emendation of the family Streptomycetaceae in the suborder Streptomycinae and the order Actinomycetales. The intrageneric phylogenetic relationships of many of the 346 recognized species in Bergey's Manual of Systematic Bacteriology inferred from the 350 complete 16S rRNA sequences, however, are clearly restricted by the limited resolving power of the method to discriminate between related species and are often in contrast with a morphologically and physiologically based classification. Though about 350 almost complete 16S rRNA sequences are available to date, the high degree of conservation within 16S rRNA genes causes problems for resolving phylogenetic relationships at the intergeneric level. Notably, the different methods used for grouping of the *Streptomyces* species often lead to contradictory results. All 376 *Streptomyces* species and subspecies with valid names (as of December 9, 2003; taken from the List of Bacterial Names with Standing in Nomenclature); and some additional species with

names not validly published (but included in taxonomic studies) are given with their grouping according to different studies.

Strains are widely distributed and abundant in soil, including composts. A few species are pathogenic for animals and man, and others are phytopathogens. The type species is *Strepto*myces albus (Kieser, 2000; Rintala et al., 2001). They are particularly active in the degradation of recalcitrant macromolecules such as lignin and cellulose (Inbar et al., 2005).

2.2.2 Morphology

Member of genus *Streptomyces* undergo a complex life cycle (Figure 2), which has been studied most intensively for strain "*S. coelicolor*" A2(3). *Streptomyces* colonies are multicellular, differentiated organisms exhibiting temporal and spatial control of gene expression, morphogenesis, metabolism and the flux of metabolites (Kieser et al., 2000). They may produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelia. In addition, colored diffusible pigments may also be formed. Note that the production of pigments largely depends on the medium composition and cultivation conditions. Many strains produce one or more antibiotics. The metabolism is oxidative and chemoorganotrophic. The catalase reaction is positive, and generally, nitrates are reduced to nitrites. Most representatives can degrade polymeric substrates like casein, gelatin,hypoxanthine, starch and also cellulose. In addition, a wide range of organic compounds is used as sole sources of carbon for energy and growth (Kampfer, 2006). The optimum temperature for most species is 25–35°C; however, several thermophilic and psychrophilic species are known. The optimum pH range for growth is 6.5–8.0.

The surface of the conidial wall often has convoluted projections which, together with the shape and the arrangement of the spore-bearing structures, are characteristic of each species and were often used for the separation of *Streptomyces* species. They can be distinguished from other actinomycetes by their cell wall type which is characterized as Type I sensu. The presence of LL-diaminopimelic acid (LL-A2pm) and

glycine and the absence of characteristic sugars are typical of this cell wall type. In addition to these traits the acyl type of the muramyl residues in the cell-wall peptidoglycans is acetyl (Anderson and Wellington, 2001; Kampfer, 2006).

Genus members lack mycolic acids, contain major amounts of saturated, iso- and anteiso-fatty acids, possess either hexa- or octahydrogenated menaquinones with nine isoprene units as the predominant isoprenolog, and have complex polar lipid patterns that typically contain diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides. In addition to these traits, the acyl type of the muramyl residues in the cell-wall peptidoglycans is acetyl (Kieser et al, 2000).

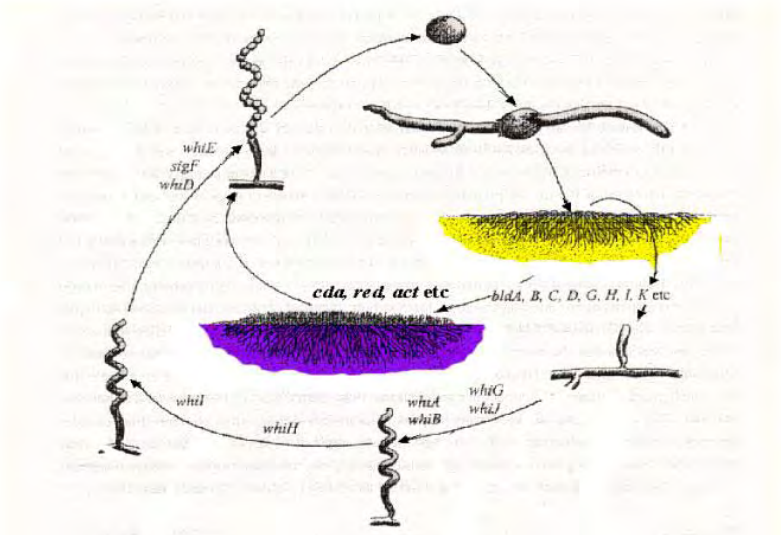


Figure 2 Life cycle of *Streptomyces coelicolor* A3(2). Under favourable conditions, one or two germ tubes emerge form a spore and grow by tip extension and branch formation to give rise to a substrate mycelium. After about two days, aerial mycelium grow up in a process that involves the action of a large number of *bld* genes. The apical compartment of individual aerial hyphae form a spiral syncytium that contains many tens of genomes. When aerial growth stops, multiple septa subdivide the apical compartment into unigenomic pre-spore compartments. These subsequently change in shape; wall thinkening occurs and grey spore pigment is deposited, to generate dessication-resistant spores (Kieser et al, 2000).

2.3 The use of molecular data to study biodiversity of soil *Streptomyces*

2.3.1 Ribosomal RNA

There is no consistent way to classify and relate microorganisms, both prokaryotes and eukaryotes, other than the use of modern methods of molecular phylogenetic analysis. The use of macromolecular sequence comparisons to define phylogenetic relationships is now well established (Woese, 1987; Anderson and Wellington, 2001). Protein sequences were most often used for phylogenetic determinations in the past as techniques for studying nucleic acid sequences were not available. Studies comparing cytochrome c, ribonucleases, globins, etc., have been rewarding, although they have proven most useful with higher eukaryotes. Among microbes, phylogenetic and biochemical diversity is such that even the identification of homologous proteins is not a straightforward task. Because they are required by all cells for protein synthesis, the nucleic acid elements of the translation apparatus - the proteinsynthesizing machinery - seem best suited for broad phylogenetic analysis. The translation of mRNAs into proteins using ribosomes and the tRNAs is an ancient mechanism. The similar architecture of the ribosomes and tRNAs in the three primary kingdoms - Archaea, Bacteria and Eucarya - means that the translation apparatus emerged largely in its modern form before the phylogenetic radiation of the three kingdoms. Thus, phylogenetic analysis of the components of the translation apparatus allow, in principle, the relationships among organisms to be traced nearly to the time of the origin of life on the earth. However, tRNAs are not very useful for phylogenetic characterizations because they are too constrained in structure. The tRNA structure is tightly locked up in a complex tertiary organization. Almost every residue in a tRNA molecule has contact with at least one other residue, and the tight interlocking of the molecule imposes conformational constraints on all residues. Another major problem is the limited number of mutable residues in homologous tRNAs. The small number of changes in compared molecules means that the statistical error in a calculated evolutionary distance is great. We are therefore left with rRNAs as the most useful tools for molecular data used to investigate diversity of *strepomyces* (Hugenholtz and Pace,

1996; Jurgens, 2002). Figure 3 and Figure 4 show bacterial ribosome and its structure, respectively.

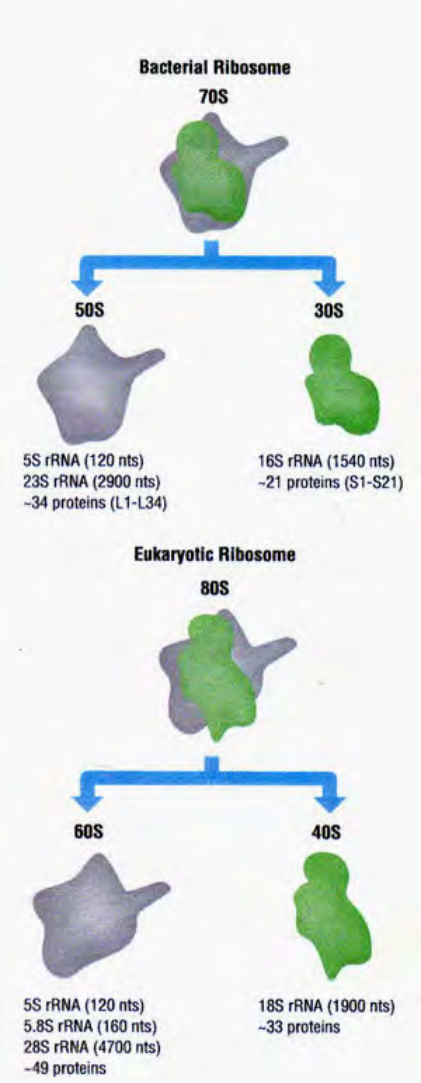


Figure 3 Bacterial ribosome (70S) consisted of 2 subunits: large subunit (50S) and small subunit (30S). The large subunit consisted of 23S rRNA (2,900 nts) and 5S rRNA (120 nts) and 34 proteins. The small subunit consited of 16S rRNA (1,500 nts) and 21 proteins. Compared to eukaryotic ribosome (80S); large subunit (60S) consisted of 28S rRNA (4,700 nts), 5.8S rRNA (160 nts), and 5S rRNA (120 nts) and 49 proteins. For the small subunit (40S) consisted of 18S rRNA (1,900 nts) and 33 proteins (http://www.newsciencepress.com).

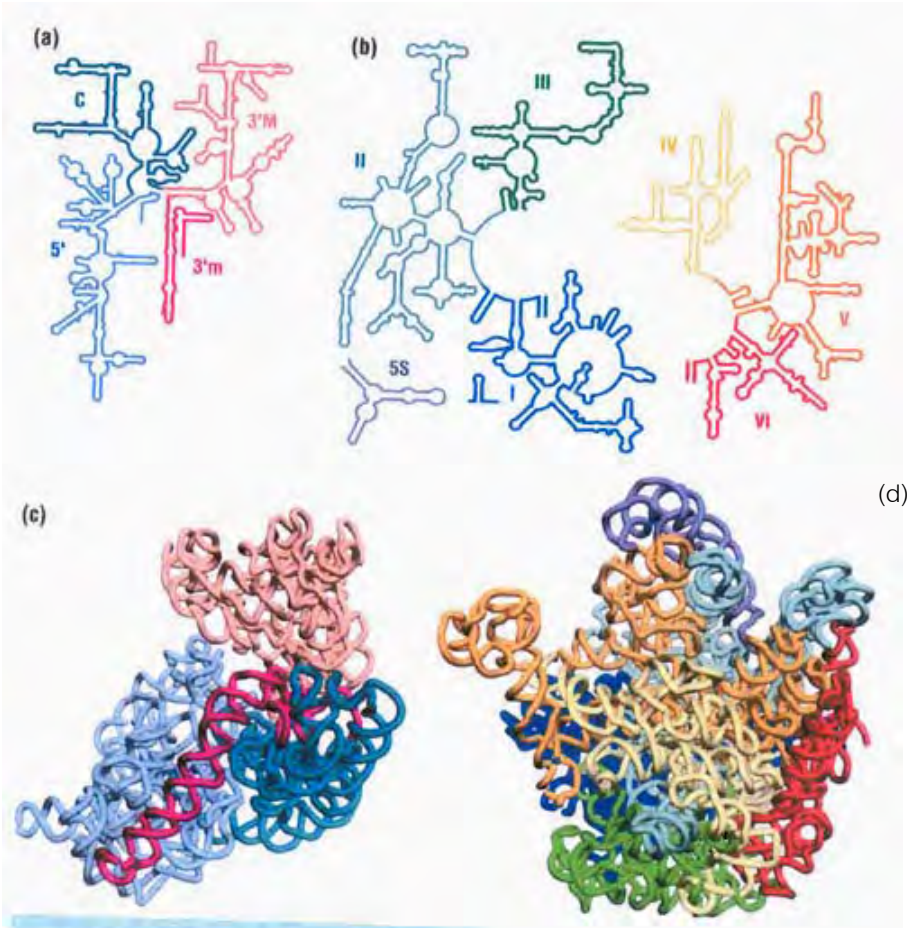


Figure 4 Secondary and tertiary structure of 16S rRNA from small subunit (a) and (c) respectively, and those of 5S rRNA and 23S rRNA (b) and (d) respectively (http://www.newsciencepress.com).

There are several explicit reasons for focusing on the ribosomal RNAs:

- the rRNAs, as key elements of the protein-synthesizing machinery, are of profound importance to all organisms.

- the rRNAs are ancient molecules and extremely conserved in overall structure. Thus, homologous forms of rRNA are readily identified by their sizes alone.

- the conserved nature of rRNA structure extends to the nucleotide sequence level. Some segments of rRNA sequences do not vary among the biological kingdoms (domains), whereas others vary to greater or lesser extents (Gutell et al., 1994; Van de Peer et al., 1996). The conserved sequences and secondary structure allow disparate sequences to be aligned, so that only homologous sequences are used in phylogenetic analysis. The highly conserved regions also provide convenient hybridization targets for cloning rRNA genes and sequencing techniques.

- in general, rRNAs are essential and conserved across all phylogenetic domains, thus "universal" tracts of sequences can be identified. In addition, it is possible to identify sequence motifs of increasing phylogenetic resolution and recognize "signature" sequences for the domains Archaea, Bacteria, and Eucarya and their subdivisions (Gutell et al.,1994).

- rRNA constitutes a significant component of cellular mass, and is generally recovered easily from all types of organisms (Tiedje et al., 1999).

- rRNA sequences are sufficiently long to provide statistically significant comparisons (Jurgens, 2002).

- rRNA genes seem to be free from artifacts of lateral transfer between phylogenetically distant organisms. Thus, relationships established by rRNA sequence comparisons represent true evolutionary relationships (Stackebrandt et al., 1993).

Taken together, these features indicate that rRNAs may be uniquely suitable for establishing phylogenetic relationships among very different organisms (Gutell et al., 1994).

Of the three ribosomal RNAs (5S, 16S/18S and 23S/28S), the 5S is too small (~ 120 nucleotides) to be used indiscriminately for phylogenetic inferences. One might expect that the 23S/28S rRNA (23S rRNA in most prokaryotes, containing approximately

2,900 nucleotides) would provide about twice the phylogenetic information compared with the 16S/18S rRNA (16S rRNA, containing approximately 1,500 nucleotides). This is true within limits - the average rate of sequence change (as reflected in frequency of differences between corresponding sequences from a pair of organisms) of 23S rRNA is significantly faster than that of 16S rRNA. Thus, for close relationships, the larger molecule can be quite valuable although it has not proved to be as proportionately useful in the deepest branches of the tree. Generally, when both sequences are available for a set of organisms, the phylogenies inferred by each rRNA tend to be similar. As 16S and 23S rRNAs are not functionally independent, it is not surprising that they give congruent pictures. We should also take into account the fact that the number of currently available complete 23S rRNA sequences in the databases is rather poor in comparison to the number of 16S rRNA sequences. Therefore, small subunit (SSU) rRNA has served as the “gold standard” in elucidating bacterial phylogeny in recent years, and the new edition of both Bergey’s Manual of Systematic Bacteriology and "Brock Biology of Microorganisms" (Jurgens, 2002) base their respective phylogenetic relationships among microorganisms upon the small subunit (SSU) rRNA tree.

More than 5,000 of 16S rDNA sequences deposited in the public database are available at Ribosomal Database Project (RDP) http://rdp.cme.msu.edu and from National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov. An important resource fro the phylogenetic studies is the Ribosomal Database Project which provides aligned rRNA sequences and a variety of sevices related to phylogenetic analysis of rRNAs, such as the calculation of phylogenetic trees, probe analysis, chumeric sequcnece testing and sequence-similarity analysis (Hugenholtz and Pace, 1996). Figure 5 shows 16S rDNA of bacteria.

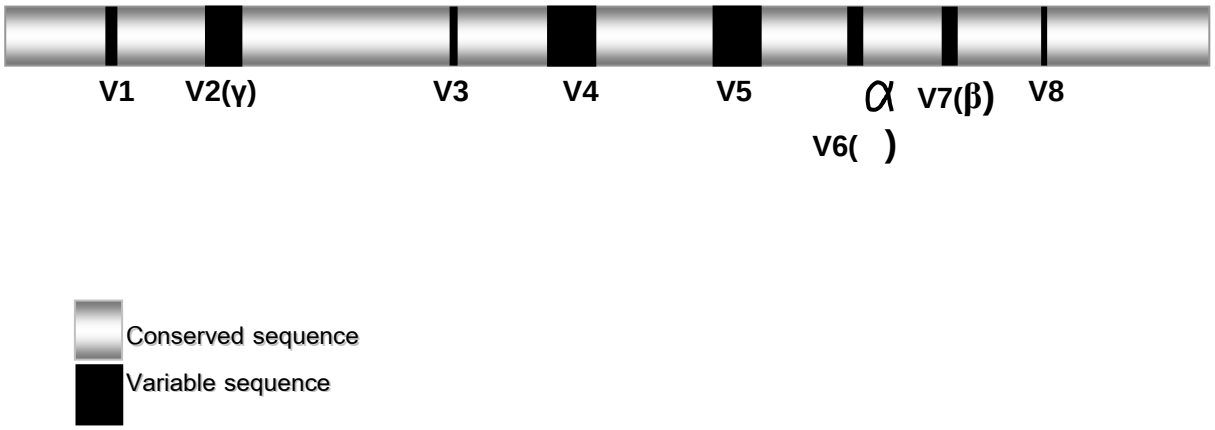


Figure 5 Map of 16S rDNA sequence of *Streptomyces ambofaciens* consisted of conserved and variable regions. The conserved regions, which conserved to all bacterial domain, are useful to design primers (universal primers). For the variable regions, are vary among all bacteria, but specific to some bacterial groups. These are very useful to design primers of genus and group specific (Rintala et al., 2001).

2.4 Molecular techniques

Due to the limitations of traditional culture-dependent methods the use of molecular techniques has become of growing importance for the study of microbial communities in various ecosystems (Yeates et al., 1997). The extraction of total DNA from soil samples enables microbial ecologists to obtain biological material without the need to isolate microorganisms by cultivation (Tebbe and Vahjen, 1993). Environmental samples can contain a number of different bacterial species and PCR has the potential to allow rapid detection of any bacterial species for which specific amplification primers are available. Depending on source of samples, various inhibitors of PCR amplification may be present, such as humic acid and fulvic acid. Finally, DNA can be difficult to isolate. (McGregor et al., 1996). The rRNA approach has been successfully applied to reveal the existence of several novel lineages of hitherto unknown prokaryotes leading to a broadening of our view on microbial diversity. It must be stressed, however, that cultivation-independent, PCR-based methods can also have inherent biases preventing a reliable assessment of the structure of bacterial populations which may lead to a misinterpretation of the abundance of certain phylogenetic groups. Such pitfalls may be avoided by hybridizing whole cells or extracted rRNA from the studied habitat with specific oligonucleotide probes in order to verify the initial results. Furthermore, the retrieval of a novel 16S rRNA sequence reveals very little about the phenotypic traits of the respective organism and its metabolic activity. It is only when the retrieved sequence can be clearly affiliated to a monophyletic lineage which is characterized by a common phenotypic trait that some conclusions may be drawn about the function of the corresponding microorganism. In most cases, however, the simple knowledge of the phylogenetic diversity in an environment is not very helpful in understanding the interacting metabolic processes and factors which control them. Nevertheless, a molecular approach can help in the identification of microorganisms which are ecologically relevant because of their abundance or activity. These microorganisms can then be the subject of detailed studies or a target of directed cultivation experiments. The majority of prokaryotes living in natural environments are rather inconspicuous. Therefore, several molecular techniques were developed in order to overcome the lack

of information about the function of bacteria identified by cultivation-independent methods. Despite the progress which has been made in linking the identification of distinct microorganisms with their functions in situ, it may still be necessary to isolate or enrich novel bacteria to reveal their metabolic potential under various environmental conditions. The results of molecular ecology research has established that experimental strategies based on the combination of molecular techniques with traditional cultivation-dependent methods have great potential in revealing some of the hidden complexity of natural microbial ecosystems (Jurgens, 2002).

2.4.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) invented by (Mullis et al., 1986). It has become a profound impact on molecular biology as well as has great potential as an important tool in detecting genetic polymorphism. PCR is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA. Like molecular cloning, PCR seems infinite and is still growing. The theoretical basis of PCR is outlined in figure 6. There are three nucleic acid segments: the segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking it. Additionally, there is a protein component (a DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTPs), a buffer, and salts. PCR cycle consists of three steps. The first step is called deneturation. In the step, the double stranded DNA molecules are separated into single-stranded ones which are used as templates for the next two steps, the second strep is called annealing. Primers anneal to the templates. During the third step which is called polymerase extension, DNA polymerase extends the annealed primers using the single-stranded molecular as templates. The process is repeated for many cycles. After extraction of soil DNA, the initial step is PCR amplification of the 16S rRNA genes, from the community DNA using universal, domain or group specific primers. The resulting products are separated in different ways, depending on the technique (Tiedje et al., 1999).

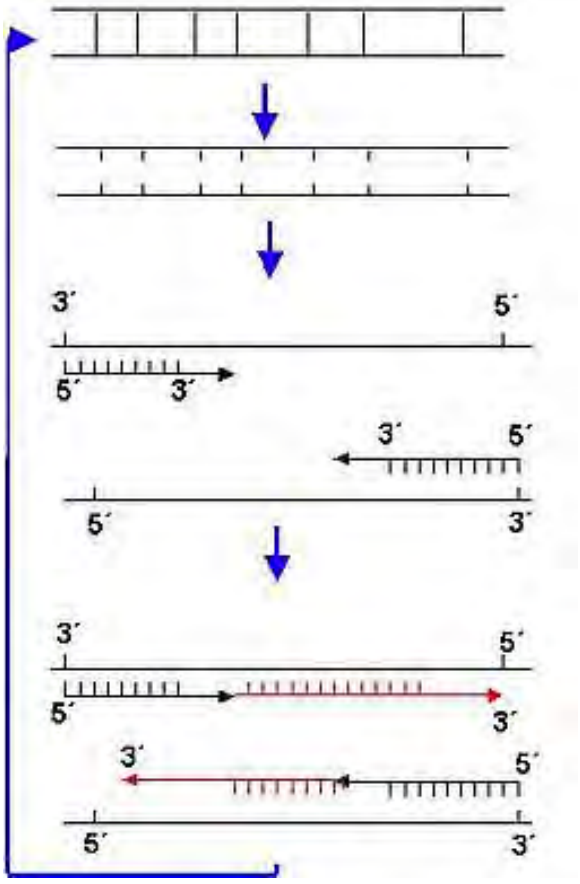


Figure 6 Principle of PCR amplification. First, the DNA template is denature at 94 °C to 95 °C. The annealling (50-65 °C) of primer to template DNA by Extension (72 ° C) by addtioning of nucleotide bases (dNTPs) catalysed by Taq DNA polymerase. (http://www.bioron.net)

2.4.2 Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

Genotypic variation can also be monitored using denaturing gradient gel electrophoresis (DGGE) and Thermal gradient gel electrophoresis (TGGE) (Muyzer et al., 1993). The technique is based on the separation of polymerase chain reaction-amplified fragments, all the some length but different base-pair sequences. Separation of DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacylamide gels, which is decreased compared with that of the completely helical form of the molecule, the melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt (Figure 7). Sequence variation with such domains causes their melting temperatures to differ. Sequence variants of the particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE.

This technique has been successfully applied to identifying sequence variations in a number of genes from several different organisms. PCR can be used to selectively amplify the sequence of interest before DGGE is used. G-C-rich sequences can be incorporated into one of the primers to modify the melting behavior of the fragment of the interested to the extent to which close to 100% of all possible sequence variations can be detected. However, a limitation of this method is the fact that only partial sequences of up to about 500 bp are separated well. Most studies of microbial community diversity so far have been based on the analysis of only one to three variable regions (Muyzer et al., 1993). Moreover, there is no comparative sequence database that would provide insight into the relationship of Tm values to sequence.

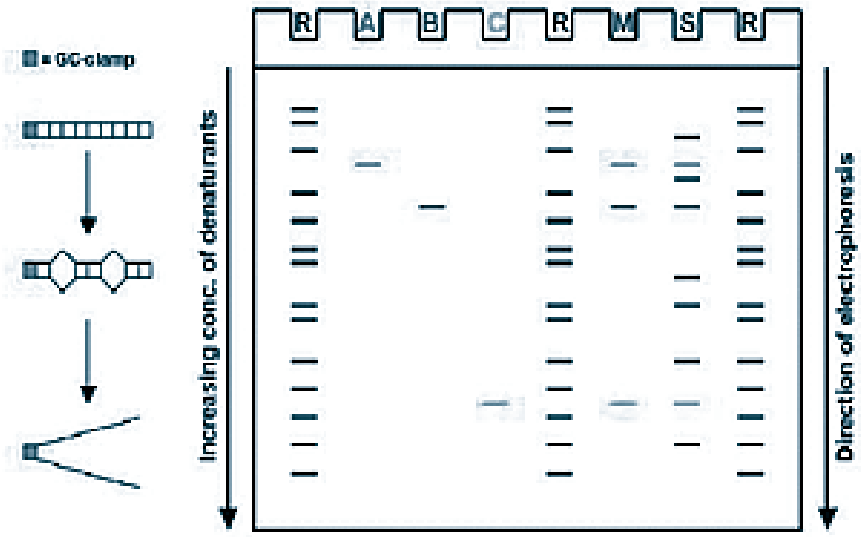


Figure 7 DGGE and TGGE principle. (http://bccm.belspo.be)

2.4.3 Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) is the easiest and most employed technique in detecting mutation and analyzing variation. After using the technique PCR in amplification of the specific place of interest, the aftermath DNA will be worked more. It will be denatured to create single-stranded molecules and loaded on non-denaturing gel. Single-stranded DNA folds differently, depending on each single base (Sunnucks et al., 2000). Also, some scientists believe that mutation-induced changes of tertiary structure of the DNA bring about differences in terms of mobility for each DNA strand. As for the non-denaturing gel, the composition and the running conditions of its can be varied: alteration of the temperature or the degree of cross-linking or the adding of glycerol or sucrose. However, some single strand nucleic acids can exist in several netastable electrophoretically resolvable conformations. Thus, the assignment of an apparently unique conformer to a population or 'ribotype' is made with and element of faith (Tiedje et al., 1999).

2.4.4 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Amplified ribosomal DNA Restriction analysis (ARDRA),

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of 16S rDNA otherwise known as Amplified ribosomal DNA Restriction analysis (ARDRA) are simple method based, consisted of PCR amplification of 16S rDNA with primers pair (Domain, genus, or strain specific) and the product is digested with appropriate restriction endunuclease (usually 4 bp recognition sites which called tetrameric restriction enzyme) and separating the DNA fragment by gel electrophoresis (Urakawa et al., 1999; Gich et al., 2000) as shown in Figure 8. This approach has been used most frequently on isolates as part of a clones screening step (Liu et al., 1997) prior to sequencing or some cases, to provide a level of insight into phylogeny (Moyer et al., 1996). More recently, the technique has been used to probe community structure and useful as a means to detect changes in communities (Tiedje et al., 1999) and each RFLP pattern is representative of a ribotybe or operational taxonomic unit (Moyer et al., 1996). This technique provides a higher level of resolution than T-RFLP because the sizes of all restriction fragments are used in the analysis fro each enzyme, therefore sampling a larger portion of rDNA sequence. A computer analysis of digestion sites of over 100 environmental stains in the ribosomal database showed that the median sequence difference detected by the use of four tetrameric restriction endonucleases was 97.4% (Moyer et al., 1996). The 16S rRNA clones differentiated by restriction enzymes are often termed OTUs or phylotypes (Moyer et al., 1994).

2.4.5 Ribosomal Intergenic Spacer Analysis (RISA)

In the majority of characterizaed microorganisms, the 16S rRNA gene is adjacent to the 23S rRNA gene and is separated by and intervening region of variable length. Robosomal intergenic spacer analysis (RISA) separates PCR products that space the 5' end of the 16S rRNA gene, through the spacer, and into the 3' end of the 23S rRNA genes (Lanoot et al., 2005).

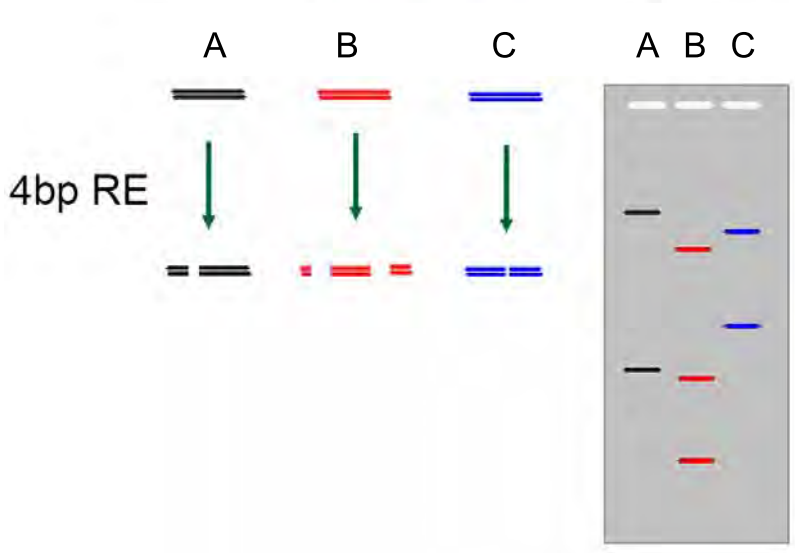


Figure 8 Principle of Restriction Fragment Length Polymorphism (RFLP).
(www.campus.skelleftea.se)

2.4.6 Terminal-Restriction Fragment Length Polymohphism (T-RFLP)

In T-RFLP, near complete genes such as 16S rRNA genes in a sample are amplified using a fluorescently-labeled primer to yield a mixture of labeled 16S rRNA genes. These amplification products are digested with restriction enzymes to produce labeled terminal restriction enzyme fragments (T-RFs).These T-RFs are then denatured, and the single stranded DNA is separated by electrophoresis under denaturing conditions (e.g. at high temperature) (Figure 9). The size of the fragments can be accurately determined using an internal standards labelled with a different fluorochrome. Ideally, each T-RF represents a single microorganism, though in practice microorganisms of different species often share one T-RF. Therefore, digestion with up to three different restriction enzymes is usually necessary to accurately identify a microorganism on the basis of T-RF size. The relative abundance of microorganisms represented by a T-RF can be determined by measuring the fluorescence of each T-RF relative to the sum of the fluorescence. Computer analysis of gene sequences can be

carried out to determine the theoretical T-RFs of known microorganisms. Alternatively, a database of experimentally-derived T-RFs from known microorganisms can be created to identify microorganisms in environmental samples (Liu et al., 1997).

T-RFLP is a rapid method for analyzing microbial communities. It can be automated to process multiple samples in a short time-span. However, T-RFLP is susceptible to biases common for all PCR-based community analysis techniques. The method is only semi-quantitative due to the variation in 16S rRNA gene copy number in different microbes. The reliable lower limit of detection of PCR products in a mixture is about 1%.

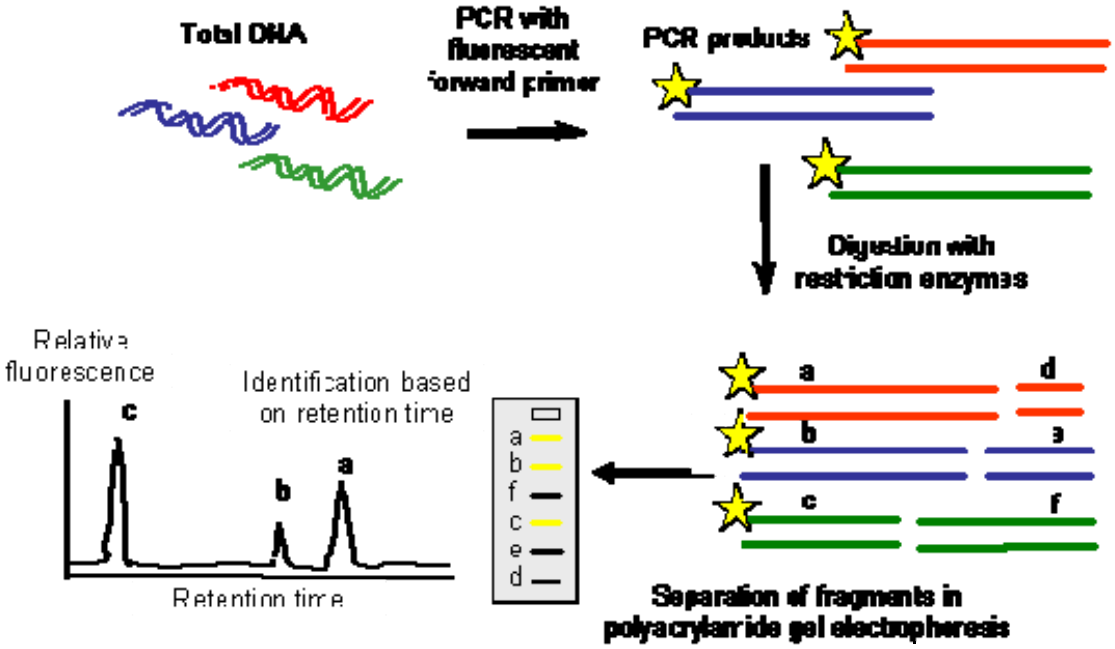


Figure 9 Principle of Terminal fragment length polymorphism (T-RFLP).

(www.campus.skelleftea.se)

2.4.7 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) analysis, was first developed in 1990, is usually employed in genetic diversity studies. This technique is not base an amplification of 16S rDNA but, based on the use of a single arbitrary primer, commonly a 10-mer or 20-mer, in a PCR reaction to synthesize multiple copies of random genomic DNA sequences. The amplification products are derived from regions of the genome that contain two shot segments on opposite strands with homology to the primer and situated sufficiently close together for the amplification to work. The advantage of this technique required no DNA probes, sequence information, and use small amount of DNA therefore quick, simple and efficient. Also, the technique can be used for species identification of *Streptomyces* strains.

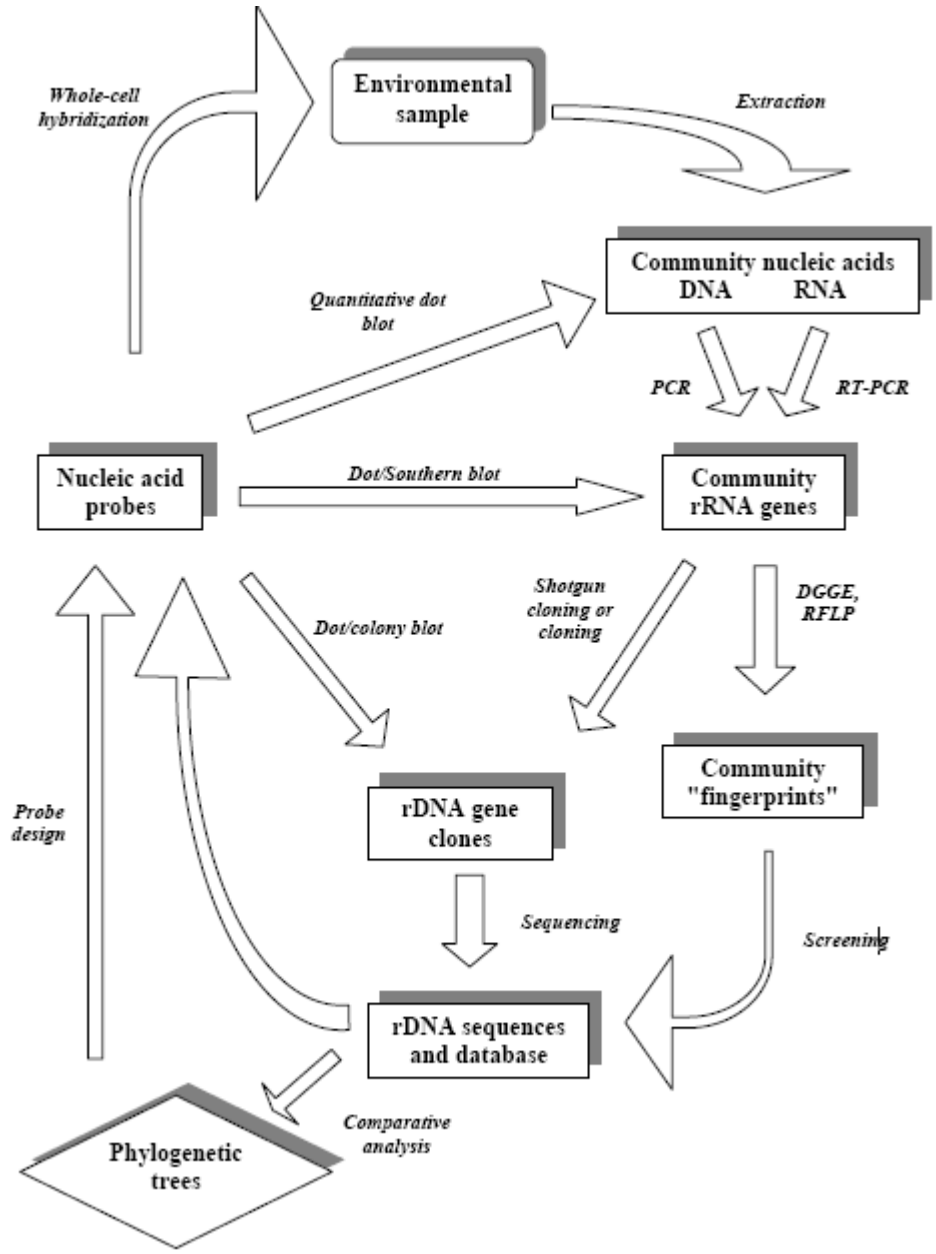


Figure 10 Full-cycle rRNA approach - strategies for characterizing microbial communities without the need for cultivation (Hugenholtz and Pace, 1996; Jurgens, 2002).

2.5 Phylogenetic analysis

The phylogenetics is the study of evolutionaty relationships. As alluded to above, phylogenetic analysis is the means of inferring or estimating these relationships. The evolutionary history inferred from phylogenetic analysis usually is depicted as branching (treelike) diagrams that represent an estimated prediction of the inheried relationships amng molecules, organisms, or both. Phylogenetics sometimes is called cladistics, because the word clade, a set of descendants form a single ancestor, is derived from the greek word for branch. However, cladistics is a particular method of hypothesizing about evolutionary relationships (Bazevanis and Ouelette, 2005).

2.5.1 Method for phylogenetic tree construction

Phylogenetic three-building methods implemented in available software are discussed in detail both in the literature (Swofford, 2002) and on the Internet. This section briefly describes a selection of the most popular mehthods. Tree-building methods can be sorted into distance-based versus character-based methods. Much of the discussion in molecular phylogenetics dwells on the usefulness of the distance-based and character-based methods. Distance methods compute pairwise distances according to some measure, and then discard the actual data, using only the fixed distances to derive trees. Character-based methods derive tree that optimize the distribution of the actual data patterns for each character. Pairwise distances thus are not fixed, because they are determined by the tree topolygy. The most commonly applied distance-based methods include NJ and Fitch-Margoliash (FM), whereas the most common character-based methods include maximum parsimony and ML.

2.5.1.1 Distance-based method

Distance-based methods use the amount of dissimilarity (the distance) between two aligned sequences to derive trees. A distance method would reconstruct the true tree if all genetic divergence events were recorded accurately in the sequence. However, divergence encounters an upper bound as sequences become mutationally satuated. After one sequence of a diverging pair has mutated at a particular site, subsequent muattions in either sequence cannot render the sites any more “different”. In

fact, subsequenct mutations can make them equal again (for example, if a valine mutates to an isoleucine, which mutates back to a valine again). There fore, most distance-based methods correct for such “unseen” substitution. In practice, application of the rate matrix dffectively presumes that some proportion of observed pariwise base identities actually represents multiple mutations, and that this proportion increases with increasing overall sequence divergence. The fragment pattern obtained form RFLP analysis consisted of presence or absence of individual bands on a gel. The present of band is indicated by “1” and for the absence by “0” transform to binary data (0 and 1). The most commonly applied distance-based methods are as follows:

- **UPGMA.** UPGMA is a clustering or phonetic algorithm; it jouns tree brances on the criterion of greatest similarity among pairs oand averages of joined pairs. It is not strictly on evolutionary distance methods. UPGMA is expected to generate an accurate topology with true branch lengths only when the divergence is according to a molecular clock (ultrameric) or is approximately equal to raw sequence dissimilarity. As mentioned earlier, these conditions rarely are met in practice.

- **NJ.** The NJ algorithm commonly is applied with distance tree building, regardless of the optimization criterion. The fully resoled tree is “decomposed” from a fully unresolved “star” tree by successively inserting brances between pa pair of closest (actually, most isolate) neighbors and the remaining terminals in the tree. The closest neighbor pair then is consolidated, effectively reforming a star tree, and the process is repeated. The method is comparative rapid.

Simulation studies indicate that UPGMA performs poorly over a broad range of tree-shape space. The use of this method is not recommended; it is mentioned here only because its application seems to persist, as evidenced by UPGMA gene tree appearing in pubications. NJ is clearly the fastest procedure and generally yields a tree close to, if not the actual, ME Tree. However, it yields only one tree. Depending on the structure of the data, numerous different tree may be as good or significantly better that NJ tree. However, the speed of NJ and its ability to produce results very similar to other slower methods ensures that this method continues to be one of the most popular used to date.

2.5.1.2 Character-based method

The character based methods attempt to infer the phylegeny based on all the individual characters sush as nucleotide or amino acids. Two commonly used method are Maxmum parsimony and Maxmum likelihood.

2.5.2 Computer software

- PHYLIP (Phylogeny Inference Package) is a package comprising approximately 30 programs that cover most of aspect of phylogenetic analysis and freely available for a wide variety of operating system written by Falsenstein. A vesion 3.68 is now release and available. PHYLIP is a command line program and does not have a point-and-click like PAUP do.

- PAUP* (Phylogenetic Analysis Using Parsimony methods*) written by Swofford. PAUP contain menu functions (including tree graphics) as possible in a single, platform-independent program with a menu interface.

2.6 The study of bacterial and *streptomyces* diversity in environment

Moyer et al. (1994) examined a microbial mat community at a deep-sea hydrothermal vent by ARDRA. They obtained 12 RFLP types from 48 clones by using four four-base-specific restriction enzymes and found two dominant clone types.

Rintala et al. (2002) examined the occurrence of streptomycetes in house dust by 16S rRNA gene sequences directly PCR amplified from DNA and found that Streptomycetes were detected in 81 % of the dust samples by PCR and actinomycetes in 36 % of the samples by culture. Thus, PCR based detection seems to be a more sensitive and accurate method for detection of streptomycetes in house dust than culture.

Atalan (2000) was separated the unknown Streptomyces strains isolated from soil samples, the interspacer regions of 16S-23S rDNA of 14 isolates were amplified with PCR (polymerase chain reaction) and digested with three restriction endonucleases, namely, Bsp143I, HaeIII and MnlI. The restriction patterns were used for RFLP analysis. A dendrogram were constructed using the unweighed pair group method using UPGMA

after analysis restriction patterns. Five RFLP groups were obtained and one test strain was left as a single member group. RFLP profile indicated that unknown strains could be identified with data based on the interspacer region of 16S-23S DNA rapid and quickly.

Inbar et al. (2005) examined the distribution of Stretpomycetes in soil amendment with compost using a stretpomycetes-specific PCR-DGGE method coupled with band excision and sequence analysis and found the community compostion of stretpomycetes in rhizosphere, as in the bulk soil, was strongly influenced by the addition of compost amendment.

CHAPTER III

MATERIALS AND METHODS

Materials

Equipments

PCR Thermal cycler

- Mycycler (Bio-rad)

Microcentrifuge Beckman Coulter, USA and Hettich)

Refrigerated centrifuge

- Allegra 25R (Beckman Coulter, USA)

Electrophoresis apparatus

- Mini-Run (Bioer)
- Maxicell (Electrophoresis system)
- Mini sub DNA cell (Bio-rad)
- Power supply: Bio-rad and Amersham

Microcentrifuge tubes 0.2 ml, 0.6 ml, 1.5 ml, 2 ml (AxyGen, Inc, USA)

Pipette tips 10 µl, 200 µl, 1000 µl (AxyGen, Inc, USA)

Orbital shaker (Innova 2100, New Brunswick scientific, co. Inc, USA)

-20 ^{o}C Freezer (Astina)

-80 ^{o}C Freezer (Sanyo Ultralow)

4 ^{o}C Refrigerator (Sharp and Sandenintercool)

Incubator (Gallenkamp)

Orbital Incubator shaker (MRC)

Refrigerated incubator (FOC225I)

Water bath shaker (Gyrotory, New Brunswick scientific, co. Inc, USA)

Laminar flow (Biohazard, model V5, Lab Service)

UV transiluminator (UVP)

Gel documentation (Syngene)

Vortex (Genie)

Water bath (Buchi)

Spectrophotometer DU 800 (Beckman Coulter, USA)

Microscope (Olympus)

Sonicater (Ney, Ultra sonic)

Autoclave (Sanyo, Labo autoclave)

pH meter (Mettler Toledo)

Balance (Mettler Toledo)

Micropipettes: P2, P20, P100, P200, P1000 (Gilson Medical Electrical S.A., France) and P20, P200, P1000 (Scolex)

Chemical reagents

Oligonucleotide primers (BSU)

dNTPs mix (New England Biolab)

BSA (New England Biolab)

DMSO (Fermentas)

DNA ladders (Fermentas)

Agarose (Seakem)

Tris-(hydroxyl methyl)-aminomethane (USB)

Glacial acetic acid (Merk, Germany)

Boric acid (Fulka)

Ethidium bromide (Sigma, USA)

Ethylene diamine tetraacetic acid, disodium salt dehydrate (Sigma)

Absolute ethanol (Merk, Germany)

Isopropanol (Merk, Germany)

Chloroform (Merk, Germany)

Isoamyl alcohol (Sigma)

N,N'-dimethyl formamide (Fulka)

IPTG (Sigma)

X-gal (Sigma)

Ampicillin (USB)

Sodium docecyl sulphate (USB)

CTAB (USB)

Yeast extract (Difco, USA)

Tryptone (Difco, USA)

NaCl (Carlo Erba)

Soluble starch (Difco, USA)

Bacto agar (Difco, USA)

Nutrient broth (Difco)

Ennzymes

Taq DNA polymerase (New England Biolab)

Restriction enzymes

- *Msp*I (New England Biolab)

RNase A (amresco)

ProteinaseK (USB)

Lysozyme (Sigma, USA)

Reagent kits

QiAquick Gel Extraction kit (Qiagen, Germany)

Gene aid gel extraction kit (USA)

RBC T/A cloning kit (Taiwan)

Methods

3.1 Sequence retrieval, alignment and DNA distance analysis

79 Bacterial 16S rDNA sequences were online downloaded from Ribosomal Database Project (RDP) website at http://rdp.cme.msu.edu or from National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov. The sequences were grouped by genera according to Buchanan and Gibbons (1974) and the RDP Hierarchical Browser (http://rdp.cme.msu.edu). Seventy sequences were *Streptomyces* 16S rDNA sequences, six were *Actinomyces* and three were outgroup bacteria. All sequences used were longer than 1,200 bp. The most recent submitted sequence to RDP database for each bacterial sequence was used, and based on reported for the following bioactive activities: antibacterial (AB), antifungal (AF), antiparasitic (AP), antiviral (AV), antitumor (AT), immunosuppressant (IM), growth promotant (GP), and herbisidal (HB). (Buchanan and Gibbons, 1974; Strohl, 1997; Kieser et al., 2000) as listed in Table 2. All sequences were aligned in Clustal X program Version 1.83. The sequence were trimmed at the priming site of primers pair StrepB and StrepF (Rintala et al., 2001) in BioEdit program version 7.0.9.0 and complied in FASTA format. The post-amplified 16S rDNA sequence file about 1 kb in length (termed StrepBF region hereafter) were realigned, and the final alignment was converted to NEXUS formats for phylogenetic tree reconstruction. Tree construction in PAUP* (Phylogenetic Analysis Using Parsimoy methods*) version 4 beta 10 program using DNA distance analysis of Kimura's 2-parameter method and Neighbor Joining were used to construct the tree. Analysis of reliability was subjected to a bootstrap test with 100 replicates and >50% bootstrap values were report.

Table 2 16S rDNA sequences of *Streptomyces*, *Actinomyces* and out group bacteria used in this study

Species	Code	Accession no.	Bioactivity*	Compound name	Ref.
S. achromogenes subsp. *rubradiris*	S01	AB184561	AT	Aclacinomycin A	Strol (1997)
S. albofaciens	S02	AB184179	AB	Oxytetractcline	Buchanan et al. (1974)
S. alboniger	S03	AB184331	AB	Puromycin	Kieser et al. (2000)
S. albolongus	S04	AB184425	AB	Proceomycin	Buchanan et al. (1974)
S. albovinaceus	S05	AB249958	AB	-	Buchanan et al. (1974)
S. almquistii	S06	AB184258	AB	Moenamycin	Buchanan et al. (1974)
S. ambofaciens	S07	AM238663	AB	Spiramycin	Kieser et al. (2000)
S. antibioticus	S08	EF063450	AB, AV, AT	Oleandomycin, Vidarabene	Kieser et al. (2000)
S. aureocirculatus	S09	AB184260	AB, GP	-	Buchanan et al. (1974)
S. aureofaciens	S10	EF063459	AB	Chlotetracycin, Narasin	Kieser et al. (2000)
S. bobili	S11	AB249925	AB	Cinerubin	Buchanan et al. (1974)
S. cattleya	S12	AB184571	AB	Thienamycin	Buchanan et al. (1974)
S. clavuligerus	S13	DQ026628	AB	Clavulanic acid	Strol (1997)
S. ederensis	S14	AB184658	AB	Moenomycin	Strol (1997)
S. fradiae	S15	EU367982	AB, GP	Neomycin, Actinomycin Z	Strol (1997)
S. fulvoviolaceus	S16	AB184573	AB, AV	Rubomycin	Buchanan et al. (1974)
S. gibsonii	S17	AB184663	AB	-	Buchanan et al. (1974)

* antibacterial (AB), antifungal (AF), antiparasitic (AP), antiviral (AV), antitumor (AT), immunosuppressant (IM), growth promotant (GP), and herbisidal (HB)

Table 2 (*continued*).

Species	Code	Accession no.	Bioactivity*	Compound name	Ref.
S. graminofaciens	S18	AB184416	AB	Streptogramin	Kieser et al. (2000)
S. griseus	S19	EU048540	AF, AT	Daunorubicin, Candicidin	Strol (1997)
S. kanamyceticus	S20	EU367975	AB	Kanamycin	Kieser et al. (2000)
S. lincolnensis	S21	DQ462654	AB, GP	Lincomycin	Strol (1997)
S. narbonensis	S22	DQ445794	AB	Josamycin, Narbomycin	Kieser et al. (2000)
S. ochraceiscleroticus	S23	DQ442533	AB	-	Buchanan et al. (1974)
S. rimosus subsp. *paromomycinus*	S24	AB184680	AB, AF, AP	Paromomycin, Streptimidone	Kieser et al. (2000)
S. rimosus	S25	EF371440	AB, GP	Oxytetracycline	Strol (1997)
S. spectabilis	S26	EU521694	AB	Spectinomycin	Strol (1997)
S. spiroverticillatus	S27	AB249921	AB, AF	-	Buchanan et al. (1974)
S. venezuelae	S28	EU367976	AB	Chloramphenical	Kieser et al. (2000)
S. xantholiticus	S29	AB184349	AB, AF	Xanthalycin A, B	Buchanan et al. (1974)
S. tenebrarius	S30	AB297962	AB	Tobramycin	Strol (1997)
S. albidoflavus	S31	EF620361	AF	3-phynyl proionic acid	Narayana et al.
S. cacaoi subsp. *asoensis*	S32	DQ026644	AF	Polyoxin	Kieser et al. (2000)
S. chrestomyceticus	S33	DQ026633	AF	Neomycin E, F	Buchanan et al. (1974)
S. nodosus	S34	EU273535	AF	Amphotericin B	Strol (1997)

Table 2 (*continued*).

Species	Code	Accession no.	Bioactivity*	Compound name	Ref.
S. noursei	S35	EF017717	AB	Nystamycin	Kieser et al. (2000)
S. tendae	S36	AB184172	AF	Nikkomycin	Strol (1997)
S. varsoviensis	S37	DQ026653	AF	Oxytetracycline	Buchanan et al. (1974)
S. natalensis	S38	AB184356	AF	Natamycin	Kieser et al. (2000)
S. albus	S39	EF059751	AP, GP	Salinomycin	Kieser et al. (2000)
S. avermitilis	S40	DQ768097	AP	avermectin	Kieser et al. (2000)
S. cinnamonensis	S41	DQ462657	AP, GP	Monensin	Kieser et al. (2000)
S. albus subsp. *pathocidicus*	S42	AB184501	AT	Pathocidin	Buchanan et al. (1974)
S. albosporeus subsp. *labilomyceticus*	S43	EF626593	AT	Labilomycin	Buchanan et al. (1974)
S. argillaceus	S44	AB045885	AT	Mithramycin	Kieser et al. (2000)
S. caespitosus	S45	AB184320	AT	Mithramycin C	Kieser et al. (2000)
S. coeruleorubidus	S46	AB184849	AT	Daunorubicin	Strol (1997)
S. galilaeus	S47	EU273538	AT	Ferrimycins A1, Cinerubicins A	Buchanan et al. (1974)
S. peucetius	S48	AB249907	AT	Daunorubicin	Strol (1997)
S. peucetius subsp. *caesius*	S49	AB184611	AT	Doxorubicin	Kieser et al. (2000)
S. verticillus	S50	EF017713	AT	Mitomycin C, Pleomycin	Buchanan et al. (1974)
S. parvulus	S51	AB184326	AT	Daetinomycin	Strol (1997)

Table 2 (*continued*).

Species	Code	Accession no.	Bioactivity*	Compound name	Ref.
S. azureus	S52	EF178674	GP	Thiostrpton	Kieser et al. (2000)
S. bambergiensis	S53	EF654096	GP	Bambermycins	Kieser et al. (2000)
S. flocculus	S54	DQ442498	GP	Ferrioxamine	Buchanan et al. (1974)
S. lavendulae	S55	EU367977	GP, AB	Streptothricin	Strol (1997)
S. virginiae	S56	EU285473	GP	Virginiamycin	Strol (1997)
S. lactamdurans	S57	AF214482	GP	Efrotomycin	Strol (1997)
S. hygroscopicus	S58	EU841547	AP, IM, HB	Bialaphos, FK506, Rapamycin	Strol (1997),
				Hygromycin B, Vilidamycin	Kieser et al. (2000)
S. tsukubaensis	S59	AB217600	IM	Tacrolimus (FK506)	Kieser et al. (2000)
S. viridochromogenes	S60	DQ442555	GP	Phosphinotricin	Strol (1997)
S. scabiei	S61	DQ861637	-	-	Kieser et al. (2000)
S. acidiscabies	S62	AB301488	-	-	Kieser et al. (2000)
S. ipomoeae	S63	AB184857	-	-	Kieser et al. (2000)
S. turgidiscabies	S64	EU593696	-	-	Kieser et al. (2000)
S. coelicolor	S65	EF371438	AB	Actinorhodin	Kieser et al. (2000)
S. lividans	S66	AB184826	AB	Actinorhodin	Kieser et al. (2000)
S. halstedii	S67	EF178695	AT	Vicenistatin	Buchanan et al. (1974)
S. baanensis	S68	EF178688	-	-	Buchanan et al. (1974)

Table 2 (*continued*).

Species	Code	Accession no.	Bioactivity*	Compound name	Ref.
S. clavifer	S69	FJ547108	-	-	Buchanan et al. (1974)
S. indigoferus	S70	EU054371	-	-	Buchanan et al. (1974)
Amycolatopsis mediterranei	A01	EF017716	AB	Rifamycin	Kieser et al. (2000)
Amycolatopsis orientalis subsp. *orientalis*	A02	FJ455098	AB	Vancomycin	Kieser et al. (2000)
Saccharopolyspora erytraea	A03	AM420293	AB	Erythromycin	Kieser et al. (2000)
Micromonospora olivasterospora	A04	EU274360	AB	Fortimicin	Kieser et al. (2000)
Sporichthya polymorpha	A05	AB025317	-	-	Buchanan et al. (1974)
Thermomonospora chromogena	A06	AF116563	-	-	Buchanan et al. (1974)
Escherichia coli	E01	CP000970	-	-	Buchanan et al. (1974)
Pseudomonas putida	P02	EU661866	-	-	Buchanan et al. (1974)
Baciilus subtilis	B03	EU686584	-	-	Buchanan et al. (1974)

3.2 *In silico* endonuclease digestions and restriction fragment length polymorphism (RFLP) analysis

Each trimmed sequence was exported to NEB Cutter Version 2.0 offered from New England Biolab Incorporation (http://tools.neb.com/NEBcutter2/index.php) for *in silico* digestion with 33 restriction endonucleases that confermed to have the dual requirements of being commercially available and recognizing a specific sequence (4 or 6 bp) in which every nucleotide position is defined (Table 3). After *in silico* restriction digestion, a simulated 2% agarose gel electrophoresis image was plotted and captured as a device-independent files in the PDF or JPG format. Restriction patterns of each enzyme for S*treptomyces* species from NEB Cutter were arranged and transferred as TIFF format using spreadsheet program (Microsoft EXEL 2003) and Adobe Photoshop version 8.0 programs, respectively for analyzing in AlphaEase program V. 5.0 to calculate distances of DNA fragments. Bands were scored manually by their presence (1) or absence (0) for restriction distance analysis.

Table 3 Restriction enzyme used for *in silico* endonuclease digestion

Restriction enzymes (isoschizomer)	Sequence	Reaction buffer NEBuffer	Optimum incubation temperature (°C)
Bfa I	C'TAG	4	37
Dpn I[a] (*Dpn* II[b], *Mbo* I[b], *Sau*3A I, *Bfu*C I)	GA'TC	4	37
*Bst*U I	CG'CG	2	60
Hha I (*Hind*P I)	GCG'C	4 + BSA	37
*Mse*I	T'TAA	2 + BSA	37
Nla III	CATG'	4 + BSA	37
Rsa I	GT'AC	1	37
Taq I	T'CGA	Taq I + BSA	65
Alu I	AG'CT	2	37
Hae III	GG'CC	2	37
Mnl I	CCTC (7/6)	2 + BSA	37
Hpa II (*Msp* I)	C'CGG	4	37
Aci I	CCGC (-3/-1)	3	37
Aat II (*Zra* I)	GACGT'C	4	37
*Acc*65I (*Kpn* I)	G'GTACC	3	37
Age I	A'CCGGT	1	37
*Apa*I (*Psp*OM I)	GGGCC'C	4 + BSA	25
*Bmg*B I	CACGTC (-3/-3)	1	37
*Bse*Y I	CCCAGC (-5/-1)	1	37
*Bsp*E I	T'CCGGA	3	37
*Bsr*G I	T'GTACA	2 + BSA	37
*Bss*S I	CACGAC (-5/-1)	3	37
Eag I	C'GGCCG	3	37
*Eco*RI	G'AATTC	EcoRI	37
*Eco*RV	GAT'ATC	3 + BSA	37

Table 3 (continued).

N0. Restriction enzymes (isoschizomer)	Sequence	Reaction buffer NEBuffer	Optimum incubation temperature (°C)
Fsp I	TGC'GCA	4	37
Nae I (*Ngo*MI V)	GCC'GGC	1	37
Pst I	CTGCA'G	3	37
Sac I	GAGCT'C	1 + BSA	37
Sac II	CCGC'GG	4	37
*Sna*B I	TAC'GTA	4 + BSA	37
Ssp I	AAT'ATT	*Ssp* I	37
Sma I (*Tsp*M I, *Xma* I)	CCC'GGG	4	25

[a] cleaves only when its regconition site is methylated

[b] Block my *dam* methylation

3.3 Restriction distance analysis

Restriction distance analysis was performed by PAUP* (Phylogenetic Analysis Using Parsimoy methods*) version 4 beta 10 program using restriction distance analysis from Nei- Li method. A resulting NJ tree was used to investigate the relationship of species and bioactivity producing.

3.4. Samples

3.4.1 Soil samples and soil sampling

Thirty six soil samples were collected from different part of Thailand. 50 -100 g of each samples were collected. Soil type, color and pH were recorded. Twenty one of the samples were from forest soil (Fifteen of the samples were taken from Doi Phuka National Park in Nan province and six form Khao Keaw National Park in Chonburi province). Seven were from mangrove forest and 8 were from paddy field (Table 4 – 6). During collection and transportation, samples were stored on ice upon arriving at the laboratory, the samples were stored at -20°C.

Table 4 Soil sample from forest mountain (MT) used in RFLP analysis

Location	Code	Soil type	pH	Location	Code	Soil type	pH
Nan province, Pua district	A01	Loam	5.87	Chonburi province	D01	Sandy soil	8.53
(Doi Phuka National Park)	A02	Loam	5.69	(Kao Keaw National Park)	D02	Sandy soil	8.22
	A03	Loam	5.09		D03	Sandy soil	6.58
	A04	Loam	3.88		D04	Sandy soil	7.02
	A05	Loam	3.93		D05	Sandy soil	5.98
	A06	Loam	3.62		D06	Sandy soil	8.34
	A07	Loam	4.04				
	A08	Loam	4.52				
	A09	Loam	3.80				
	A10	Loam	3.58				
	A11	Loam	4.26				
	A12	Loam	3.97				
	A13	Loam	4.40				
	A14	Loam	4.94				
	A15	Loam	4.60				

Table 5 Soil sample from mangrove forest (MG) used in RFLP analysis

Location	Code	Soil type	pH	Location	Code	Soil type	pH
Samutprakarn province	E01	Clay	5.88	Bangkok, Bangkhuntian	F01	Clay	7.25
(King Chulalongkorn Fort)	E02	Loam	6.81		F02	Loam	7.13
	E03	Loam	6.89		F03	Loam	7.24
	E04	Loam	7.63				

Table 6 Soil sample from paddy field (PD) used in RFLP analysis

Location	Code	Soil type	pH	Location	Code	Soil type	pH
Mukdaharn province,	G01	Sandy soil	5.29	Ubon Ratchathani province,	H01	Sandy soil	5.20
Nikhomkhamsoi district	G02	Sandy soil	4.43	Varinchamrab district	H02	Sandy soil	4.79
	G03	Sandy soil	5.25	Karnchanaburi province	I01	Clay	8.51
	G04	Sandy soil	5.20	Muang, district	I02	Loam	7.93

3.4.2 Bacterial samples and culture conditions

All *Streptomyces* (*S. venezualae, S. narbonensis, and S. lividans*) and non-Streptomycetes bacteria (*Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas putida*) from culture collection of Asst. Prof. Suchart Chanama, Ph.D. from Department of Biochemistry, Faculty of Science, Chulalongkorn University. *Streptomyces* were isolated on NS agar to produce a vegetative mycelium. A single colony was inoculated to a NS broth at 28 °C for 48-72 hr. Out group bacteria (*Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas putida*) was isolated on Luria-Bertani or nutrient agar. A single colony was grown in LB or nutrient broth for 16-18 hr. 15% glycerol stock preparation was made and stored at -70 °C to all bacterial strains.

Table 7 Bacteria and cultivation media used in this study

Species	Medium	Temperature (°C)
Streptomycetes		
Streptomyces venezuelae	NS	29
Streptomyces narbonensis	NS	29
Streptomyces lividans	NS	29
Streptomyces hygroscopicus	NS	29
Streptomyces rimosus	NS	29
Outgroup bacteria		
Escherichia coli	LB	37
Bacillus subtilus	LB	37
Psudomonas putida	NA	37

3.5 DNA isolation

3.5.1 Soil DNA extraction and purification

Crude DNA was isolated from a soil sample by direct lysis procedure (Saano and Lindstrom, cited in Trevor and Elsas, 1995) with slight modifications. First, 0.25 g of soil was mixed vigorously for 30 min at 37 ^{o}C with 625 µl lysis buffer (120 mM Na_2HPO_4 pH8, proteinase K 100 µg/ml and 1% SDS) with occationally shaking during incubation time. Subsequently, 112.5 µl of 5 M NaCl was added and vortexed. Then 93.75 µl of 10% cetyltrimethyl ammonium bromide (CTAB) in 0.7 M NaCl was added, thoroughly mixed and incubate at 65 ^{o}C for 20 min. After that samples were centrifuged at 12,000 xg for 1 min at room temperature. Equal volume of chloroform was added and mixed. The aqueous phase was transfer to a new tube after centrifugation at 12,000 xg for 5 min. An Equal volume of isopropanol was added and the mixture was left at -20^{o}C for 30 min, followed by centrifugation (12,000 xg, 5 min). Then the DNA pellet was washed with 70% ethanol and resuspended TE buffer (10 mM Tris-HCl and 1 mM EDTA) pH 8.0. If occurrence of humic acid and other phenolic compounds from soil were co-extract resulting in yellowish to brown color of DNA pellet was observed, crude DNA was purified (as these compounds interfered with the PCR) using QIAgen gel extraction kit (Qiagen, Hilden, Germany), according to the manufacturere's instructions. The purified DNA was then applied to 1% agarose gel in 0.5 X TAE buffer to checked for quality and the size of the DNA. Test of soil purity was checked by spectrophotometry at A260/230 and A260/280 which indicate the contamination of humic acid and protein, respectively (Trevors and Elsas, 1995; Yeates et al., 1997).

3.5.2 Bacterial genomic DNA extraction

3.5.2.1 Preparation of Streptomycetes genomic DNA

DNA extraction was extracted according to Kutchma et al. (1998). First, cells (1.5 ml) were harvested from cell culture by centrifugation (12,000 xg for 1 min) 50 mg of cell pellet was washed with 500 µl acetone and incubate on ice 5 min. Centrifuged at 12,000 xg 4 min and discard acetone. Resuspended chill in 500 µl TE buffer [10 mM Tris-Cl, pH 8.0 and 1 mM EDTA (with 1 mg/ml lysozyme)] and incubated 37 ^{o}C 1 hr. Add 75 µl of 10% SDS and 125 µl of 5M NaCl, inverted gently and subjected to freeze-thawing method by incubated in liquid nitrogen 3 min and placed in water bath (65 ^{o}C). Repeat 5 times and incubated on ice for 10 min. Centrifuged at 12,000 xg 5 min. Transferred clear lysate to a new tube. Added RNaseA 200 µg/ml and incubated 37 ^{o}C 30 min. Added ProteinaseK 50 µg/ml and incubated 37 ^{o}C 30 min. Added equal volume of Phenol: chloroform: isoamyl alcohol (25:24:1) inverted gently. Centrifuged at 12,000 xg 4 min. Transfered upper aqueous phase to a new tube. Added 2 volume of absolute ethanol and incubated at -20 ^{o}C 1 hr. Centrifuged at 12,000 xg 10 min. Washed DNA pellet with 500 µl of 70% ethanol. Centrifuged at 12,000 xg 5 min and let DNA dried at room temperature for 5 min and added 30 µl of TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA). Genomic DNA was then applied to 1% agarose gel in 0.5X TAE buffer to check for quality and the size of the DNA compared to a known DNA amount of DNA ladder. Spectrophotmetry measuring of DNA were employed. The OD 260 for DNA of 1.0 corresponds to concentration of 50 µg/ml. The concentration of DNA samples was estimated in µg/ml by employing the following formula: [DNA] = OD260 x dilution factor x 50.

Purity of DNA samples were evaluated from OD260/280 ratio. The ratio of the purified DNA was approximately 1.8 to 2.0 respectively (Sambrook et al., 1989).

3.5.2.2 Preparation of genomic DNA from outgroup bateria

Preparation of genomic DNA from *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas putida* performed according to Ausubel et al. (2002). First, a single colony of each bacteria was grown in 5 ml LB broth overnight. Then, transfered cultured cells to 1.5 ml microcentrifuge tube and centrifuged at 12,000 xg for 1 min. Discarded the supernatant and resuspended cells in 567 µl TE buffer. Added 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K, mixed well, and incubated 1 hr at 37 ^{o}C. Added 100 µl of 5 M NaCl and mixed thoroughly and added 80 µl CTAB/NaCl solution, mixed thoroughly, and incubated 10 min at 65 ^{o}C. Then, added an equal volume of chloroform: isoamyl alcohol (24:1) inverted gently. Then, centrifuged at 12,000 xg 4 min. Transfer upper aqueous phase to a new tube. Added 0.6 volume of isopropanol. Centrifuge at 12,000 xg 10 min. Wash DNA pellet with 500 µl of 70% ethanol. Centrifuge at 12,000 xg 5 min and let DNA stand for 5 min and added 30 µl of TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA) pH 8.0. Genomic DNA was then applied to 1% agarose gel in 1X TAE buffer to checked for quantity and the size of the DNA compared to a known DNA amount of DNA ladder.

3.6 PCR amplification of 16S rDNA

3.6.1 Primers

PCR primers used in this study were synthesized from BSU and BioDesign Co., Ltd. as listed in Table 8 which specifically designed for specific amplification of eubateria and *streptomyces*. Figure 11 illustrated amplification region of 16S rDNA.

Table 8 PCR primers

Primer	Sequence 5' – 3'	Length	Position	Specificity	Ref.
fD1	GAATTCAGAGTTTGA TCCTGGCTCAG	26	8-27 *	most eubacteria	Weisburg, 1991
rP2	GGATTCACGGCTACCTTGTTACGACTT	27	1492-1513*	most eubacteria	Weisburg, 1991
StrepB	ACAAGCCCAGGAGGCGGGGT	20	139-158 **	*Streptomyces*	Rintala, 2001
StrepF	ACGTGTGCAGCCCAAGCACA	19	1194-1212 **	*Streptomyces*	Rintala, 2001

* 16S rDNA position from *E. coli* and ** 16S rDNA position from *S. ambofaciens*

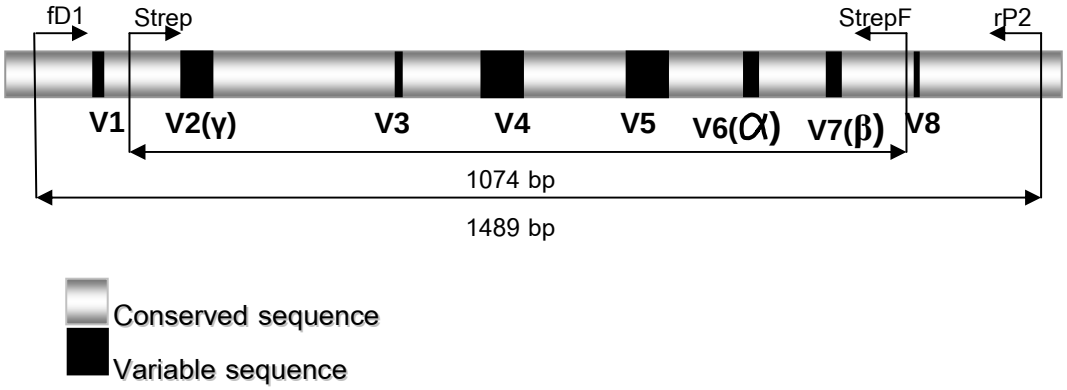


Figure 11 Map of 16S rDNA sequence of *Streptomyces ambofaciens* (Rintala et al,, 2001)

3.6.2 PCR amplification

Nearly full length of 16S rRNA gene fragments were amplified from pure culture and soil DNA extract by first round PCR performed with a MYcycler Thermal cycler (Bio-rad). For the bacterial specific amplification of 16S rDNA fragments of streptomycetes and non-streptomycetes, the reaction mixture was as follows: 1 µl of template DNA (5-100 ng), 0.5 U Taq DNA polymerase (NEB, USA),1X PCR buffer supply with 2.5 mM $MgCl_2$, 200 µM deoxynucleoside triphophates, 10 µM fD1 (5'-GAATTCAGAGTTTGATCCTGGCTCAG -3) and rP2 (5'-GGATTCACGGCTACCTTGTT ACGACTT-3') from Weisburg et al. (1991). Primer fD1 binds to base positions 8-27 and primer rP2 to base positions 1492-1513 of *E. coli* 16S rRNA gene and rP2 primers, and 5% (vol/vol) dimethyl sulfoxide was added to the reaction mixture when used of *Steptomyces*. DNA as a template to facilitate the denaturation of double-stranded DNA and to circumvent the formation of secondary structures. For amplification of soil DNA, 400 ng of bovine serum albumin per µl was used to prevent inhibition (Kreader, 1996). Twenty five microliters of the mixture was added to a 0.2 ml reaction tube. Amplification was performed by pre-denaturation at 95 °C for 2.10 min. Followed by 30 cycles of 1 min denaturation at 94 °C , 30 sec at 55 °C for primer annealing, and extension at 72 °C for 2 min. Followed by 72 °C 10 min for final extension and hold at 4°C. The PCR was set up listed in Table 9 and Figure 12 a. PCR products were only accepted for further analysis when a simultaneous negative control PCR (water instead of DNA) showed no amplification. The PCR products were electrophoresed on 1% agarose gels in TAE buffer and stained with ethidium bromide (1 mg/ml). The DNA bands were visualized by UV transillumination and photographed using Genesnap (Syngene) to ensure that a fragment of the correct size had been amplified. First round PCR products from soil sample were then used as template in a nested PCR using primer StrepB (5' – ACAAGCCCAGGAGGCGGGGT) + StrepF (5' – ACGTGTGCAGCCCAAGCACA) (Rintala et al., 2001). This *Streptomyces*-specific primer hybridized at positions internal to those targeted of *Streptomyces ambofaciens* ATCC 23877T (rrnD operon; GenBank accession no. M27245) and generated fragments approximately 1,074 bp in length. The reaction mixture was as follows: 1 µl of template DNA (1:10 dilution of first round PCR

product or DNA from pure culture), 0.5 U Taq DNA polymerase (NEB, USA),1x PCR buffer supply with 2.5 mM $MgCl_2$, 200 uM deoxynucleoside triphophates, and 10 uM StrepB and StrepF. Amplification was performed by pre-denaturation at 95 °C for 5 min. Followed by 30 cycles of 45 sec denaturation at 94 °C, 64 °C for 40 sec or primer annealing, and extension at 72 °C for 2 min. Followed by 72 °C 10 min for final extension and hold at 4°C (Table 9 and Figure 12 b). Two microliters of each PCR products were then applied to 1% agarose gel electrophoresis in 0.5X TAE buffer to check for exsistance of the amplification products and correction of the fragment.

Table 9 PCR reaction set up

Components	Volume	Final concentration
10X PCR buffer (supply with Mg)	2.5 µl	1X
10 mM dNTP mixture	0.5 µl	200 µM
Forward primer (10 µM)	0.5 µl	0.2 µM
Reverse primer (10 µM)	0.5 µl	0.2 µM
DMSO*	1.25 µl	5 %
BSA** (10mg/ml)	0.4 µl	400 ng
DNA***	1 µl	5-100 ng
Taq (NEB) (1 U)	0.5 µl	0.5 U
Sterile distilled water	To 25 µl	

* DMSO was used if DNA template was from *Streptomyces* Genomic DNA to facilitate the denaturation of double-stranded DNA and to circumvent the formation of secondary structures

** BSA was used if DNA template was from soil DNA to prevent inhibition

*** DNA template could be from total genomic DNA extraction of bacterial culture, boiled colony of *Streptomyces* on agar plate, or purified soil DNA 5-100 ng

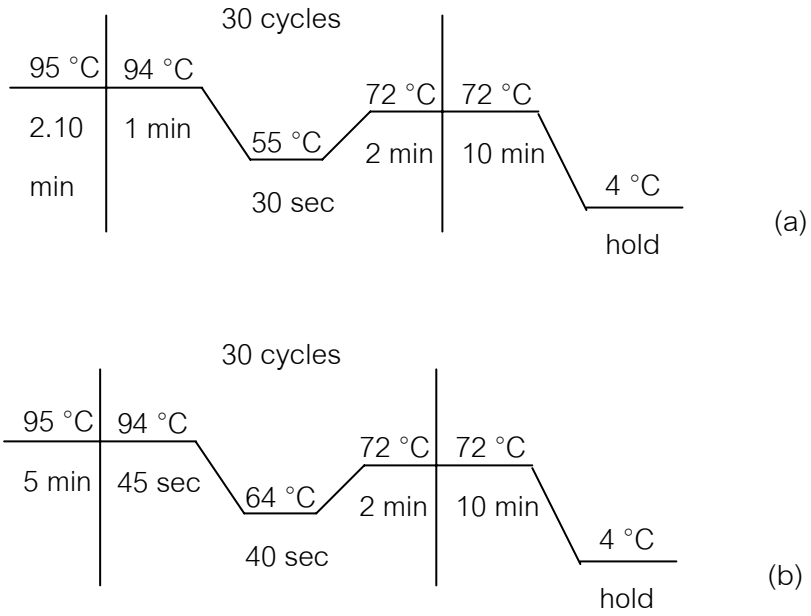


Figure 12 Thermal cycling condition for amplification of eubacterial 16S rDNA approximate size 1.5 kb (a) and amplification of *Streptomyces* 16S rDNA approximate size 1 kb (b)

3.7 Cloning of 16S rDNA library

3.7.1 Purification of PCR product from agarose gel

Single or pooled PCR products (1 kb) from soil samples were purified from 1% agarose gels with the QIAquick gel extraction kit (Qiagen). According to manufacturer's protocol, a preparative agarose gel electrophoresis using 1% agarose gel in 0.5 X TAE buffer and electrophored at 100 Volt for 25 min. The PCR fragment were excised from the gel using sterile razor blade and added equal volume of QG buffer of a gel (i.e. 100 mg gel equal 100 µl QG) and incubated at 50 °C for 10 min with occational inverting until the gel was completely melt. Then the melt solution was loaded into a column and centrifuged at max. speed form 1 min. Discarded the flow through and added 750 µl of PE buffer to a spin column and centrifuge for 1 min. Discarded flow though and assembled the collection tube back to the spin column and centrifuged again for 1 min to get rid of remaining PE solution. Then, added 30 µl of pre-warmed (50 °C) steriled or EB buffer to a spin column and let it stood for 5 min and centrifuged for 1 min at max. speed. The purified PCR product was then applied to gel electrophoresis to checked for amount of DNA by compared with the known DNA amount of DNA maker.

3.7.2 Ligation of PCR product to a vector

The purified PCR products were ligased into the T/A cloning vector (RBC, Taiwan). The ligation reaction mixture contained 56 ng (1-3 µl) of insert and 50 ng of vector (3:1 molar ratio) using T4 DNA ligase in the final reaction volume 10 µl, incubated at 4 ^{o}C overnight (18 hr).

3.7.3 Host cell preparation

A colony of *E. coli* XL1blue was cultured as the starter in 5 ml LB broth at 37 °C overnight. 1% of cultured was added to 50 ml LB broth and shacked at 250 rmp, 37 °C for 3 hrs (OD600~0.3-0.4). The cells were harvested by centrifuged at 4,000 xg, 4°C for 10 min using refrigerated centrifuge Allergra 25R (Beckman Coulter, USA). Discard the supernatant and resuspended cell pellet with 25 ml cold 100 mM $CaCl_2$ solution. Mixed gently by pipetting up and down. After chilled on ice for 30 min, centrifuged at 4,000 xg, 4 °C for 10 min. Resuspended cells in 2 ml 100 mM $CaCl_2$ containing 15% glycerol. Aliquot (100 µl) into 1.5 ml centrifuge tubes and stored at -80 °C until used.

3.7.4 Transformation of ligation product to E.coli host cells

Prior to transformation the water bath was set at 42°C. 9 µl of ligation mixture was added to a 100 µl aliquot competent cell and mixed well. Then, chilled on ice for 25 min and heat shocked at 42 °C water bath for 45 sec. Added 900 µl of LB broth and incubated at 37 °C for 1 hr with shacking. Then the cultured cell suspension was centrifuged at 10,000 xg for 15 sec. The cell pellet was resuspended in 300 µl of LB and spreaded on LB agar plates supplement with 50 µg/ml ampicillin, 20 µl 50 mg/ml X-Gal (5- bromo-4-chloro-3-indoly-b-D-galactopyranoside), and 100 µl 100 mM IPTG (isopropyl-b-D-thiogalactopyranoside). After incubation at 37 °C for 16 hr, the positive recombinants were screened on alpha-complementation of (blue-white screening). The white colonies were positive clones which contain insert DNA whereas the blue colonies contained no insert DNA. 100-200 colonies were picked for single or pooled soil samples.

3.8 Restriction endonuclease digestions and analysis

3.8.1 DNA preparation

Positive clones were amplified by PCR amplification with primer pairs StrepB + StrepF. The PCR reaction contained 0.5 U Taq DNA polymerase (NEB, USA),1x PCR buffer supply with 2.5 mM $MgCl_2$, 200 uM deoxynucleoside triphophates, and 10 µM StrepB and StrepF. The template DNA was prepared from single colony by heated at 95 °C in 100 µl dH_2O for 5 min and centrifuged (12,000 xg for 2 min) and 5 µl were take as a template for PCR reaction. Amplification was performed by pre-denaturation at 95 °C for 5 min. Followed by 30 cycles of 45 sec denaturation at 94 °C, 64 °C for 40 sec or primer annealing, and extension at 72 °C for 2 min. Followed by 72 °C 10 min for final extension and hold at 4°C. The PCR products were elctrophoretically analysed by 1% agarose gel.

3.8.2 Digestion of PCR amplicon

No pre-treatment of PCR amplified DNA from positive colony was required for enzymatic digestion. 10 µl of PCR products were digested with restriction enzyme was set up as follows (Table 10) and incubated at 37 °C for 3–4 hr.

Table 10 Restriction digestion reaction

Component	Volume
PCR product	10 µl (1 µg)
Restriction enzyme (10-20 U/ µl)	1 µl
10X NEB buffer	2 µl
Sterile distilled water	To 20 µl

Samples were electrophoresed on 3% agarose gel (20 x 20 cm) in 1X TBE buffer at 80 Volts for 2.30 hr. The gels were stained for 10 min in distilled water with ethidium bromide (1 mg/ml). Destaining was done for 40 min in distilled water. A photograph of the gel was stored on hard disk as a TIFF file through a coupled camera using Genesnap software. The restriction fragment patterns were compared manually with those from the *in silico* restriction endonuclease digestions. All bands within a profile were assigned except for those with a size of less than 45 bp found within the profile which is the detection limit of agarose gel electrophoresis. Clones with similar banding patterns were grouped together. The summarization of methods showed in Figure 13.

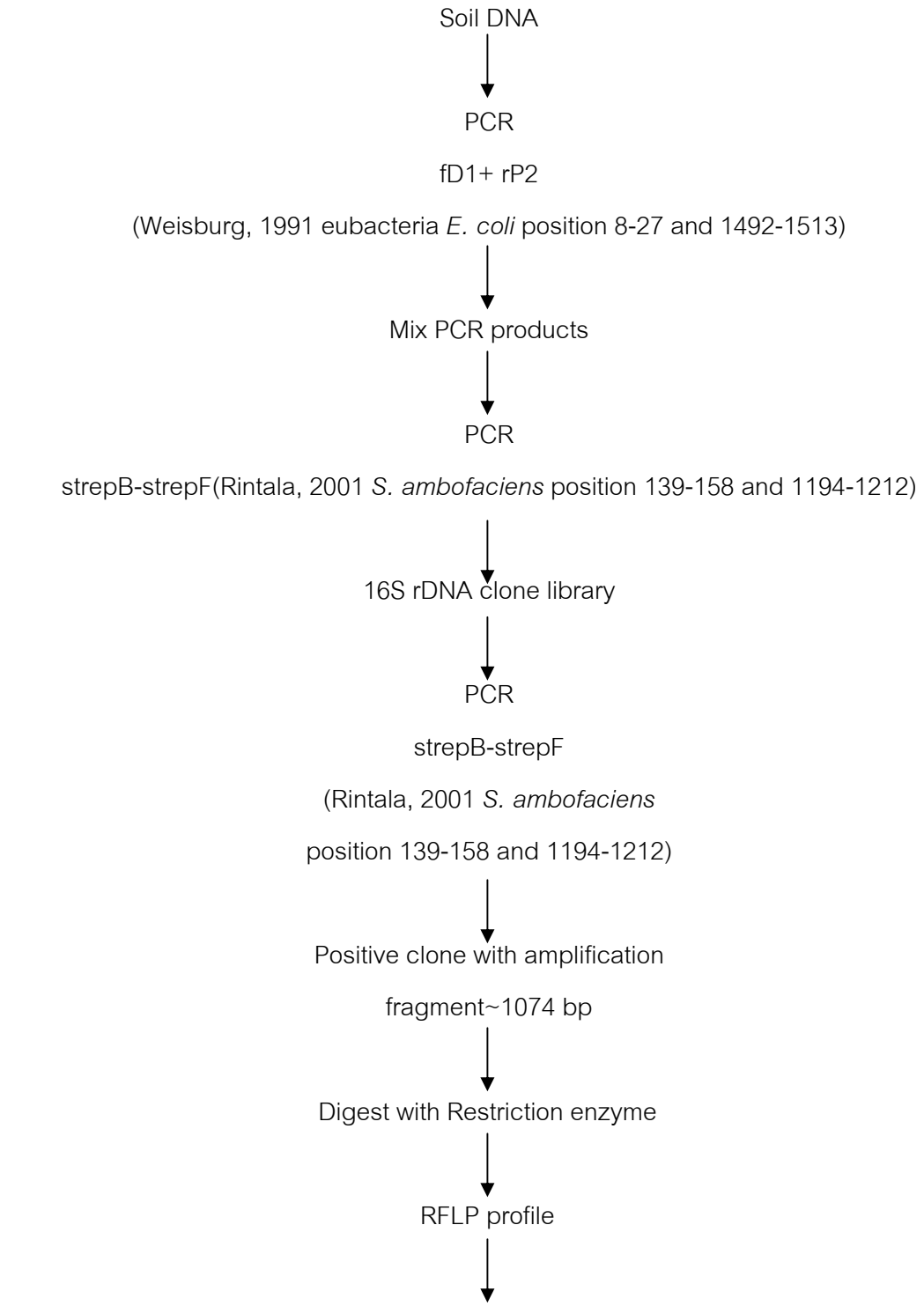


Figure 13 Flow diagram of protocols to analyze bioactive producing *Streptomyces* from soil

CHAPTER IV

RESULTS

4.1 Frequency of bioactive *Streptomyces* and *Actinomyces*

Frequency of bioactive *Streptomyces* and *Actinomyces* were listed in Table 11. Among seventy six *Streptomyces* and *Actinomyces*, thirty five have antibacterial activity, fourteen have antitumor, twelve have growth promotant activity, eleven have antifungal activity, five have antipasitic activity, two have antiviral, two have immunosuppressant activity, and two herbicidal. In particular, many *Streptomyces* reported on antibacterial activity, whereas antiviral activity, immunosuppressant activity, and herbisidal activity were only two from seventy six. Some *Sterpomyces* such as *S. hygroscopicus* was reported on many biological activity including antiparasitic, immunosuppressant, and herbisidal activity, and *S. griseus* on antifungal and antitumor activity.

Table 11 Frequency of bioactive *Stretpomyces* and *Actinomyces*

Code	Name of Bacteria	Bioactivity *							
		AB	AF	AP	AV	AT	IM	GP	HB
S01	*S. acromogens* subs. *rubradirus*	-	-	-	-	+	-	-	-
S02	*S. albofaciens*	+	-	-	-	-	-	-	-
S03	*S. alboniger*	+	-	-	-	-	-	-	-
S04	*S. albolongus*	+	-	-	-	-	-	-	-
S05	*S. albovinaceus*	+	-	-	-	-	-	-	-
S06	*S. almquistii*	+	-	-	-	-	-	-	-
S07	*S. ambofacines*	+	-	-	-	-	-	-	-
S08	*S. antibioticus*	+	-	-	+	+	-	-	-
S09	*S. aureocirculatus*	+	-	-	-	-	-	-	-
S10	*S. aureofaciens*	+	-	-	-	-	-	+	-
S11	*S. bolili*	+	-	-	-	-	-	-	-
S12	*S. cattleya*	+	-	-	-	-	-	-	-
S13	*S. clavuligerus*	+	-	-	-	-	-	-	-
S14	*S. ederensis*	+	-	-	-	-	-	-	-
S15	*S. fridiae*	+	-	-	-	-	-	+	-
S16	*S. fulvoviolaceus*	+	-	-	+	-	-	-	-
S17	*S. gibsonii*	+	-	-	-	-	-	-	-
S18	*S. graminofaciens*	+	-	-	-	-	-	-	-
S19	*S. griseus*	-	+	-	-	+	-	-	-
S20	*S. kanamyceticus*	+	-	-	-	-	-	-	-
S21	*S. lincolnensis*	+	-	-	-	-	-	+	-
S22	*S. narbonensis*	+	-	-	-	-	-	-	-
S23	*S. ochraceiscleroticus*	+	-	-	-	-	-	-	-
S24	*S. rumosus* subs. *paromomycinus*	+	+	+	-	-	-	-	-
S25	*S. rimosus*	+	-	-	-	-	-	+	-
S26	*S. spectabilis*	+	-	-	-	-	-	-	-
S27	*S. spiroverticillatus*	+	+	-	-	-	-	-	-
S28	*S. venezuelae*	+	-	-	-	-	-	-	-
S29	*S. xantholiticus*	+	+	-	-	-	-	-	-
S30	*S. tenebrarius*	+	-	-	-	-	-	-	-
S31	*S. albidoflavus*	-	+	-	-	-	-	-	-
S32	*S. cacaoi* subs. *asoensis*	-	+	-	-	-	-	-	-
S33	*S. chrestomyceticus*	-	+	-	-	-	-	-	-
S34	*S. nodosus*	-	+	-	-	-	-	-	-
S35	*S. noursei*	+	-	-	-	-	-	-	-
S36	*S. tendae*	-	+	-	-	-	-	-	-
S37	*S. varsoviensis*	-	+	-	-	-	-	-	-
S38	*S. natalensis*	-	+	-	-	-	-	-	-
S39	*S. albus*	-	-	+	-	-	-	+	-

Table 11 (continued).

Code	Name of Bacteria	Bioactivity *							
		AB	AF	AP	AV	AT	IM	GP	HB
S40	*S. avermitilis*	-	-	+	-	-	-	-	-
S41	*S. cinnamonensis*	-	-	+	-	-	-	+	-
S42	*S. albus subs. pathodicus*	-	-	-	-	+	-	-	-
S43	*S. albosporeus subs. labilomyceticus*	-	-	-	-	+	-	-	-
S44	*S. argillaceus*	-	-	-	-	+	-	-	-
S45	*S. caespetosus*	-	-	-	-	+	-	-	-
S46	*S. coeruleorubidus*	-	-	-	-	+	-	-	-
S47	*S. galilaceus*	-	-	-	-	+	-	-	-
S48	*S. peucetius*	-	-	-	-	+	-	-	-
S49	*S. peucetius subs. caesius*	-	-	-	-	+	-	-	-
S50	*S. verticillus*	-	-	-	-	+	-	-	-
S51	*S. parvulus*	-	-	-	-	+	-	-	-
S52	*S. azureus*	-	-	-	-	-	-	+	-
S53	*S. bambergiensis*	-	-	-	-	-	-	+	-
S54	*S. flocculus*	-	-	-	-	-	-	+	-
S55	*S. lavendulae*	-	-	-	-	-	-	+	-
S56	*S. virginiae*	-	-	-	-	-	-	+	-
S57	*S. lactamdurans*	-	-	-	-	-	-	+	-
S58	*S. hygroscopicus*	-	-	+	-	-	+	-	+
S59	*S. tsukabaensis*	-	-	-	-	-	+	-	-
S60	*S. viridochromogens*	-	-	-	-	-	-	-	+
S61	*S. sacbiei*	-	-	-	-	-	-	-	-
S62	*S. acidiscabie*	-	-	-	-	-	-	-	-
S63	*S. ipomoeae*	-	-	-	-	-	-	-	-
S64	*S. turgidiscabies*	-	-	-	-	-	-	-	-
S65	*S. coelicolor*	+	-	-	-	-	-	-	-
S66	*S. lividans*	+	-	-	-	-	-	-	-
S67	*S. halstedii*	-	-	-	-	+	-	-	-
S68	*S. baanensis*	-	-	-	-	-	-	-	-
S69	*S. clavifer*	-	-	-	-	-	-	-	-
S70	*S. indigoferus*	-	-	-	-	-	-	-	-
A01	*Amycolatopsis mediterranei*	+	-	-	-	-	-	-	-
A02	*Amycolatopsis orientalis* subsp. *orientalis*	+	-	-	-	-	-	-	-
A03	*Saccharopolyspora erytraea*	+	-	-	-	-	-	-	-
A04	*Micromonospora olivasterospora*	+	-	-	-	-	-	-	-
A05	*Sporichthya polymorpha*	-	-	-	-	-	-	-	-
A06	*Thermomonospora chromogena*	-	-	-	-	-	-	-	-
		35	11	5	2	14	2	12	2

* Bioactivities: antibacterial (AB), antifungal (AF), antiparasitic (AP), antiviral (AV), antitumor (AT), immunosuppressant (IM), growth promotant (GP), and herbicidal (HB)

4.2 Phylogenetic tree reconstruction from sequence data

The results of the phylogenetic tree of *Streptomyces*, *Actinomyces*, and outgroup bacteria based on the 16S rRNA gene sequences (StrepBF region) and the bioactivities of the microorganisms are shown in Figure 14. The microorganisms that had antibacterial were relatively consistent with the microorganisms had antifungal activity. A Phylogenetic tree was reconstructed using PAUP* program, neighbor-joining distance method Kimura's 2-parameters. The bootstrap values > 50% were shown in Figure 15 indicated the reliable of the tree from DNA sequence.

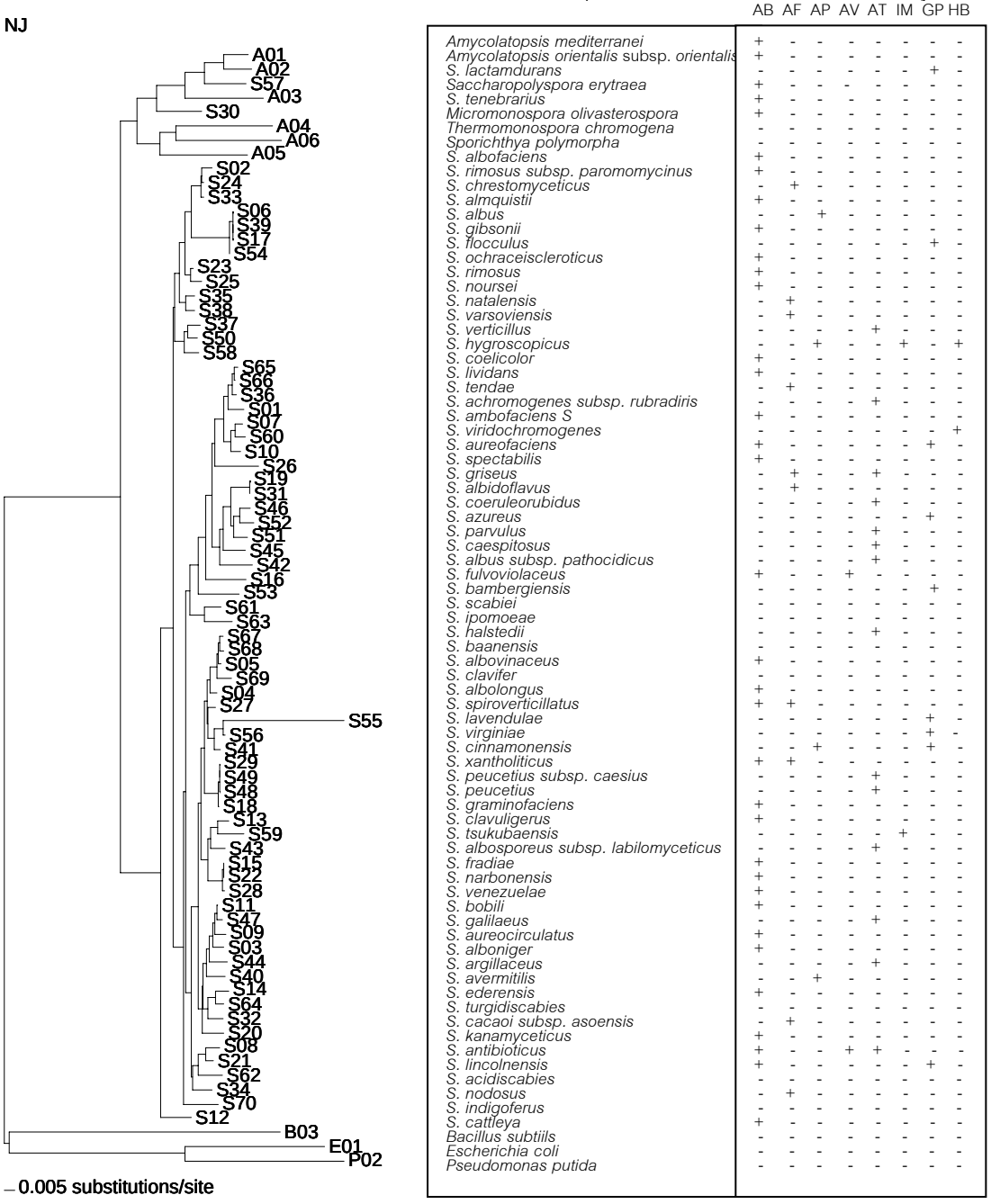


Figure 14 Phylogenetic tree of *Streptomyces*, *Actinomyces*, and outgroup bateria based on 16S rDNA sequences (StrepBF region ~ 1 kb) and bioactivity of the microorganisms. Bioactivities: antibacterial (AB), antifungal (AF), antiparasitic (AP), antiviral (AV), antitumor (AT), immunosuppressant (IM), growth promotant (GP), and herbicidal (HB)

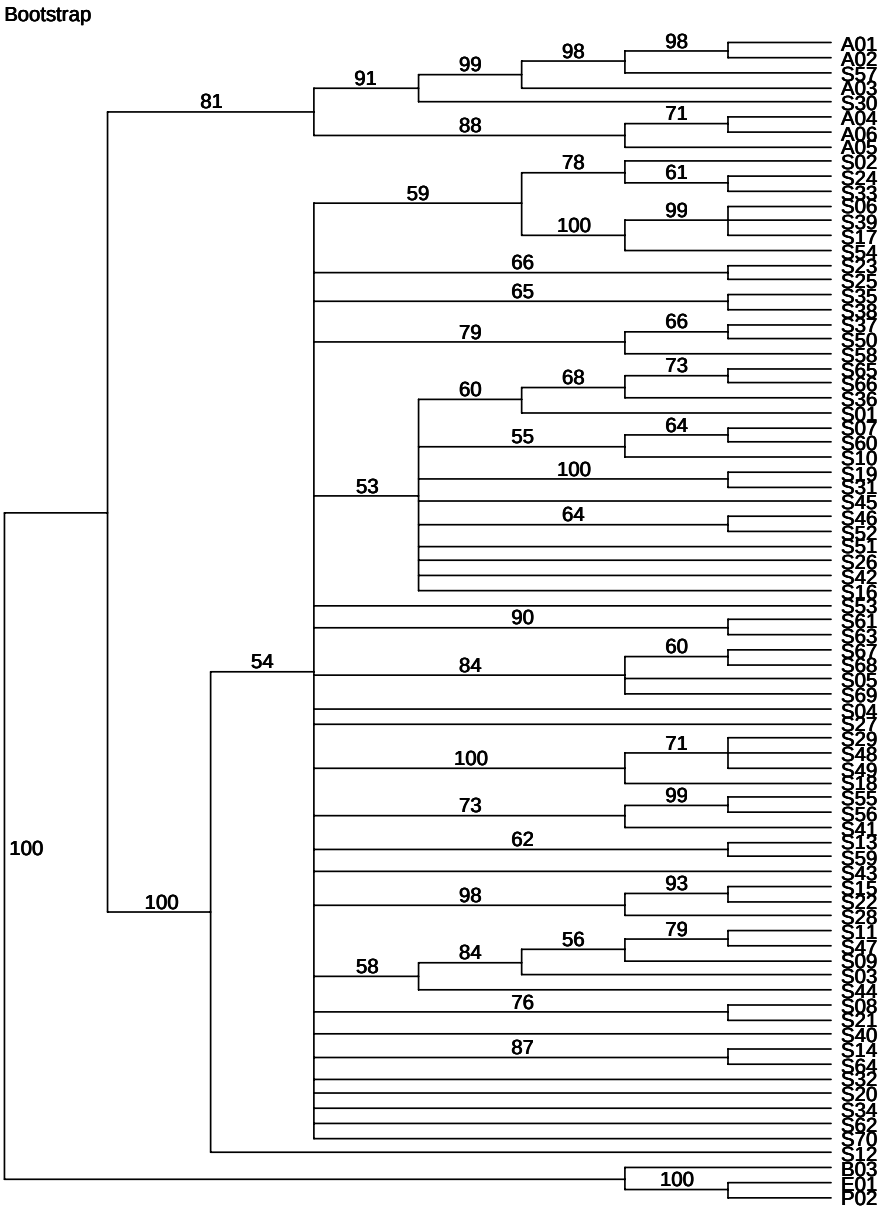


Figure 15 Bootstrap tree with a 100 replication of data set of *Streptomyces*, *Actinomyces*, and outgroup bateria based on 16S rDNA sequence (StrepBF region ~ 1 kb). The bootstrap values more than 50% were shown.

4.3 *In silico* endonuclease digestions and restriction fragment length polymorphism (RFLP) analysis

Thirty three restriction enzymes were used for *in silico* restriction enzyme digestion. Thirteen were 4 –bp cutters (tetrameric restriction enzymes) and twenty were 6-bp cutters (hexameric restriction enzymes). Restriction enzymes were retrieved from REBASE (Restriction Enzyme Database) at http://rebase.neb.com as listed in Table 3. Resulting RFLP Patterns of 79 16S rDNA sequences from *Streptomyces*, *Actinomyces*, and outgroup bacteria digested *in silico* with each restriction enzyme using NEBcutter Version 2.0 (New England Biolab) were group according to pattern similarity, numerical distribution of microorganisms were also shown in Figure 16. The similarity of patterns among *Streptomyces* species were grouped as listed in Table 12 to 44.

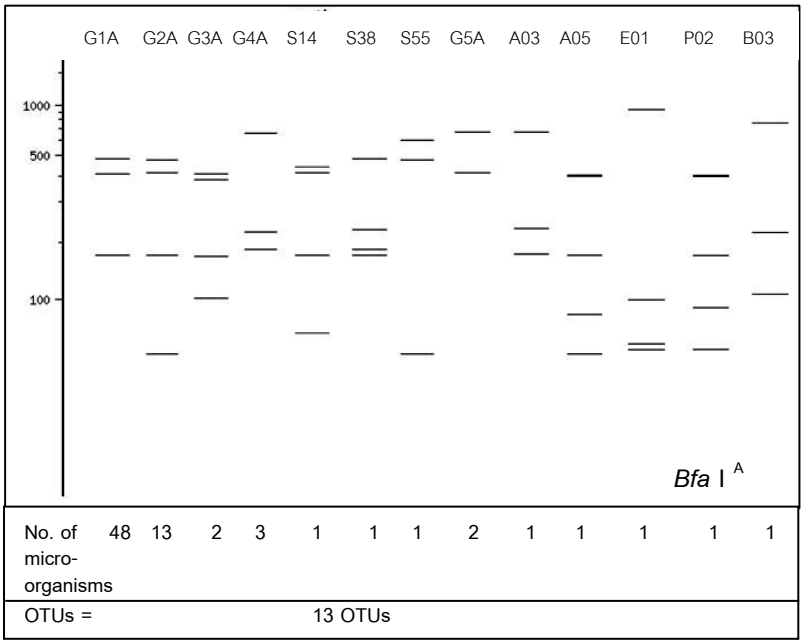
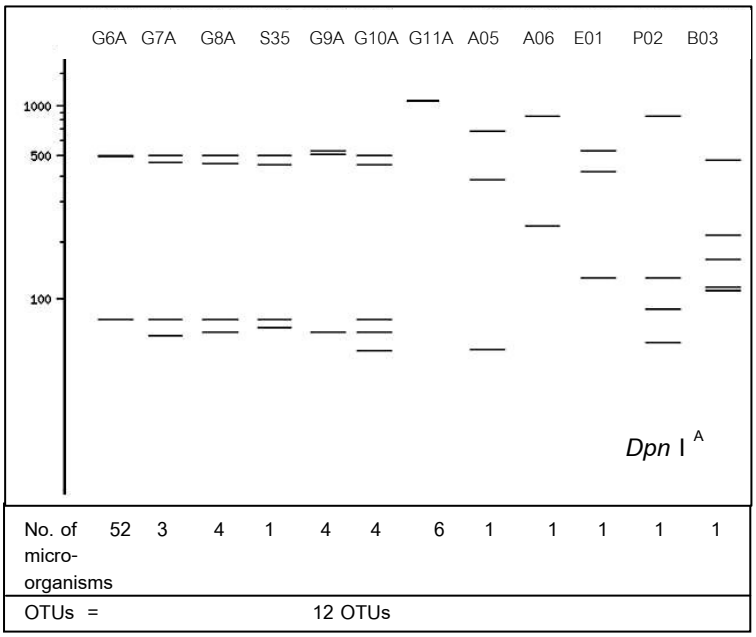


Figure 16 2% agarose gel from computer-simulated RFLP pattern from *in silico* digestion of 16S rDNA StrepBF region with single restriction enzyme. Thirty three enzymes were used; *Bfa*I, *Dpn*I, *Bst*UI, *Hha*I, *Mse*I, *Nla*III, *Rsa*I, *Taq*I, *Alu*I, *Hae*III, *Mnl*I, *Hpa*II, and *Aci*I. The unique RFLP pattern was indicated by code of each microorganism as listed in the table at the top of the figure. The same RFLP pattern obtained from the same restriction enzyme digestion of different microorganisms were indicated as G listed in Table 12-44. The scale bar represented the 100 bp marker. The table at the bottom of the figure showed the numerical distribution of the microorganisms containing one or more than one microorganism and number of OTUs.

[A] tetrameric restriction enzyme

[B] hexameric restriction enzyme



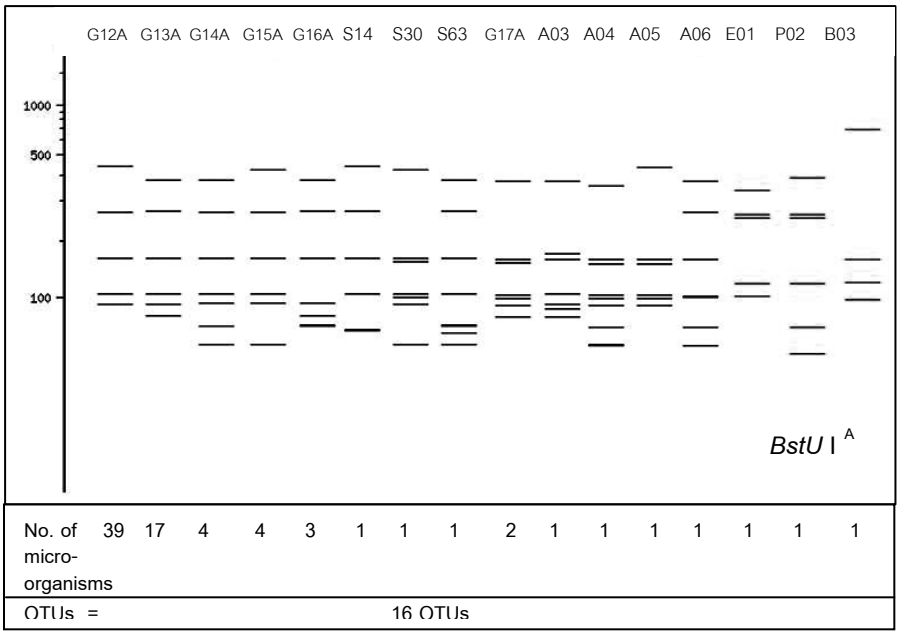
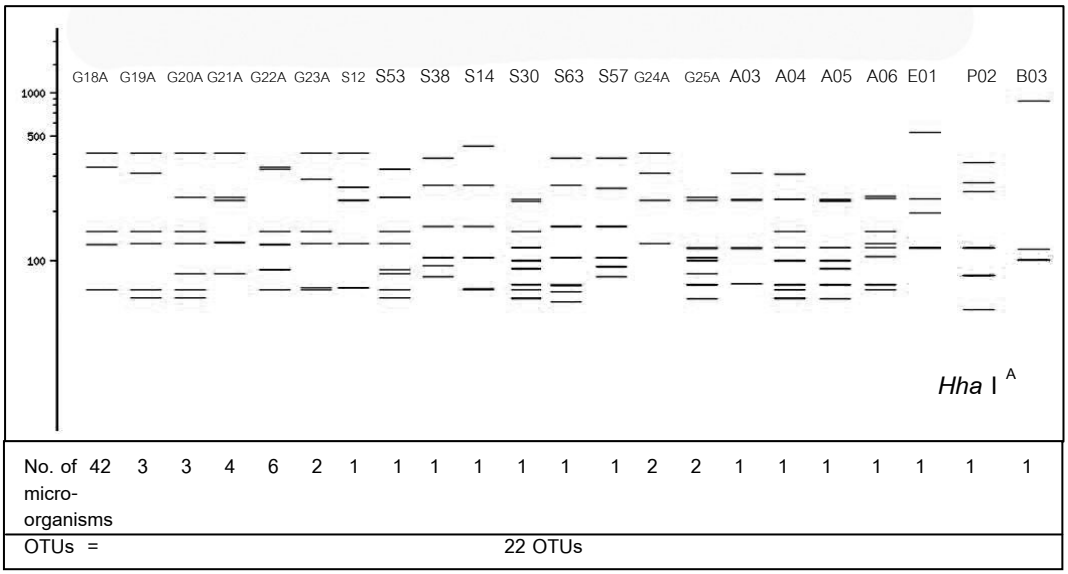
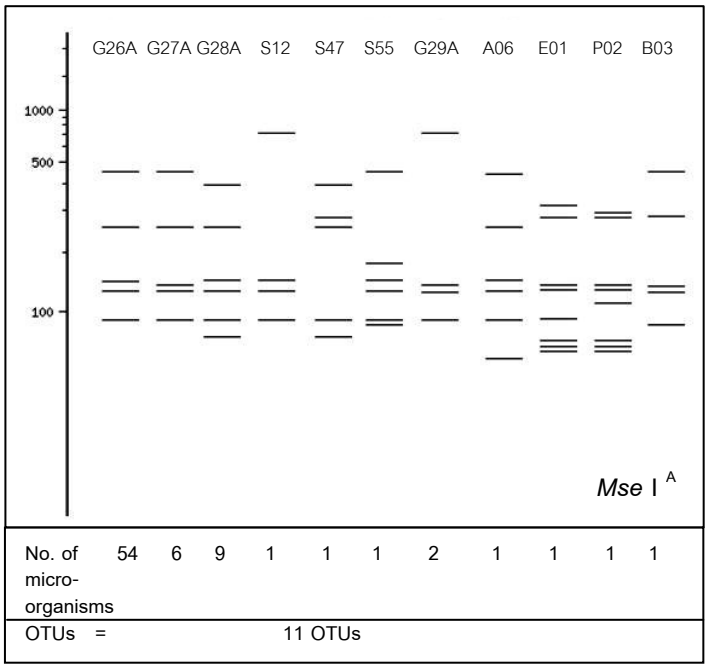


Figure 16 (continued).





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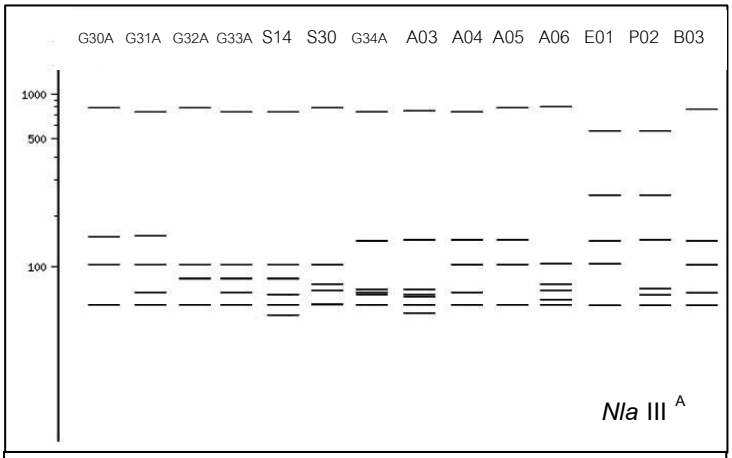
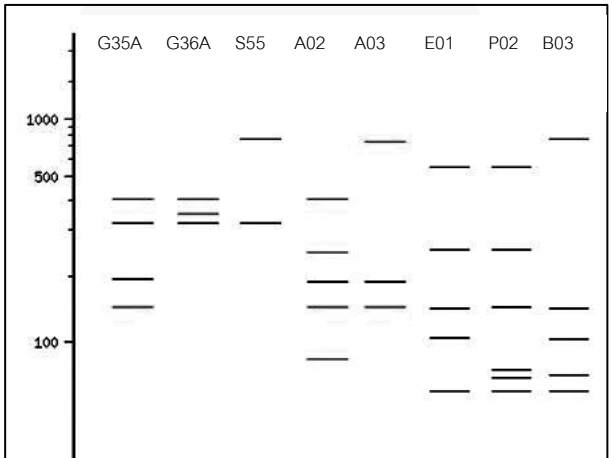
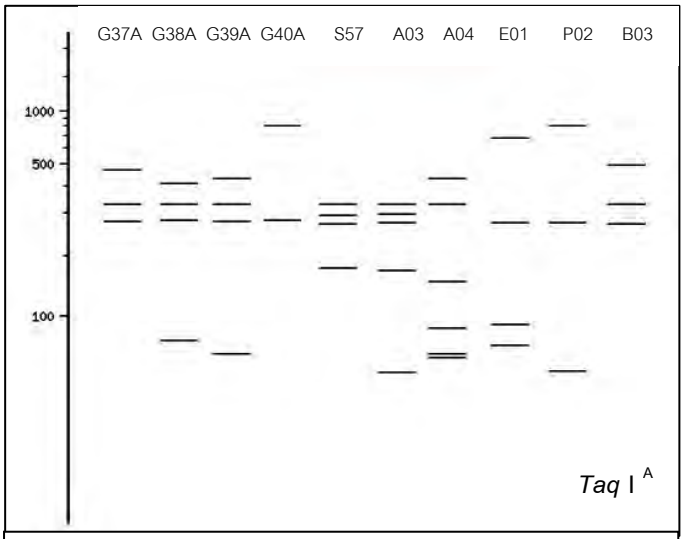


No. of micro-organisms	14	12	8	33	1	1	3	1	1	1	1	1	1	1
OTUs =	14 OTUs													

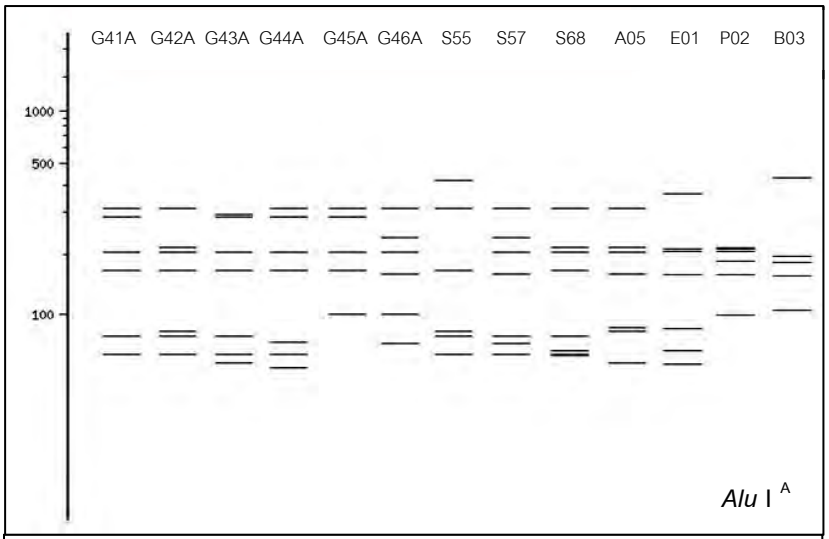


No. of micro-organisms	52	21	1	1	1	1	1	1
OTUs =	8 OTUs							

Figure 16 (continued).

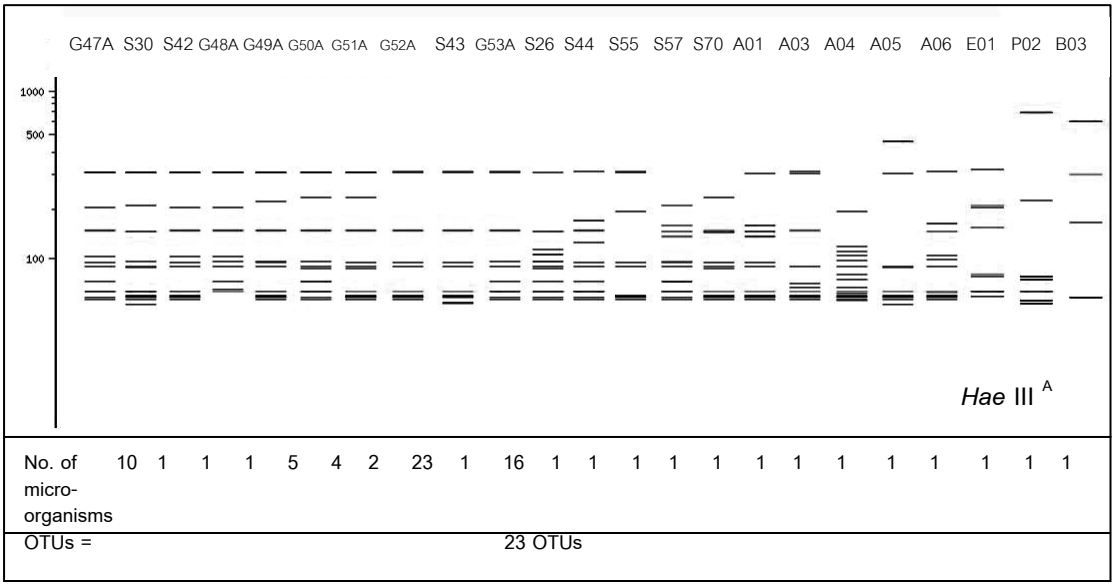


No. of micro-organisms	51	13	2	7	1	1	1	1	1	1
OTUs =	10 OTUs									



No. of micro-organisms	46	13	2	2	1	1	1	1	1	1	1	1	1
OTUs =	12 OTUs												

Figure 16 (continued).



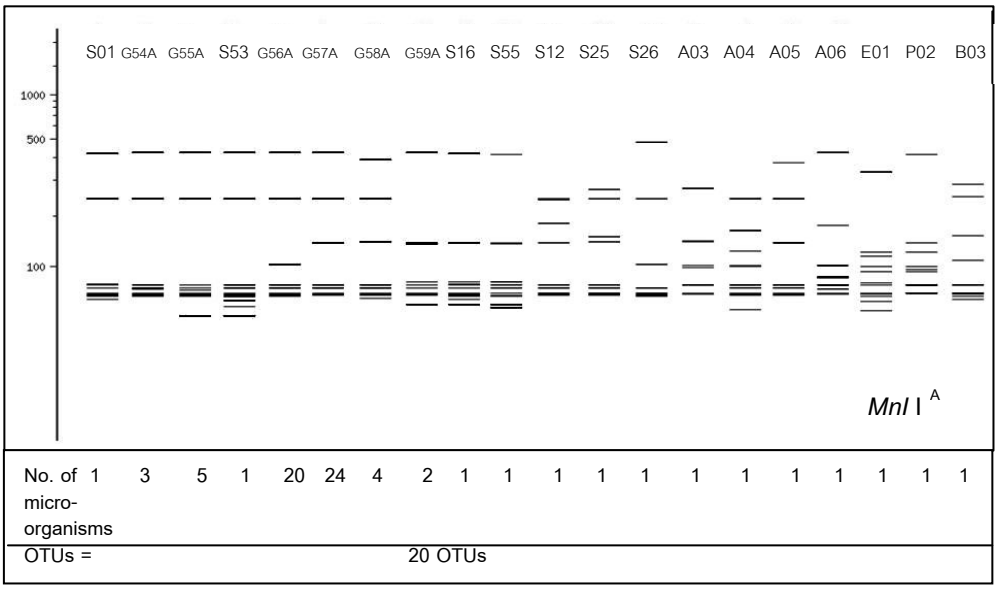
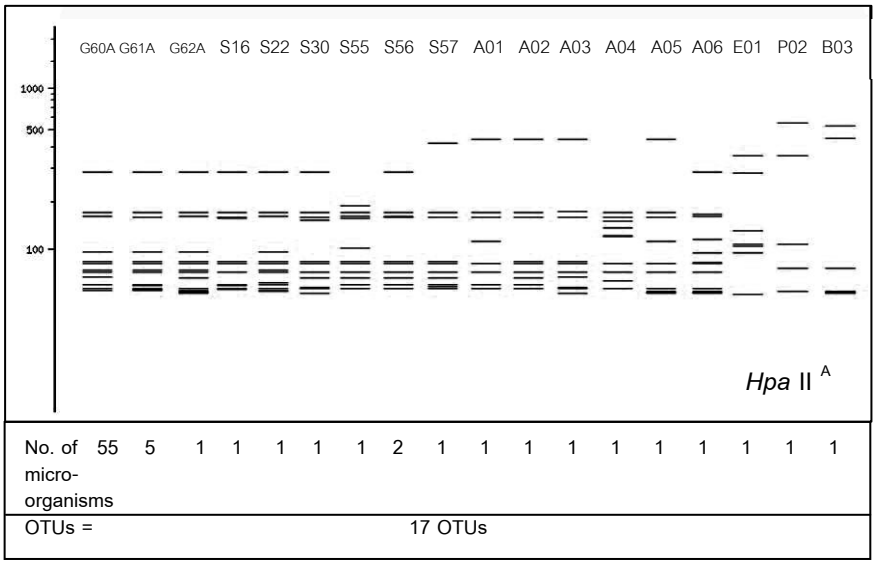


Figure 16 (continued).



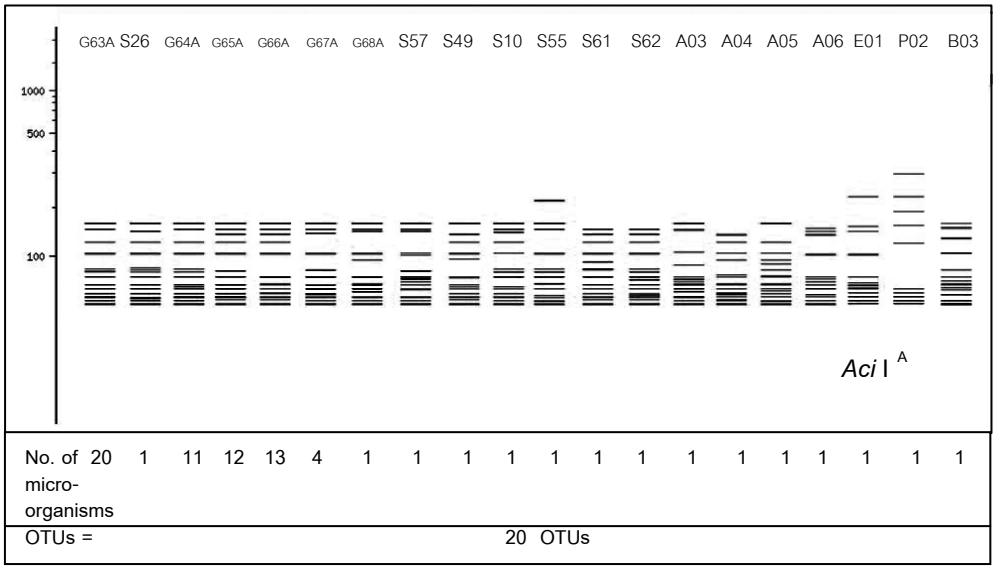


Figure 16 (continued).

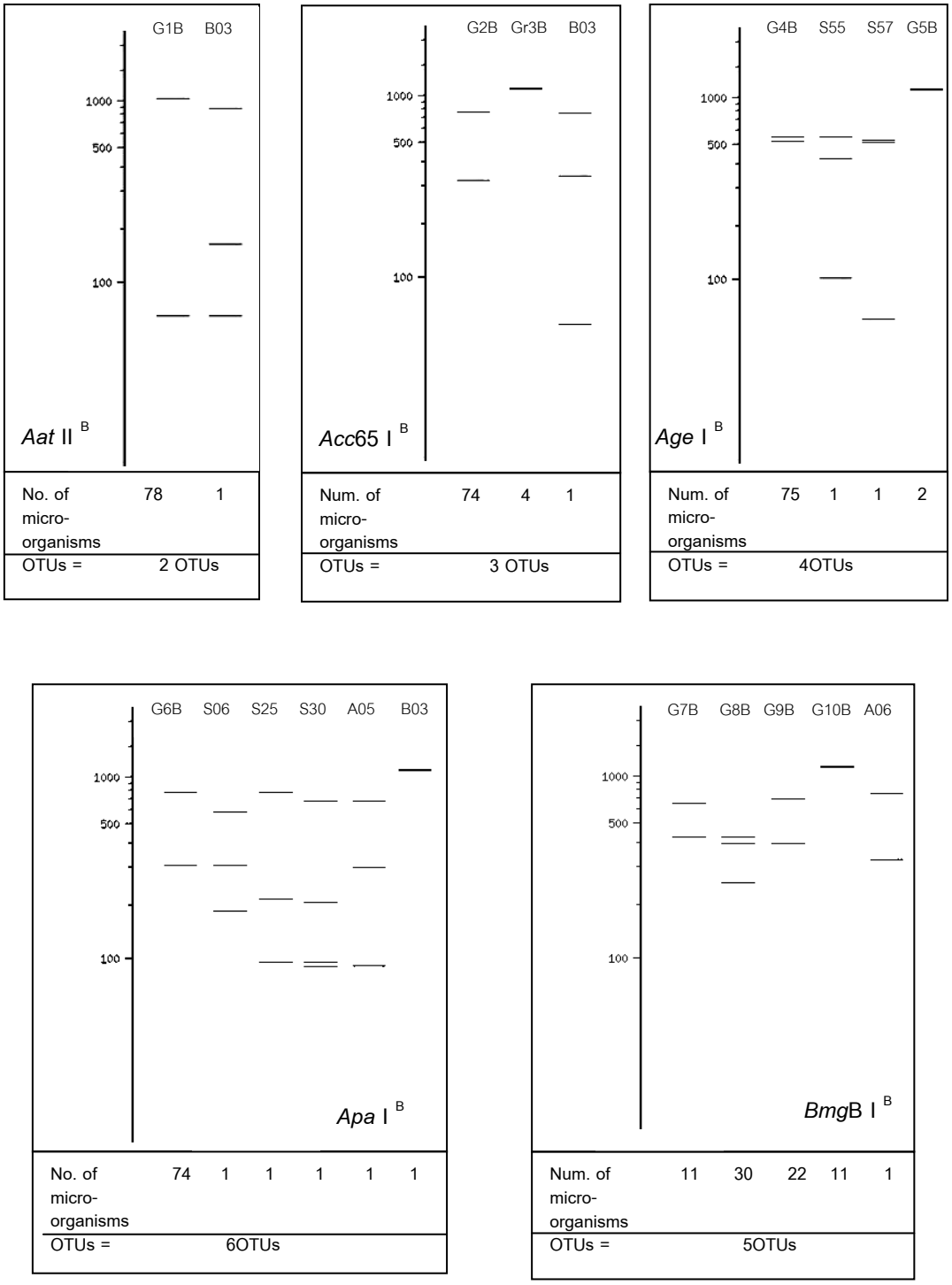
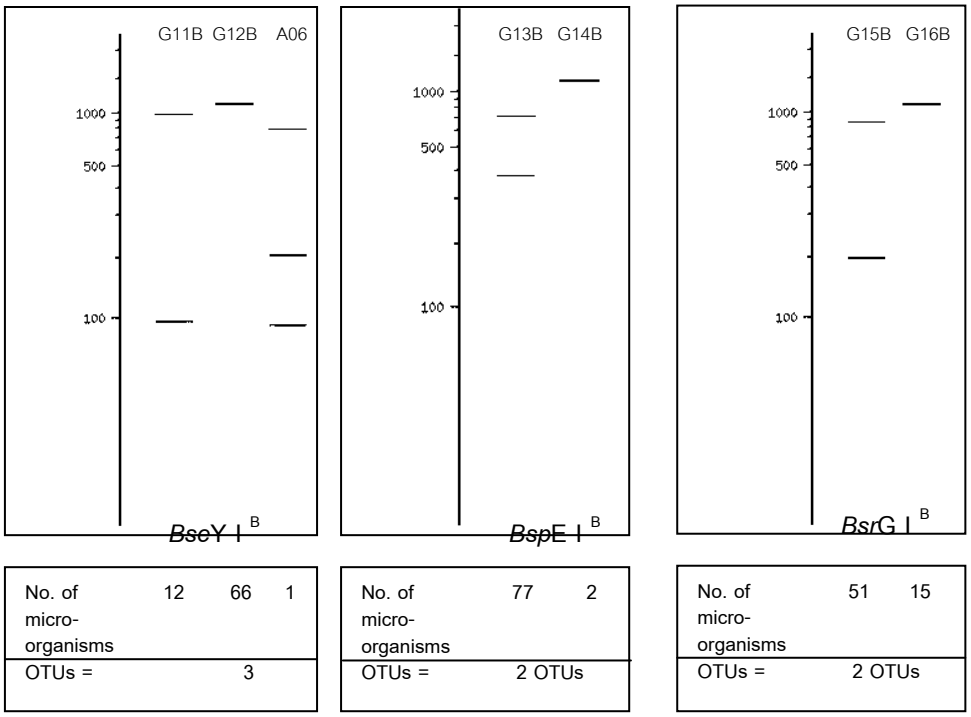


Figure 16 (continued).



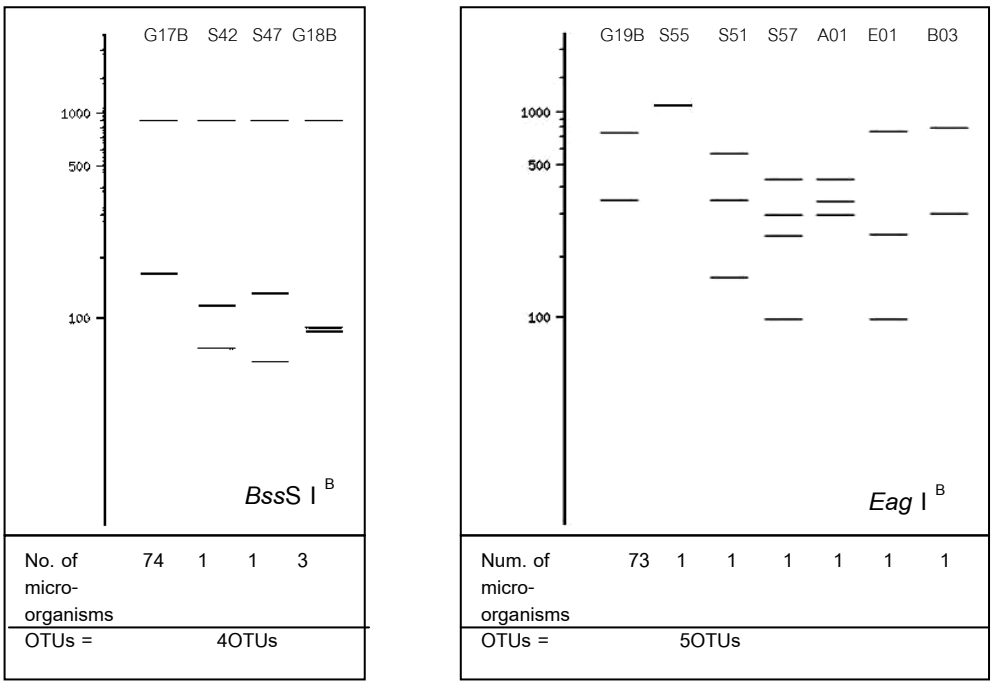
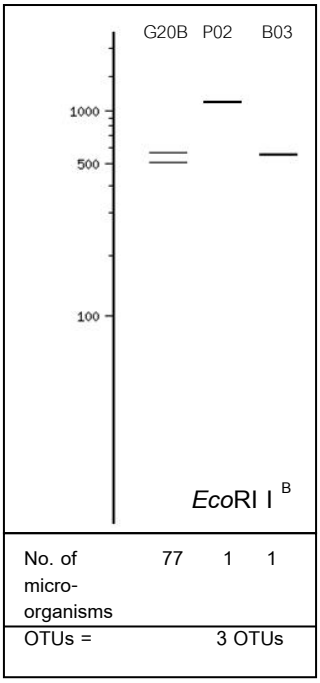
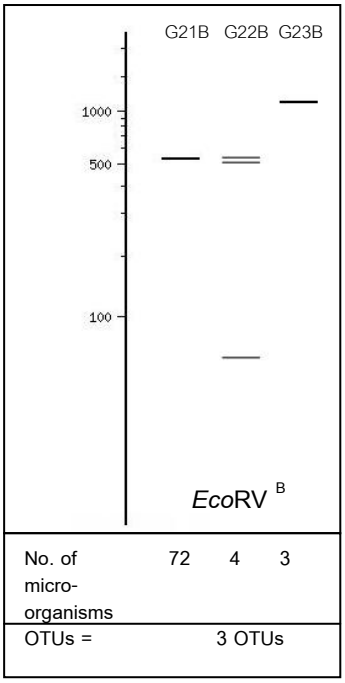


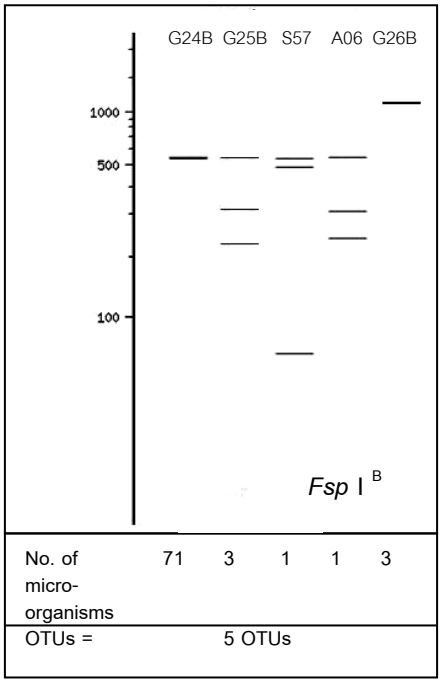
Figure 16 (continued).



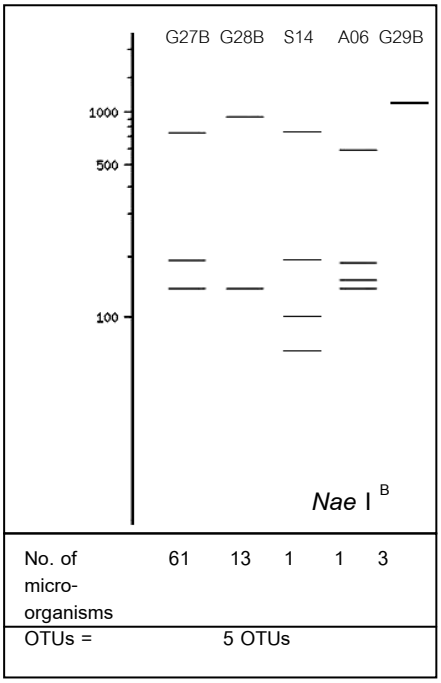
No. of micro-organisms	77	1	1
OTUs =		3 OTUs	



No. of micro-organisms	72	4	3
OTUs =		3 OTUs	



No. of micro-organisms	71	3	1	1	3
OTUs =		5 OTUs			



No. of micro-organisms	61	13	1	1	3
OTUs =		5 OTUs			

Figure 16 (continued).

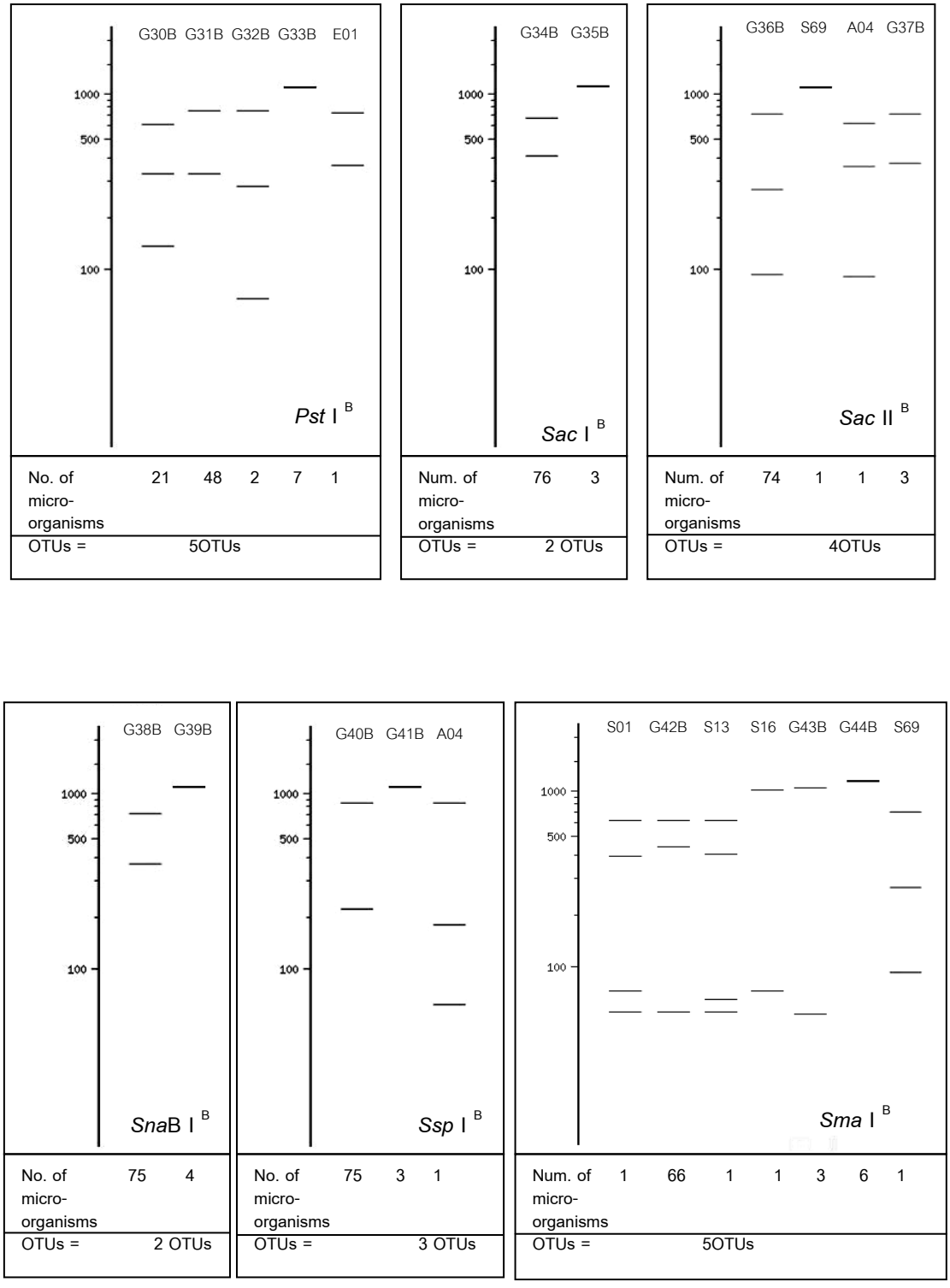


Figure 16 (continued).

Table 12 List of groups of microorganisms according to similar RFLP pattern after digested with *Bfa*I

G1A	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S06	*S. almquistii*
S07	*S. ambofacines*
S10	*S. aureofaciens*
S12	*S. cattleya*
S13	*S. clavuligerus*
S15	*S. fridiae*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S39	*S. albus*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S54	*S. flocculus*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S70	*S. indigoferus*
A04	*Micromonospora olivasterospora*
A06	*Thermomonospora chromogena*

G2A	Name of microorganisms
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S11	*S. bolili*
S16	*S. fulvoviolaceus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S40	*S. avermitilis*
S44	*S. argillaceus*
S47	*S. galilaceus*
S56	*S. virginiae*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*

G3A	Name of microorganisms
S26	*S. spectabilis*
S53	*S. bambergiensis*

G4A	Name of microorganisms
S30	*S. tenebrarius*
S57	*S. lactamdurans*
A02	*Amycolatopsis mediterranei*

G5A	Name of microorganisms
S63	*S. ipomoeae*
A01	*Amycolatopsis mediterranei*

Table13 List of groups of microorganisms according to similar RFLP pattern after digested with *Dpn*I

G6A	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*

G7A	Name of microorganisms
S02	*S. albofaciens*
S07	*S. ambofacines*
S60	*S. viridochromogens*

G8A	Name of microorganisms
S18	*S. graminofaciens*
S29	*S. xantholiticus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*

G9A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S39	*S. albus*
S54	*S. flocculus*

G10A	Name of microorganisms
S24	*S. rumosus* subs. *paromomycinus*
S26	*S. spectabilis*
S33	*S. chrestomyceticus*
S36	*S. tendae*

G11A	Name of microorganisms
S30	*S. tenebrarius*
S57	*S. lactamdurans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*

Table 14 List of groups of microorganisms according to similar RFLP pattern after digested with *BstU*I

G12A	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S11	*S. bolili*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S24	*S. rumosus* subs. *paromomycinus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S33	*S. chrestomyceticus*
S36	*S. tendae*
S37	*S. varsoviensis*
S40	*S. avermitilis*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S47	*S. galilaceus*
S51	*S. parvulus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S63	*S. ipomoeae*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*

G13A	Name of microorganisms
S12	*S. cattleya*
S23	*S. ochraceiscleroticus*
S25	*S. rimosus*
S29	*S. xantholiticus*
S32	*S. cacaoi* subs. *asoensis*
S34	*S. nodosus*
S41	*S. cinnamonensis*
S38	*S. natalensis*
S45	*S. caespetosus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S54	*S. flocculus*
S57	*S. lactamdurans*
S62	*S. acidiscabie*
S64	*S. turgidiscabies*

G14A	Name of microorganisms
S52	*S. azureus*
S53	*S. bambergiensis*
S35	*S. noursei*
S61	*S. sacbiei*

G15A	Name of microorganisms
S10	*S. aureofaciens*
S42	*S. albus subs. pathodicus*
S26	*S. spectabilis*
S46	*S. coeruleorubidus*

G16A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S39	*S. albus*
S70	*S. indigoferus*

G17A	Name of microorganisms
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*

Table 15 List of groups of microorganisms according to similar RFLP pattern after digested with *Hha*I

G18A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S02	*S. albofaciens*
S03	*S. alboniger*
S05	*S. albovinaceus*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S15	*S. fridiae*
S18	*S. graminofaciens*
S20	*S. kanamyceticus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S32	*S. cacaoi* subs. *asoensis*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S52	*S. azureus*
S55	*S. lavendulae*
S56	*S. virginiae*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*

G19A	Name of microorganisms
S04	*S. albolongus*
S23	*S. ochraceiscleroticus*
S33	*S. chrestomyceticus*

G20A	Name of microorganisms
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S37	*S. varsoviensis*

G21A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S39	*S. albus*
S54	*S. flocculus*

G22A	Name of microorganisms
S16	*S. fulvoviolaceus*
S19	*S. griseus*
S31	*S. albidoflavus*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S58	*S. hygroscopicus*

G23A	Name of microorganisms
S13	*S. clavuligerus*
S50	*S. verticillus*

G24A	Name of microorganisms
S69	*S. clavifer*
S70	*S. indigoferus*

G25A	Name of microorganisms
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*

Table 16 List of groups of microorganisms according to similar RFLP pattern after digested with *Mse*I

G26A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S02	*S. albofaciens*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S42	*S. albus subs. pathodicus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
zS60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*

G27A	Name of microorganisms
S30	*S. tenebrarius*
S34	*S. nodosus*
S57	*S. lactamdurans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*

G28A	Name of microorganisms
S03	*S. alboniger*
S11	*S. bolili*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S43	*S. albosporeus subs. labilomyceticus*
S68	*S. baanensis*
S69	*S. clavifer*

G29A	Name of microorganisms
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*

Table 17 List of groups of microorganisms according to similar RFLP pattern after digested with *Nla*III

G30A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S02	*S. albofaciens*
S07	*S. ambofacines*
S10	*S. aureofaciens*
S16	*S. fulvoviolaceus*
S36	*S. tendae*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S52	*S. azureus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S63	*S. ipomoeae*
S65	*S. coelicolor*
S66	*S. lividans*

G31A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S19	*S. griseus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S31	*S. albidoflavus*
S39	*S. albus*
S42	*S. albus subs. pathodicus*
S45	*S. caespetosus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S62	*S. acidiscabie*

G32A	Name of microorganisms
S03	*S. alboniger*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S21	*S. lincolnensis*
S40	*S. avermitilis*
S43	*S. albosporeus subs. labilomyceticus*
S61	*S. sacbiei*
S64	*S. turgidiscabies*

G33A	Name of microorganisms
S04	*S. albolongus*
S05	*S. albovinaceus*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S15	*S. fridiae*
S18	*S. graminofaciens*
S20	*S. kanamyceticus*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S41	*S. cinnamonensis*
S44	*S. argillaceus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*

G34A	Name of microorganisms
S57	*S. lactamdurans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*

Table 18 List of groups of microorganisms according to similar RFLP pattern after digested with *Rsa*I

G35A	Name of mcroorganisms	G36A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*	S04	*S. albolongus*
S02	*S. albofaciens*	S05	*S. albovinaceus*
S03	*S. alboniger*	S14	*S. ederensis*
S06	*S. almquistii*	S15	*S. fridiae*
S07	*S. ambofacines*	S18	*S. graminofaciens*
S08	*S. antibioticus*	S20	*S. kanamyceticus*
S09	*S. aureocirculatus*	S22	*S. narbonensis*
S10	*S. aureofaciens*	S27	*S. spiroverticillatus*
S11	*S. bolili*	S28	*S. venezuelae*
S12	*S. cattleya*	S29	*S. xantholiticus*
S13	*S. clavuligerus*	S41	*S. cinnamonensis*
S16	*S. fulvoviolaceus*	S48	*S. peucetius*
S17	*S. gibsonii*	S49	*S. peucetius subs. caesius*
S19	*S. griseus*	S56	*S. virginiae*
S21	*S. lincolnensis*	S67	*S. halstedii*
S23	*S. ochraceiscleroticus*	S68	*S. baanensis*
S24	*S. rumosus* subs. *paromomycinus*	S69	*S. clavifer*
S25	*S. rimosus*	S70	*S. indigoferus*
S26	*S. spectabilis*		
S30	*S. tenebrarius*		
S31	*S. albidoflavus*		
S32	*S. cacaoi* subs. *asoensis*		
S33	*S. chrestomyceticus*		
S34	*S. nodosus*		
S35	*S. noursei*		
S36	*S. tendae*		
S37	*S. varsoviensis*		
S38	*S. natalensis*		
S39	*S. albus*		
S40	*S. avermitilis*		
S42	*S. albus subs. pathodicus*		
S43	*S. albosporeus subs. labilomyceticus*		
S44	*S. argillaceus*		
S45	*S. caespetosus*		
S46	*S. coeruleorubidus*		
S47	*S. galilaceus*		
S50	*S. verticillus*		
S51	*S. parvulus*		
S52	*S. azureus*		
S53	*S. bambergiensis*		
S54	*S. flocculus*		
S57	*S. lactamdurans*		
S58	*S. hygroscopicus*		
S59	*S. tsukabaensis*		
S60	*S. viridochromogens*		
S61	*S. sacbiei*		
S62	*S. acidiscabie*		
S63	*S. ipomoeae*		
S64	*S. turgidiscabies*		
S65	*S. coelicolor*		
S66	*S. lividans*		
A01	*Amycolatopsis mediterranei*		
A04	*Micromonospora olivasterospora*		
A05	*Sporichthya polymorpha*		
A06	*Thermomonospora chromogena*		

Table 19 List of groups of microorganisms according to similar RFLP pattern after digested with *Taq*I

G37 A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S09	*S. aureocirculatus*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S61	*S. sacbiei*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*

G38A	Name of microorganisms
S02	*S. albofaciens*
S07	*S. ambofacines*
S08	*S. antibioticus*
S10	*S. aureofaciens*
S32	*S. cacaoi* subs. *asoensis*
S36	*S. tendae*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S52	*S. azureus*
S60	*S. viridochromogens*
S62	*S. acidiscabie*
S65	*S. coelicolor*
S66	*S. lividans*

G39A	Name of microorganisms
S16	*S. fulvoviolaceus*
S30	*S. tenebrarius*

G40A	Name of microorganisms
S15	*S. fridiae*
S22	*S. narbonensis*
S26	*S. spectabilis*
S28	*S. venezuelae*
A02	*Amycolatopsis orientalis subsp. orientalis*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*

Table 20 List of groups of microorganisms according to similar RFLP pattern after digested with *Alu*I

G41A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S02	*S. albofaciens*
S06	*S. almquistii*
S07	*S. ambofacines*
S10	*S. aureofaciens*
S13	*S. clavuligerus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S70	*S. indigoferus*
A02	*Amycolatopsis orientalis subsp. orientalis*

G42A	Name of microorganisms
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S11	*S. bolili*
S16	*S. fulvoviolaceus*
S21	*S. lincolnensis*
S40	*S. avermitilis*
S44	*S. argillaceus*
S47	*S. galilaceus*
S56	*S. virginiae*
S67	*S. halstedii*
S68	*S. baanensis*

G43A	Name of microorganisms
S14	*S. ederensis*
S20	*S. kanamyceticus*

G44A	Name of microorganisms
S15	*S. fridiae*
S22	*S. narbonensis*

G45A	Name of microorganisms
S12	*S. cattleya*
A01	*Amycolatopsis mediterranei*
A04	*Micromonospora olivasterospora*
A06	*Thermomonospora chromogena*

G46A	Name of microorganisms
S30	*S. tenebrarius*
A03	*Saccharopolyspora erytraea*

Table 21 List of groups of microorganisms according to similar RFLP pattern after digested with *Hae*III

G47A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S10	*S. aureofaciens*
S16	*S. fulvoviolaceus*
S19	*S. griseus*
S26	*S. spectabilis*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S36	*S. tendae*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S52	*S. azureus*
S55	*S. lavendulae*
S57	*S. lactamdurans*

G48A	Name of microorganisms
S04	*S. albolongus*
S05	*S. albovinaceus*
S12	*S. cattleya*
S13	*S. clavuligerus*
S15	*S. fridiae*
S18	*S. graminofaciens*
S23	*S. ochraceiscleroticus*
S22	*S. narbonensis*
S58	*S. hygroscopicus*
S24	*S. rumosus* subs. *paromomycinus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S33	*S. chrestomyceticus*
S38	*S. natalensis*
S41	*S. cinnamonensis*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S56	*S. virginiae*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S67	*S. halstedii*

G49A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S25	*S. rimosus*
S39	*S. albus*
S54	*S. flocculus*

G50A	Name of microorganisms
S08	*S. antibioticus*
S21	*S. lincolnensis*
S61	*S. sacbiei*
S62	*S. acidiscabie*

G51A	Name of microorganisms
S34	*S. nodosus*
S37	*S. varsoviensis*

G52A	Name of microorganisms
S22	*S. narbonensis*
S68	*S. baanensis*
S69	*S. clavifer*
A02	*Amycolatopsis orientalis subsp. orientalis*

G53A	Name of microorganisms
S02	*S. albofaciens*
S03	*S. alboniger*
S07	*S. ambofacines*
S11	*S. bolili*
S14	*S. ederensis*
S09	*S. aureocirculatus*
S20	*S. kanamyceticus*
S32	*S. cacaoi* subs. *asoensis*
S35	*S. noursei*
S40	*S. avermitilis*
S47	*S. galilaceus*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S53	*S. bambergiensis*

Table 22 List of groups of microorganisms according to similar RFLP pattern after digested with *Mnl*I

G54A	Name of microorganisms
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S40	*S. avermitilis*
G55A	Name of microorganisms
S03	*S. alboniger*
S36	*S. tendae*
S45	*S. caespetosus*
S65	*S. coelicolor*
S66	*S. lividans*
G56A	Name of microorganisms
S02	*S. albofaciens*
S07	*S. ambofacines*
S10	*S. aureofaciens*
S11	*S. bolili*
S14	*S. ederensis*
S19	*S. griseus*
S21	*S. lincolnensis*
S60	*S. viridochromogens*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S35	*S. noursei*
S44	*S. argillaceus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S51	*S. parvulus*
S52	*S. azureus*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S69	*S. clavifer*
S70	*S. indigoferus*

G57A	Name of microorganisms
S04	*S. albolongus*
S05	*S. albovinaceus*
S13	*S. clavuligerus*
S15	*S. fridiae*
S18	*S. graminofaciens*
S23	*S. ochraceiscleroticus*
S33	*S. chrestomyceticus*
S24	*S. rumosus* subs. *paromomycinus*
S34	*S. nodosus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S37	*S. varsoviensis*
S38	*S. natalensis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S67	*S. halstedii*
S68	*S. baanensis*
A02	*Amycolatopsis orientalis subsp. orientalis*
G58A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S39	*S. albus*
S54	*S. flocculus*
G59A	Name of microorganisms
S56	*S. virginiae*
S57	*S. lactamdurans*

Table 23 List of groups of microorganisms according to similar RFLP pattern after digested with *Hpa*II

G60A	Name of microorganisms
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*

G61A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S28	*S. venezuelae*

G62A	Name of microorganisms
S12	*S. cattleya*
S70	*S. indigoferus*

S67	*S. halstedii*
S68	*S. baanensis*

Table 24 List of groups of microorganisms according to similar RFLP pattern after digested with *Aci*I

G63A	Name of microorganisms
S01	*S. acromogens* subs. *rubradirus*
S03	*S. alboniger*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S11	*S. bolili*
S13	*S. clavuligerus*
S16	*S. fulvoviolaceus*
S36	*S. tendae*
S40	*S. avermitilis*
S43	*S. albosporeus subs. labilomyceticus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S65	*S. coelicolor*
S66	*S. lividans*

G64A	Name of microorganisms
S02	*S. albofaciens*
S07	*S. ambofacines*
S15	*S. fridiae*
S19	*S. griseus*
S21	*S. lincolnensis*
S28	*S. venezuelae*
S31	*S. albidoflavus*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S44	*S. argillaceus*
S56	*S. virginiae*

G65A	Name of microorganisms
S04	*S. albolongus*
S12	*S. cattleya*
S20	*S. kanamyceticus*
S24	*S. rumosus* subs. *paromomycinus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S35	*S. noursei*
S38	*S. natalensis*
S50	*S. verticillus*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*

G66A	Name of microorganisms
S05	*S. albovinaceus*
S14	*S. ederensis*
S18	*S. graminofaciens*
S23	*S. ochraceiscleroticus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S29	*S. xantholiticus*
S34	*S. nodosus*
S37	*S. varsoviensis*
S48	*S. peucetius*
S58	*S. hygroscopicus*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*

G67A	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S39	*S. albus*
S54	*S. flocculus*

G68A	Name of microorganisms
S30	*S. tenebrarius*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*

Table 25 List of groups of microorganisms according to similar RFLP pattern after digested with *Aat*II

G1B	Name of microorganisms	G1B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*	S41	*S. cinnamonensis*
S02	*S. albofaciens*	S42	*S. albus subs. pathodicus*
S03	*S. alboniger*	S43	*S. albosporeus subs. labilomyceticus*
S04	*S. albolongus*	S44	*S. argillaceus*
S05	*S. albovinaceus*	S45	*S. caespetosus*
S06	*S. almquistii*	S46	*S. coeruleorubidus*
S07	*S. ambofacines*	S47	*S. galilaceus*
S08	*S. antibioticus*	S48	*S. peucetius*
S09	*S. aureocirculatus*	S49	*S. peucetius subs. caesius*
S10	*S. aureofaciens*	S50	*S. verticillus*
S11	*S. bolili*	S51	*S. parvulus*
S12	*S. cattleya*	S52	*S. azureus*
S13	*S. clavuligerus*	S53	*S. bambergiensis*
S14	*S. ederensis*	S54	*S. flocculus*
S15	*S. fridiae*	S55	*S. lavendulae*
S16	*S. fulvoviolaceus*	S56	*S. virginiae*
S17	*S. gibsonii*	S57	*S. lactamdurans*
S18	*S. graminofaciens*	S58	*S. hygroscopicus*
S19	*S. griseus*	S59	*S. tsukabaensis*
S20	*S. kanamyceticus*	S60	*S. viridochromogens*
S21	*S. lincolnensis*	S61	*S. sacbiei*
S22	*S. narbonensis*	S62	*S. acidiscabie*
S23	*S. ochraceiscleroticus*	S63	*S. ipomoeae*
S24	*S. rumosus* subs. *paromomycinus*	S64	*S. turgidiscabies*
S25	*S. rimosus*	S65	*S. coelicolor*
S26	*S. spectabilis*	S66	*S. lividans*
S27	*S. spiroverticillatus*	S67	*S. halstedii*
S28	*S. venezuelae*	S68	*S. baanensis*
S29	*S. xantholiticus*	S69	*S. clavifer*
S30	*S. tenebrarius*	S70	*S. indigoferus*
S31	*S. albidoflavus*	A01	*Amycolatopsis mediterranei*
S32	*S. cacaoi* subs. *asoensis*	A02	*Amycolatopsis orientalis subsp. orientalis*
S33	*S. chrestomyceticus*	A03	*Saccharopolyspora erytraea*
S34	*S. nodosus*	A04	*Micromonospora olivasterospora*
S35	*S. noursei*	A05	*Sporichthya polymorpha*
S36	*S. tendae*	A06	*Thermomonospora chromogena*
S37	*S. varsoviensis*	E01	*Escherichia coli*
S38	*S. natalensis*	P02	*Pseudomonas putida*
S39	*S. albus*		
S40	*S. avermitilis*		

Table 26 List of groups of microorganisms according to similar RFLP pattern after digested with *Acc*65I

G2B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*

G3B	Name of microorganisms
A03	*Saccharopolyspora erytraea*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*

Table 27 List of groups of microorganisms according to similar RFLP pattern after digested with *Age*I

G4B	Name of microorganisms	G4B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*	S41	*S. cinnamonensis*
S02	*S. albofaciens*	S42	*S. albus subs. pathodicus*
S03	*S. alboniger*	S43	*S. albosporeus subs. labilomyceticus*
S04	*S. albolongus*	S44	*S. argillaceus*
S05	*S. albovinaceus*	S45	*S. caespetosus*
S06	*S. almquistii*	S46	*S. coeruleorubidus*
S07	*S. ambofacines*	S47	*S. galilaceus*
S08	*S. antibioticus*	S48	*S. peucetius*
S09	*S. aureocirculatus*	S49	*S. peucetius subs. caesius*
S10	*S. aureofaciens*	S50	*S. verticillus*
S11	*S. bolili*	S51	*S. parvulus*
S12	*S. cattleya*	S52	*S. azureus*
S13	*S. clavuligerus*	S53	*S. bambergiensis*
S14	*S. ederensis*	S54	*S. flocculus*
S15	*S. fridiae*	S56	*S. virginiae*
S16	*S. fulvoviolaceus*	S58	*S. hygroscopicus*
S17	*S. gibsonii*	S59	*S. tsukabaensis*
S18	*S. graminofaciens*	S60	*S. viridochromogens*
S19	*S. griseus*	S61	*S. sacbiei*
S20	*S. kanamyceticus*	S62	*S. acidiscabie*
S21	*S. lincolnensis*	S63	*S. ipomoeae*
S22	*S. narbonensis*	S64	*S. turgidiscabies*
S23	*S. ochraceiscleroticus*	S65	*S. coelicolor*
S24	*S. rumosus* subs. *paromomycinus*	S66	*S. lividans*
S25	*S. rimosus*	S67	*S. halstedii*
S26	*S. spectabilis*	S68	*S. baanensis*
S27	*S. spiroverticillatus*	S69	*S. clavifer*
S28	*S. venezuelae*	S70	*S. indigoferus*
S29	*S. xantholiticus*	A01	*Amycolatopsis mediterranei*
S30	*S. tenebrarius*	A02	*Amycolatopsis orientalis subsp. orientalis*
S31	*S. albidoflavus*	A03	*Saccharopolyspora erytraea*
S32	*S. cacaoi* subs. *asoensis*	A04	*Micromonospora olivasterospora*
S33	*S. chrestomyceticus*	A05	*Sporichthya polymorpha*
S34	*S. nodosus*	A06	*Thermomonospora chromogena*
S35	*S. noursei*	E01	*Escherichia coli*
S36	*S. tendae*		
S37	*S. varsoviensis*	G5B	Name of microorganisms
S38	*S. natalensis*	P02	*Pseudomonas putida*
S39	*S. albus*	B03	*Bacillus subtillis*
S40	*S. avermitilis*		

Table 28 List of groups of microorganisms according to similar RFLP pattern after digested with *Apa*I

G6B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*

Table 29 List of groups of microorganisms according to similar RFLP pattern after digested with *Bmg*BI

G7B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S07	*S. ambofacines*
S19	*S. griseus*
S31	*S. albidoflavus*
S36	*S. tendae*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S60	*S. viridochromogens*
S65	*S. coelicolor*
S66	*S. lividans*

G8B	Name of microorganisms
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S18	*S. graminofaciens*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S24	*S. rumosus* subs. *paromomycinus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S33	*S. chrestomyceticus*
S40	*S. avermitilis*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S47	*S. galilaceus*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*

G9B	Name of microorganisms
S06	*S. almquistii*
S17	*S. gibsonii*
S23	*S. ochraceiscleroticus*
S25	*S. rimosus*
S29	*S. xantholiticus*
S32	*S. cacaoi* subs. *asoensis*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S41	*S. cinnamonensis*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S70	*S. indigoferus*

G10B	Name of microorganisms
S12	*S. cattleya*
S30	*S. tenebrarius*
S42	*S. albus subs. pathodicus*
S45	*S. caespetosus*
S52	*S. azureus*
S57	*S. lactamdurans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 30 List of groups of microorganisms according to similar RFLP pattern after digested with *Bse*YI

G11B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S16	*S. fulvoviolaceus*
S19	*S. griseus*
S26	*S. spectabilis*
S31	*S. albidoflavus*
S36	*S. tendae*
S42	*S. albus subs. pathodicus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S52	*S. azureus*
S60	*S. viridochromogens*

G12B	Name of microorganisms
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

G12B	Name of microorganisms
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*

Table 31 List of groups of microorganisms according to similar RFLP pattern after digested with *Bsp*EI

G13B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*

G13B	Name of microorganisms
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*

G14B	Name of microorganisms
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 32 List of groups of microorganisms according to similar RFLP pattern after digested with *Bsr*GI

G15B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S19	*S. griseus*
S21	*S. lincolnensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*

G15B	Name of microorganisms
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*

G16B	Name of microorganisms
S04	*S. albolongus*
S05	*S. albovinaceus*
S14	*S. ederensis*
S15	*S. fridiae*
S18	*S. graminofaciens*
S20	*S. kanamyceticus*
S22	*S. narbonensis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S41	*S. cinnamonensis*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S55	*S. lavendulae*
S56	*S. virginiae*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 33 List of groups of microorganisms according to similar RFLP pattern after digested with *Bss*SI

G16B	Name of microorganisms	G16B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*	S41	*S. cinnamonensis*
S02	*S. albofaciens*	S43	*S. albosporeus subs. labilomyceticus*
S03	*S. alboniger*	S44	*S. argillaceus*
S04	*S. albolongus*	S45	*S. caespetosus*
S05	*S. albovinaceus*	S46	*S. coeruleorubidus*
S06	*S. almquistii*	S48	*S. peucetius*
S07	*S. ambofacines*	S49	*S. peucetius subs. caesius*
S08	*S. antibioticus*	S50	*S. verticillus*
S09	*S. aureocirculatus*	S51	*S. parvulus*
S10	*S. aureofaciens*	S52	*S. azureus*
S11	*S. bolili*	S53	*S. bambergiensis*
S12	*S. cattleya*	S54	*S. flocculus*
S13	*S. clavuligerus*	S55	*S. lavendulae*
S14	*S. ederensis*	S56	*S. virginiae*
S15	*S. fridiae*	S58	*S. hygroscopicus*
S16	*S. fulvoviolaceus*	S59	*S. tsukabaensis*
S17	*S. gibsonii*	S60	*S. viridochromogens*
S18	*S. graminofaciens*	S61	*S. sacbiei*
S19	*S. griseus*	S62	*S. acidiscabie*
S20	*S. kanamyceticus*	S63	*S. ipomoeae*
S21	*S. lincolnensis*	S64	*S. turgidiscabies*
S22	*S. narbonensis*	S65	*S. coelicolor*
S23	*S. ochraceiscleroticus*	S66	*S. lividans*
S24	*S. rumosus* subs. *paromomycinus*	S67	*S. halstedii*
S25	*S. rimosus*	S68	*S. baanensis*
S26	*S. spectabilis*	S69	*S. clavifer*
S27	*S. spiroverticillatus*	S70	*S. indigoferus*
S28	*S. venezuelae*	A03	*Saccharopolyspora erytraea*
S29	*S. xantholiticus*	A04	*Micromonospora olivasterospora*
S30	*S. tenebrarius*	A05	*Sporichthya polymorpha*
S31	*S. albidoflavus*	A06	*Thermomonospora chromogena*
S32	*S. cacaoi* subs. *asoensis*	E01	*Escherichia coli*
S33	*S. chrestomyceticus*	P02	*Pseudomonas putida*
S34	*S. nodosus*	B03	*Bacillus subtillis*
S35	*S. noursei*		
S36	*S. tendae*	G17B	Name of microorganisms
S37	*S. varsoviensis*	S57	*S. lactamdurans*
S38	*S. natalensis*	A01	*Amycolatopsis mediterranei*
S39	*S. albus*	A02	*Amycolatopsis orientalis subsp. orientalis*
S40	*S. avermitilis*		

Table 34 List of groups of microorganisms according to similar RFLP pattern after digested with *Eag*I

G18B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*
P02	*Pseudomonas putida*

Table 35 List of groups of microorganisms according to similar RFLP pattern after digested with *Eco*RI

G19B	Name of microorganisms	G19B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*	S41	*S. cinnamonensis*
S02	*S. albofaciens*	S42	*S. albus subs. pathodicus*
S03	*S. alboniger*	S43	*S. albosporeus subs. labilomyceticus*
S04	*S. albolongus*	S44	*S. argillaceus*
S05	*S. albovinaceus*	S45	*S. caespetosus*
S06	*S. almquistii*	S46	*S. coeruleorubidus*
S07	*S. ambofacines*	S47	*S. galilaceus*
S08	*S. antibioticus*	S48	*S. peucetius*
S09	*S. aureocirculatus*	S49	*S. peucetius subs. caesius*
S10	*S. aureofaciens*	S50	*S. verticillus*
S11	*S. bolili*	S51	*S. parvulus*
S12	*S. cattleya*	S52	*S. azureus*
S13	*S. clavuligerus*	S53	*S. bambergiensis*
S14	*S. ederensis*	S54	*S. flocculus*
S15	*S. fridiae*	S55	*S. lavendulae*
S16	*S. fulvoviolaceus*	S56	*S. virginiae*
S17	*S. gibsonii*	S57	*S. lactamdurans*
S18	*S. graminofaciens*	S58	*S. hygroscopicus*
S19	*S. griseus*	S59	*S. tsukabaensis*
S20	*S. kanamyceticus*	S60	*S. viridochromogens*
S21	*S. lincolnensis*	S61	*S. sacbiei*
S22	*S. narbonensis*	S62	*S. acidiscabie*
S23	*S. ochraceiscleroticus*	S63	*S. ipomoeae*
S24	*S. rumosus* subs. *paromomycinus*	S64	*S. turgidiscabies*
S25	*S. rimosus*	S65	*S. coelicolor*
S26	*S. spectabilis*	S66	*S. lividans*
S27	*S. spiroverticillatus*	S67	*S. halstedii*
S28	*S. venezuelae*	S68	*S. baanensis*
S29	*S. xantholiticus*	S69	*S. clavifer*
S30	*S. tenebrarius*	S70	*S. indigoferus*
S31	*S. albidoflavus*	A01	*Amycolatopsis mediterranei*
S32	*S. cacaoi* subs. *asoensis*	A02	*Amycolatopsis orientalis subsp. orientalis*
S33	*S. chrestomyceticus*	A03	*Saccharopolyspora erytraea*
S34	*S. nodosus*	A04	*Micromonospora olivasterospora*
S35	*S. noursei*	A05	*Sporichthya polymorpha*
S36	*S. tendae*	A06	*Thermomonospora chromogena*
S37	*S. varsoviensis*	E01	*Escherichia coli*
S38	*S. natalensis*		
S39	*S. albus*		
S40	*S. avermitilis*		

Table 36 List of groups of microorganisms according to similar RFLP pattern after digested with *Eco*RV

G20B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*

G21B	Name of microorganisms
S08	*S. antibioticus*
S32	*S. cacaoi* subs. *asoensis*
S62	*S. acidiscabie*
A01	*Amycolatopsis mediterranei*

G22B	Name of microorganisms
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 37 List of groups of microorganisms according to similar RFLP pattern after digested with *Fsp*I

G23B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*

G23B	Name of microorganisms
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A04	*Micromonospora olivasterospora*

G24B	Name of microorganisms
S30	*S. tenebrarius*
A03	*Saccharopolyspora erytraea*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*

G25B	Name of microorganisms
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 38 List of groups of microorganisms according to similar RFLP pattern after digested with *Nae*I

G26B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus* subs. *pathodicus*
S43	*S. albosporeus* subs. *labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius* subs. *caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
A04	*Micromonospora olivasterospora*

G27B	Name of microorganisms
S06	*S. almquistii*
S12	*S. cattleya*
S17	*S. gibsonii*
S22	*S. narbonensis*
S39	*S. albus*
S54	*S. flocculus*
S57	*S. lactamdurans*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A05	*Sporichthya polymorpha*

G28B	Name of microorganisms
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 39 List of groups of microorganisms according to similar RFLP pattern after digested with *Pst*I

G29B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S07	*S. ambofacines*
S10	*S. aureofaciens*
S16	*S. fulvoviolaceus*
S19	*S. griseus*
S20	*S. kanamyceticus*
S26	*S. spectabilis*
S31	*S. albidoflavus*
S36	*S. tendae*
S42	*S. albus subs. pathodicus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S60	*S. viridochromogens*
S65	*S. coelicolor*
S66	*S. lividans*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*

G30B	Name of microorganisms
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S15	*S. fridiae*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
E01	*Escherichia coli*

G31B	Name of microorganisms
S14	*S. ederensis*
S64	*S. turgidiscabies*

G32B	Name of microorganisms
S57	*S. lactamdurans*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A06	*Thermomonospora chromogena*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 40 List of groups of microorganisms according to similar RFLP pattern after digested with *Sac*I

G33B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*

G33B	Name of microorganisms
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A04	*Micromonospora olivasterospora*
A05	*Sporichthya polymorpha*
A06	*Thermomonospora chromogena*

G34B	Name of microorganisms
E01	*Escherichia coli*
P02	*Pseudomonas putida*
B03	*Bacillus subtillis*

Table 41 List of groups of microorganisms according to similar RFLP pattern after digested with *Sac*II

G35B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*

G35B	Name of microorganisms
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A05	*Sporichthya polymorpha*
B03	*Bacillus subtillis*

G36B	Name of microorganisms
A04	*Micromonospora olivasterospora*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*

Table 42 List of groups of microorganisms according to similar RFLP pattern after digested with *Sna*BI

G37B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*

G37B	Name of microorganisms
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A03	*Saccharopolyspora erytraea*
A05	*Sporichthya polymorpha*
B03	*Bacillus subtillis*

G38B	Name of microorganisms
A04	*Micromonospora olivasterospora*
A06	*Thermomonospora chromogena*
E01	*Escherichia coli*
P02	*Pseudomonas putida*

Table 43 List of groups of microorganisms according to similar RFLP pattern after digested with *Ssp*I

G39B	Name of microorganisms
S01	*S. acromogens* subs. *rubradiris*
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S13	*S. clavuligerus*
S14	*S. ederensis*
S15	*S. fridiae*
S16	*S. fulvoviolaceus*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S30	*S. tenebrarius*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S55	*S. lavendulae*
S56	*S. virginiae*
S57	*S. lactamdurans*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S69	*S. clavifer*
S70	*S. indigoferus*
A01	*Amycolatopsis mediterranei*
A02	*Amycolatopsis orientalis subsp. orientalis*
A05	*Sporichthya polymorpha*
E01	*Escherichia coli*
P02	*Pseudomonas putida*

G40B	Name of microorganisms
A03	*Saccharopolyspora erytraea*
A06	*Thermomonospora chromogena*
B03	*Bacillus subtillis*

Table 44 List of groups of microorganisms according to similar RFLP pattern after digested with *Sma*I

G41B	Name of microorganisms
S02	*S. albofaciens*
S03	*S. alboniger*
S04	*S. albolongus*
S05	*S. albovinaceus*
S06	*S. almquistii*
S07	*S. ambofacines*
S08	*S. antibioticus*
S09	*S. aureocirculatus*
S10	*S. aureofaciens*
S11	*S. bolili*
S12	*S. cattleya*
S14	*S. ederensis*
S15	*S. fridiae*
S17	*S. gibsonii*
S18	*S. graminofaciens*
S19	*S. griseus*
S20	*S. kanamyceticus*
S21	*S. lincolnensis*
S22	*S. narbonensis*
S23	*S. ochraceiscleroticus*
S24	*S. rumosus* subs. *paromomycinus*
S25	*S. rimosus*
S26	*S. spectabilis*
S27	*S. spiroverticillatus*
S28	*S. venezuelae*
S29	*S. xantholiticus*
S31	*S. albidoflavus*
S32	*S. cacaoi* subs. *asoensis*
S33	*S. chrestomyceticus*
S34	*S. nodosus*
S35	*S. noursei*
S36	*S. tendae*
S37	*S. varsoviensis*
S38	*S. natalensis*
S39	*S. albus*
S40	*S. avermitilis*
S41	*S. cinnamonensis*
S42	*S. albus subs. pathodicus*
S43	*S. albosporeus subs. labilomyceticus*
S44	*S. argillaceus*
S45	*S. caespetosus*
S46	*S. coeruleorubidus*
S47	*S. galilaceus*
S48	*S. peucetius*
S49	*S. peucetius subs. caesius*
S50	*S. verticillus*

G41B	Name of microorganisms
S51	*S. parvulus*
S52	*S. azureus*
S53	*S. bambergiensis*
S54	*S. flocculus*
S58	*S. hygroscopicus*
S59	*S. tsukabaensis*
S60	*S. viridochromogens*
S61	*S. sacbiei*
S62	*S. acidiscabie*
S63	*S. ipomoeae*
S64	*S. turgidiscabies*
S65	*S. coelicolor*
S66	*S. lividans*
S67	*S. halstedii*
S68	*S. baanensis*
S70	*S. indigoferus*

G42B	Name of microorganisms
S30	*S. tenebrarius*
A03	*Saccharopolyspora erytraea*
A06	*Thermomonospora chromogena*
B03	*Bacillus subtillis*

4.4 Choice of restriction enzyme to investigate *Streptomyces* diversity from soil

The choice for restriction enzymes used to investigate diversity of *Streptomyces* from soil was based on these criteria: (i) high average number of restriction sites per species and (ii) the co-relation between phylogenetic distribution and the production of bioactive compounds. The average number of restriction sites per *Streptomyces* species and OTUs detect by using each of the 33 restriction enzymes were listed in Table 45. The results showed a range of an average of 2.00 to 17.00 sites per species for tetrameric restriction enzymes (4 bp cutter enzymes) and 0.5 to 2.00 sites per species for hexameric restriction enzymes (6 bp cutter enzymes). The phylogenetic reconstruction from restriction data were shown in Figure 15.

Table 45 Average of restriction sited per taxon of restriction enzyme

Restriction enzyme	Mean no. of restriction site per taxon (70 *Strepstomyces*)	OTU detect (70 *Strepstomyces*)
Bfa I [A]	2.38	8
Dpn I [A]	3.00	7
BstU I [A]	5.50	8
Hha I [A]	5.57	14
Mse I [A]	4.17	6
Nla III [A]	4.57	7
Rsa I [A]	2.00	3
Taq I [A]	2.40	5
Alu I [A]	5.38	8
Hae III [A]	9.46	15
Mnl I [A]	8.85	13
Hpa II [A]	10.50	8
Aci I [A]	17.00	13

Table 45 (continued).

Restriction enzyme	Mean no. of restriction site per taxon (70 *Strepstomyces*)	OTU detect (70 *Strepstomyces*)
Aat II [B] (*Zra* I)	1.00	1
*Acc*65I [B] (*Kpn* I)	1.00	1
Age I [B]	1.67	3
Apa [B] I (*Psp*OM I)	2.00	4
*Bmg*B I [B]	1.00	4
*Bse*Y I [B]	0.50	2
*Bsp*E I [B]	1.00	1
*Bsr*G I [B]	0.52	2
*Bss*S I [B]	1.75	4
Eag I [B]	1.50	4
*Eco*RI [B]	1.00	1
*Eco*RV [B]	1.50	2
Fsp I [B]	1.67	3
Nae I [B] (*Ngo*MI V)	2.00	3
Pst I [B]	1.00	5
Sac I [B]	1.00	1
Sac II [B]	1.00	2
*Sna*B I [B]	1.00	1
Ssp I [B]	1.00	1
Sma I [B] (*Tsp*M I, *Xma* I)	1.70	7

[A] tetrameric restriction enzyme

[B] hexameric restriction enzyme

Parenthesis indicated the isoschizomers

4.4.1 Phylogenetic tree reconstruction from restriction fragment data

In order to examine whether the simulated RFLP fragment pattern from each restriction enzyme had the co-relation between phylogenetic distribution and the production of bioactive compounds of Streptomyces and Actinomyces, the phylogenetic trees were reconstructed. The results of the phylogenetic tree of *Streptomyces*, *Actinomyces* and outgroup bacteria based on restriction enzyme digestion of the 16S rRNA (StrepBF region) were shown in Figure16. A Phylogenetic tree was reconstructed using PAUP* program, Nei-Li distance method was used to generate the restriction distance matrix. The resulting Neighbor-joining trees.

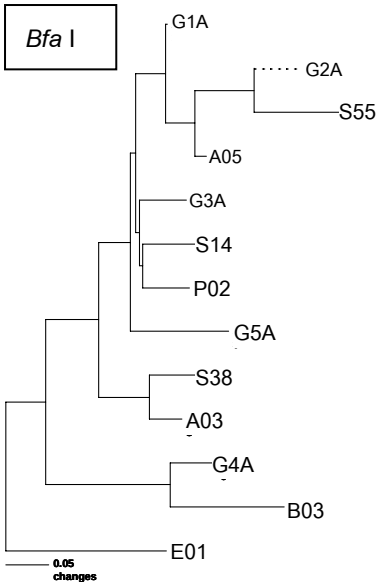


Figure 17 The Neighbor-joining tree constructed from a restriction distance matrix by Nei-Li method from PAUP* program showing phylogenetic relationships among *Streptomyces*, *Actinomyces*, and outgroup bacteria, based on PCR-RFLP analysis of each tetrameric restriction enzyme (*Bfa*I, *Dpn*I, *Bst*UI, *Hha*I, *Mse*I, *Nla*III, *Rsa*I, *Taq*I, *Alu*I, *Hae*III, *Mn*II, *Hpa*II, and *Aci*I) digestion. Similar RFLP pattern were group. The scale bar indicated 0.05 change.

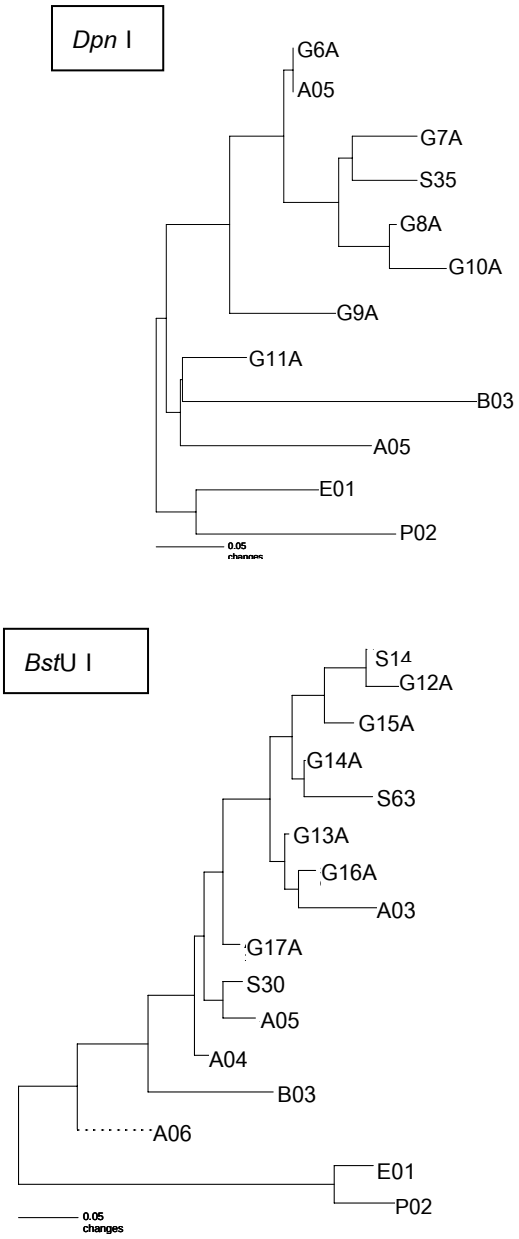
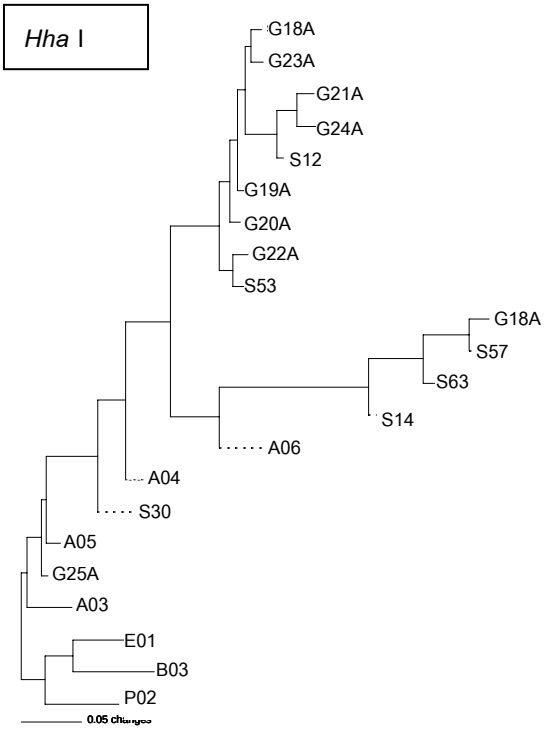


Figure 17 (continued).


Hha I
G18A
G23A
G21A
G24A
S12
G19A
G20A
G22A
S53
G18A
S57
S63
S14
A06
A04
S30
A05
G25A
A03
E01
B03
P02
0.05 changes

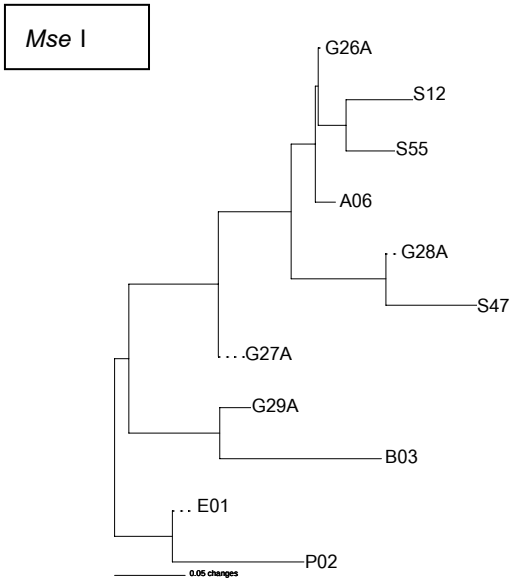
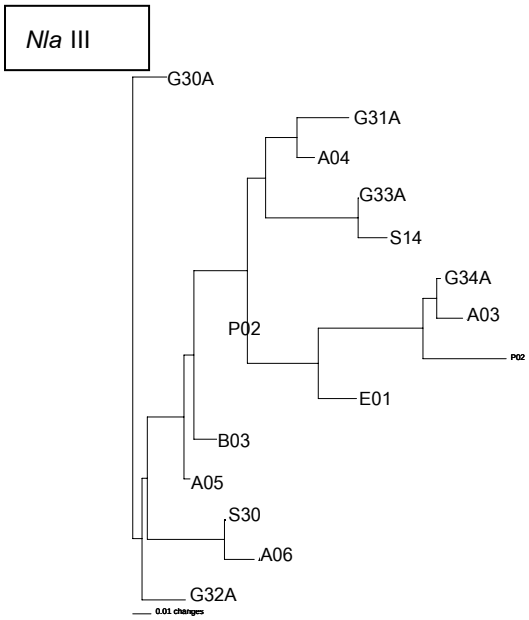

Mse I
G26A
S12
S55
A06
G28A
S47
G27A
G29A
B03
E01
P02
0.05 changes

Figure 17 (continued).



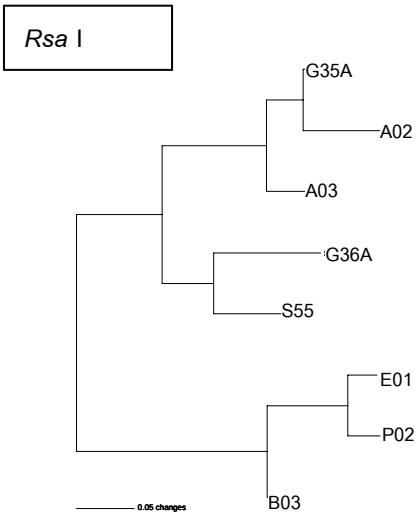


Figure 17 (continued).

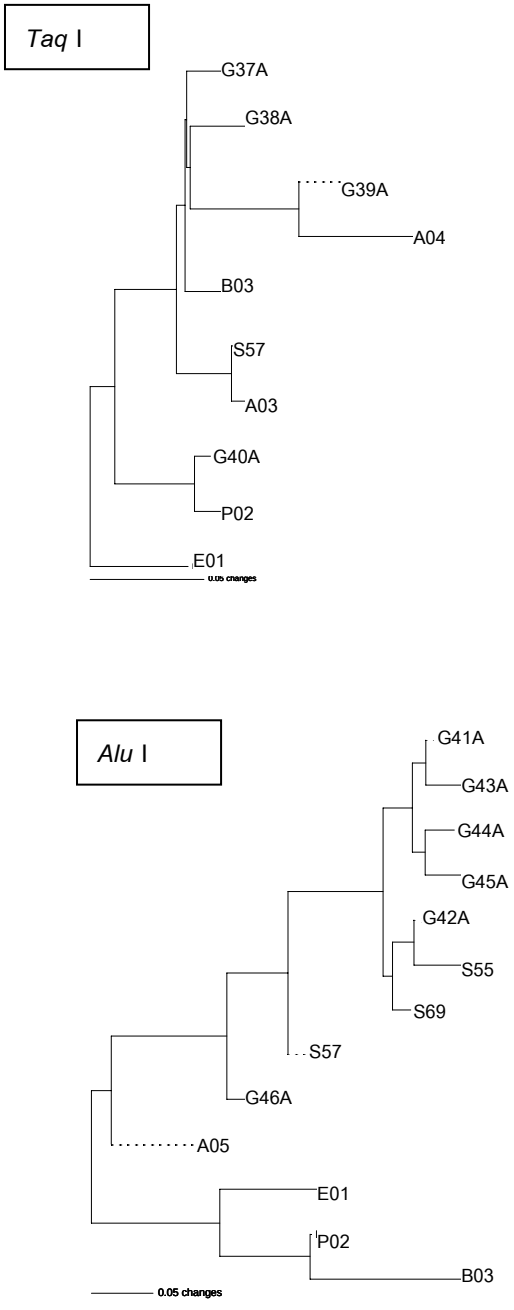
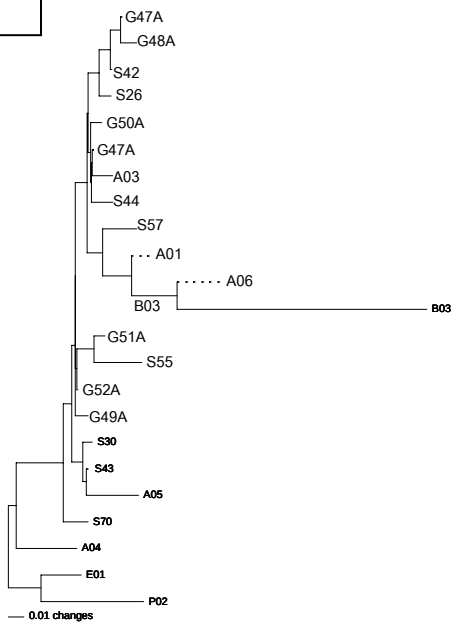


Figure 17 (continued).

Hae III


G47A
G48A
S42
S26
G50A
G47A
A03
S44
S57
A01
A06
B03
B03
G51A
S55
G52A
G49A
S30
S43
A05
S70
A04
E01
P02
0.01 changes

Mnl I

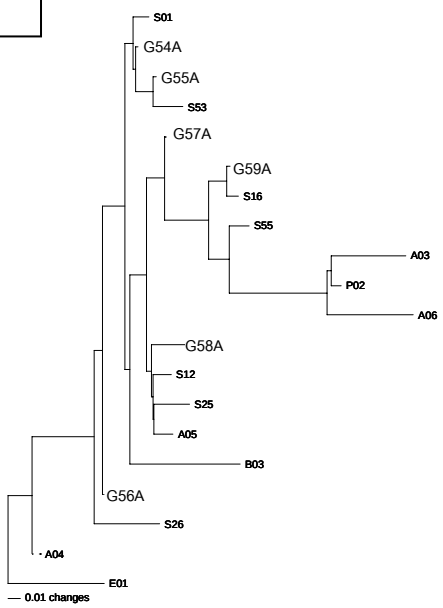
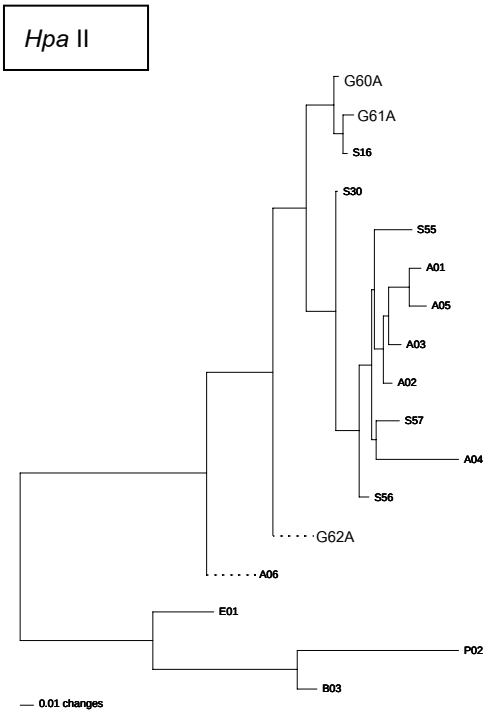

S01
G54A
G55A
S53
G57A
G59A
S16
S55
A03
P02
A06
G58A
S12
S25
A05
B03
G56A
S26
A04
E01
0.01 changes

Figure 17 (continued).



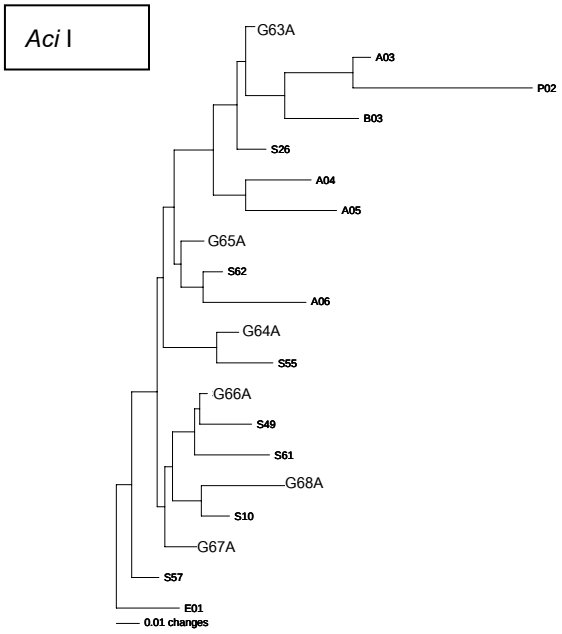


Figure 17 (continued).

4.5 Genomic DNA extraction

4.5.1 Total genomic DNA extraction from test organisms

The genomic DNA extraction from test organisms (*Streptomyces venezuelae, S. narbonensis, S. lividans, Escherichia coli, Bacillus subtillus, Pseudomonas putida*) yield a high molecular weight. A nucleic acid analysis at OD 260/280 ratio using spectrophotometer DU800 (Beckman). The DNA was dilute with TE buffer 1/200 fold. The high molecular weight of the genomic DNA was obtained about >10 kb. The OD 260/280 was approximately 1.9 - 2.0 indicated a high purity suitable for PCR amplification. Table 46 and Figure 18 showing DNA concentration from *S. venezuelae* and *S. narbonensis*.

Table 46 Concentration and purity of DNA from *Strepmyces venezuelae and S. narbonensis*

Sample	OD 260	OD260/280	DNA (ng/µl)
S. venezuelae no.1	0.3315	2.0332	3,315
S. venezuelae no.2	0.4737	1.9630	4,737
S. narbonensis no.1	0.0823	2.0060	823
S. narbonensis no.2	0.1057	1.9897	1,057

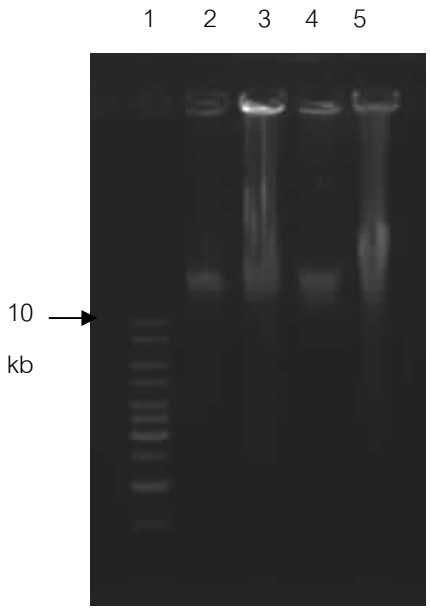


Figure 18 Genomic DNA from *Streptomyces venezuelae* (lane 2 and 3) and *S. narbonensis* (Lane 4 and 5). Lane 1: 1 Kb DNA ladder. Genomic DNA was carry on with 1% agarose gel electrophesis in 1X TAE buffer at 80 Volt 30 min

4.6 PCR amplification of 16S rDNA from model organisms

An expected size of 1.5 kb fragment from PCR amplification of 16S rDNA using eubacterial specific primer (fD1 + rP2) were observed from *Streptomyces venezuelae*, *S. lividans* and *S. narbonensis* (Figure 19). By using *Streptomyces* specific (StrepB + StrepF) amplification of 16S rDNA, the 1 kb fragment were obtained from *Streptomyces venezuelae*, *S. lividans* and *S. narbonensis* (Figure 20) whereas no amplification from those outgroup species (Figure 21).

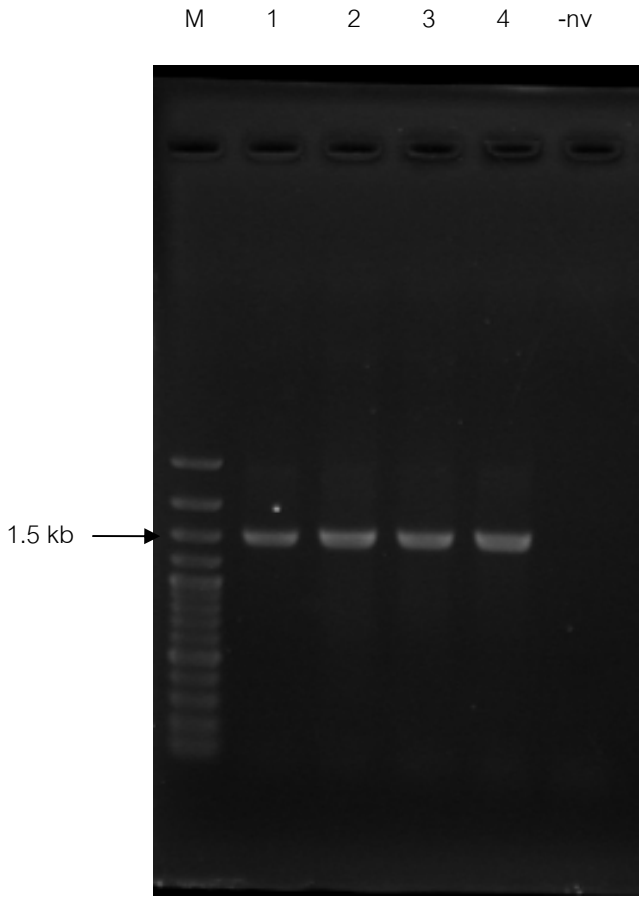


Figure 19 1% agarose gel electrophoresis of PCR product (1.5 kb). Lane M :100 bp ladder, lane 2 : *S. narbonensis*, lane 2 and lane3 : *S. venezuelae*, lane 4 :*S. lividans*. -nv was a negative control using double distilled water instead of DNA.

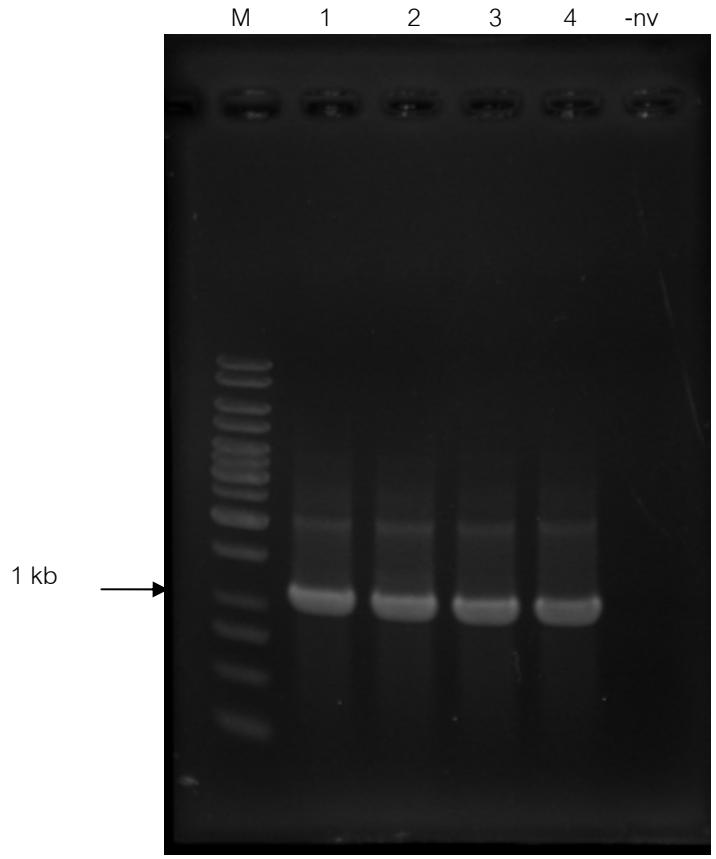


Figure 20 1% agarose gel electrophoresis of PCR product (1 kb) using StrepB and StrepF primer pair. Lane M :1 kb ladder, lane 1 : *S. narbonensis*, Lane 2 and lane3 : *S. venezuelae*, lane 4 :*S. lividans* and -nv was a negative control using double distilled water instead of DNA.

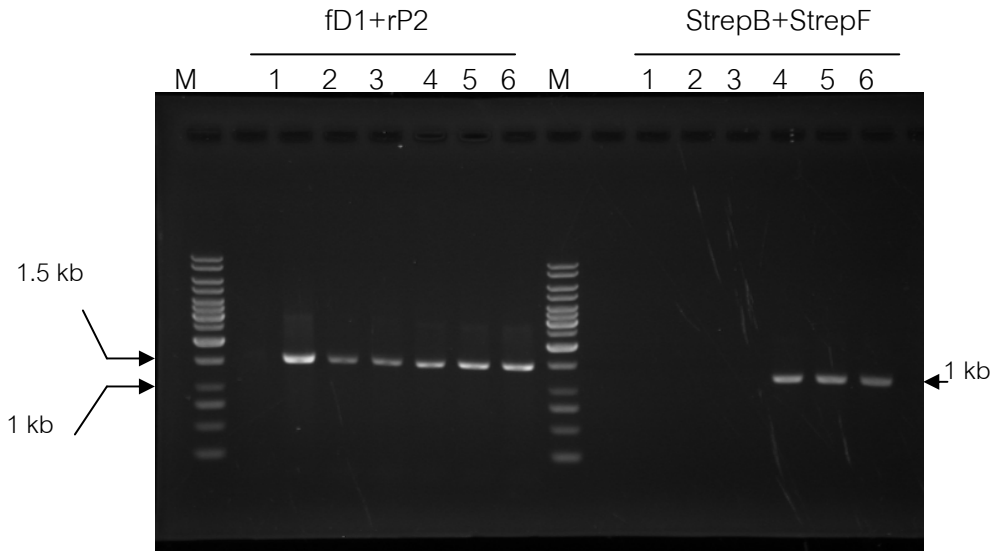


Figure 21 1% agarose gel electrophoresis of PCR product using fD1+rP2 (1.5 kb) and StrepB+StrepF (1 kb) of bacterial culture. Lane M :1 kb ladder, lane 1 : *B. subtilis*, lane 2: *P. putida*, lane 3: *E.coli* XL1Blue, lane 4: *S. narbonensis*, lane 5 : *S. venezuelae*, and lane 6: *S. lividans*.

4.7 Soil DNA extraction

The soil DNA was extracted from 33 soil samples by direct lysis method (Saano, cited in Trevor, 1995). After add 50 µl of TE buffer the Crude soil DNA were yellowish to dark brown in color indicated co-extraction of humic acid. Table 47 showed the ratio of OD260/OD230 and OD260/OD280 which indicate purity of DNA. Further purification crude DNA was obtained by gel extraction. A 2 µl of total 30 µl elution of purified DNA were then analyzed by agarose gel electrophoresis (Figure 22 – 25). A high molecular weight (> 10 kb) of both DNA extraction obtained from crude and purified soil DNA indicated that the DNA extraction method were suitable to obtained a high molecular weight DNA.

Table 47 OD 260/280 and OD 260/230 Ratio of soil sample after purified

Sample	OD260/280	OD260/230
A01	1.5858	0.5057
A02	1.8033	0.2385
A03	1.5485	0.2385
A04	1.5529	0.3423
A05	1.8016	0.0186
A06	1.5235	0.1600
A07	1.4291	0.1385
A08	1.6446	0.0929
A09	1.6415	0.1967
A10	1.5499	0.1263
A11	1.6059	0.2066
A12	1.6113	0.2517
A13	1.8919	0.5625
A14	1.9004	0.0467
A15	1.8591	0.2348

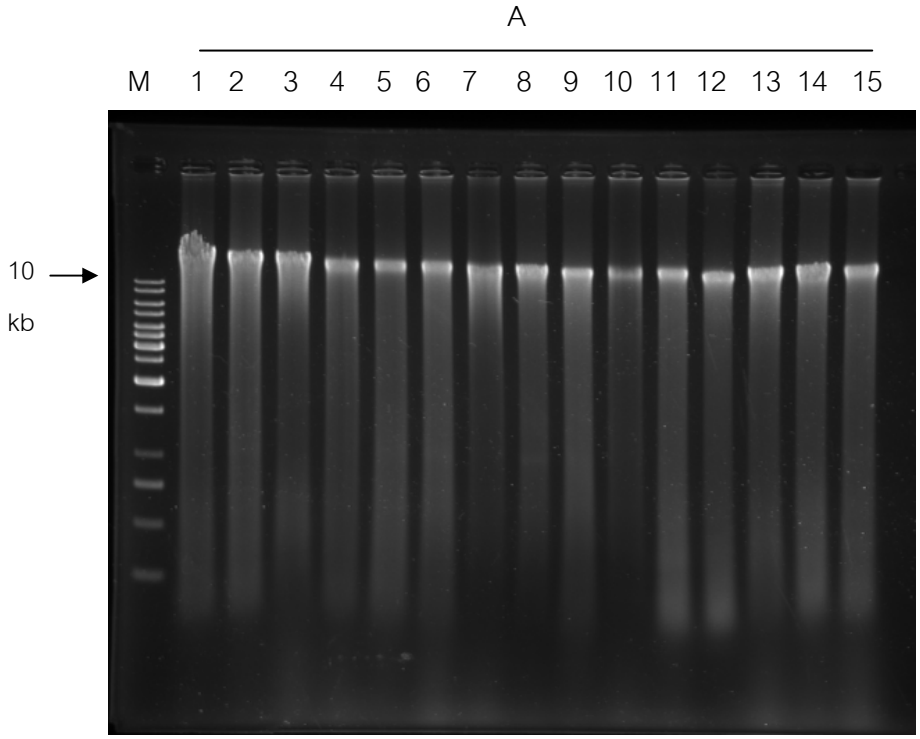


Figure 22 1% agarose gel elctrophoresis of crude soil DNA from Doi Phuka National Park of Nan province. Lane 1-15 were A01-A15 and lane M: 1 kb DNA ladder.

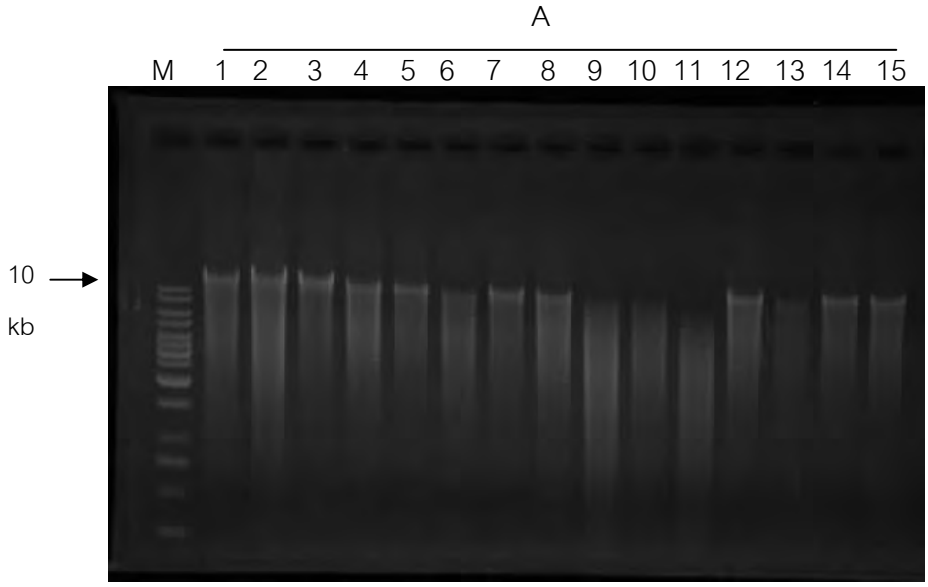


Figure 23 1% agarose gel electrophoresis of purified soil DNA from Doi Phuka National Park of Nan province. Lane 1-15 were A01-A15 and lane M: 1 kb DNA ladder.

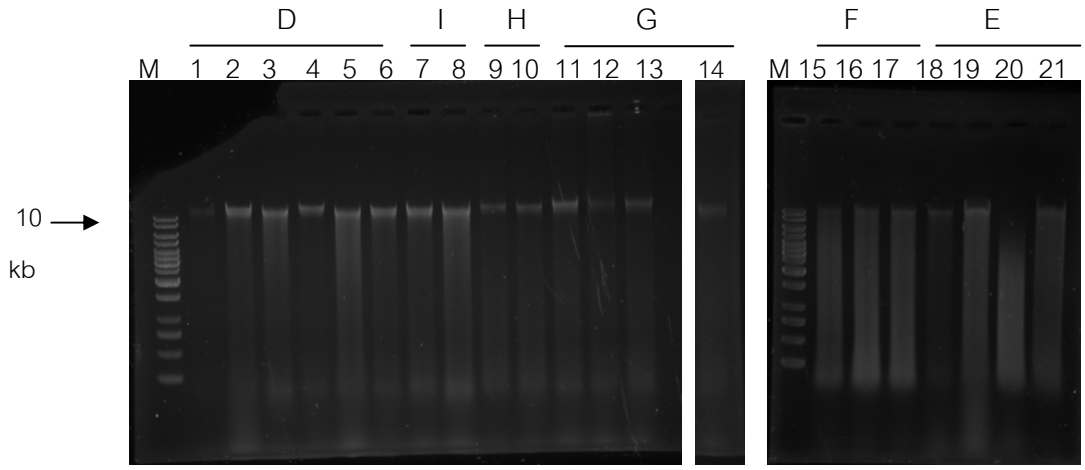


Figure 24 1% agarose gel elctrophoresis of Crude soil DNA from Kao Keaw National Park of Chonburi province (D01- D06) lane1-6, paddy field (I01-I02, H01-H02, and G01-G04) lane 7-14, and mangrove forest (F01-F03, and E01-E04) lane 15-21, respectively. Lane M: 1 kb DNA ladder.

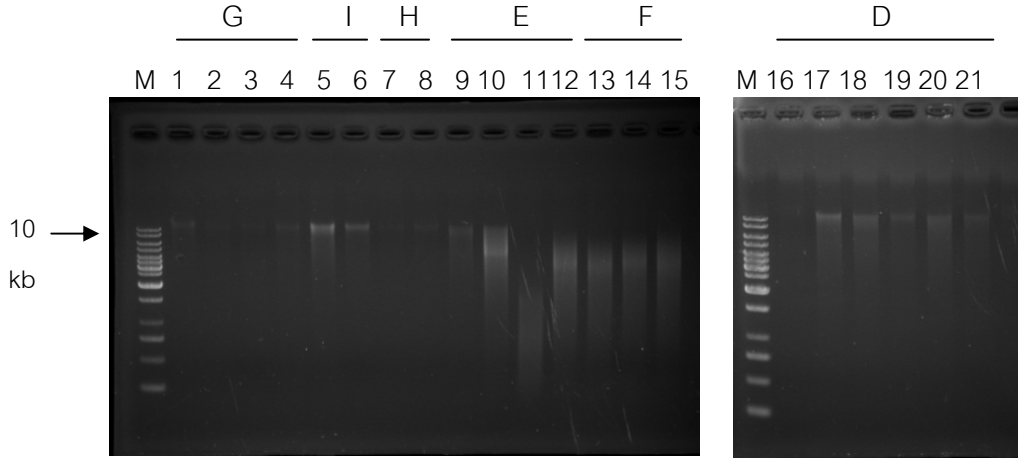


Figure 25 1% agarose gel elctrophoresis of purified soil DNA from paddy field (G01-G04 I01-I02, and H01-H02) lane 1-8, mangrove forest (E01-E04 and F01-F03) lane 9-15, and Kao Keaw National Park of Chonburi province (D01- D06) lane 16-21, respectively. lane M: 1 kb DNA ladder.

4.8 PCR amplification of soil DNA

By using eubacterial primer pair (fD1 + rP2) the expected size of 1.5 kb were obtain from some soil DNA samples from mountain forest of Nan provice. Addition of 5% DMSO in the PCR rection was shown in Figure 26 - 27 resulting in less amplification compared to the PCR reaction without DMSO. Figure 28 – 29 shown the amplification product using purified soil DNA from paddy field, mangrove forest, and Kao Keaw National Park.

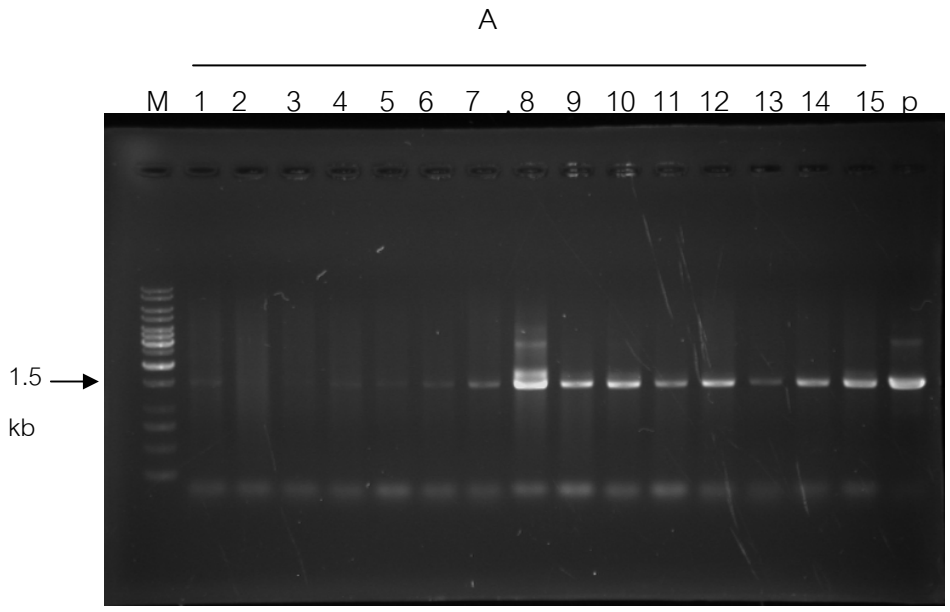


Figure 26 1% agarose gel elctrophoresis of PCR product amplified from purified soil DNA from Doi Phuka National Park of Nan province. Lane 1-15 were A01-A15, lane M: 1 kb DNA ladder, lane p was the positive control using *S. narbonensis* DNA as a template.

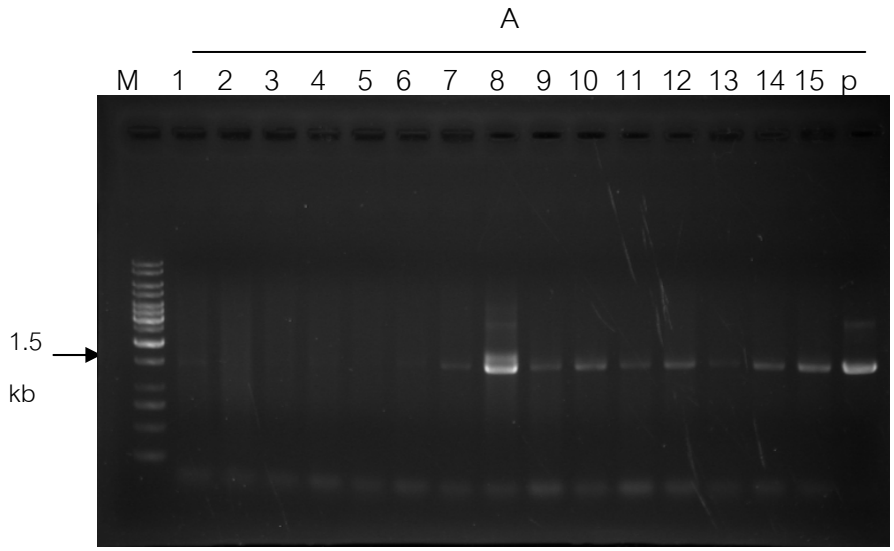


Figure 27 1% agarose gel elctrophoresis of PCR product amplified from purified soil DNA from Doi Phuka National Park of Nan province with addition of 5% DMSO to the PCR reaction. Lane 1-15 were A01-A15, lane M: 1 kb DNA ladder, lane p was the positive control using *S. narbonensis* DNA as a template.

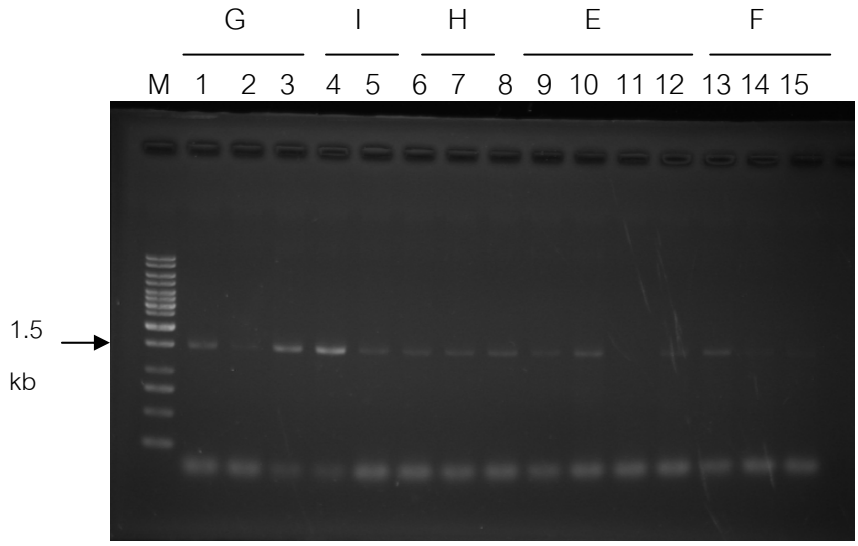


Figure 28 1% agarose gel elctrophoresis of PCR product amplified from purified soil DNA from paddy field (G01-G04, I01-I02, and H01-H02): lane 1 -8 and mangrove forest (E01-E04 and F01-F03): lane 9-15.

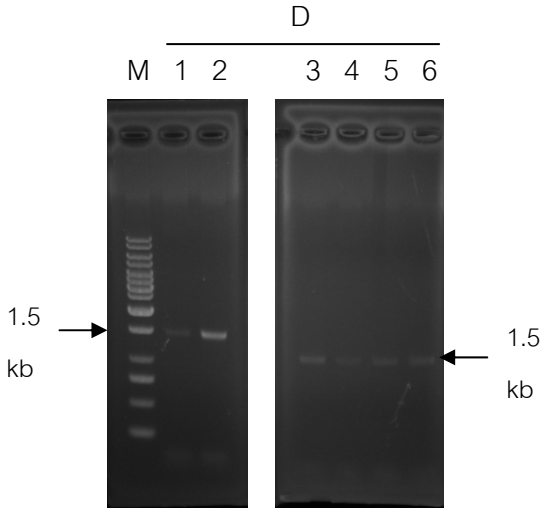


Figure 29 1% agarose gel elctrophoresis of PCR product amplified from purified soil DNA from Kao Keaw National Park of Chonburi province (D01-D06): lane 1-6.

4.9 Nested PCR amplification of 16S rDNA (StrepBF region)

A nested PCR performed by using *Stretomyces* specific primer (StrepB + StrepF) and PCR diluted (5 to 50 fold) from fist amplification products were used as a DNA template. Figure 30 – 32 were amplification product about 1 kb obtained using purified soil DNA from Doi Phuka Nationalpark, paddy field, and mangrove forest, respectively.

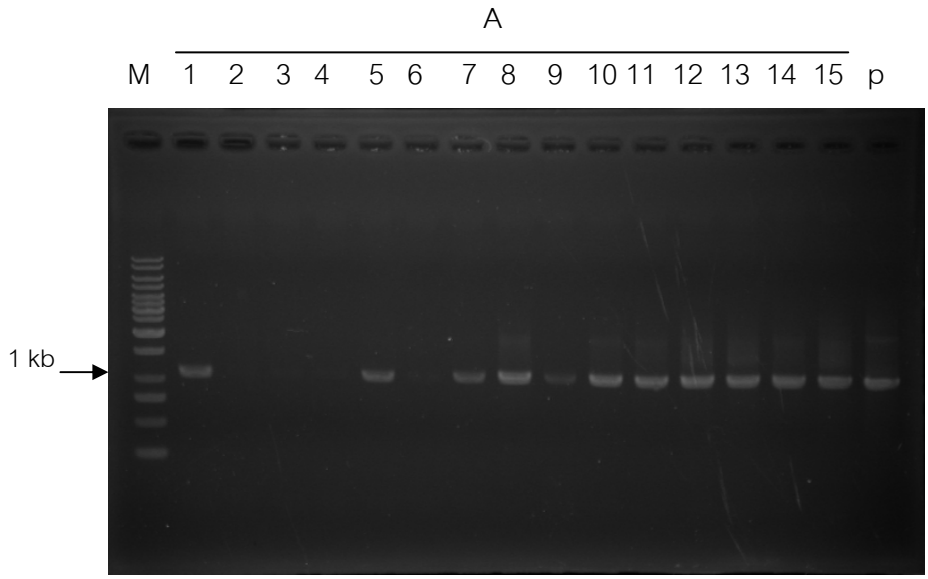


Figure 30 1% agarose gel elctrophoresis of PCR product amplified from nested PCR, first amplification product was used as a template from Doi Phuka National Park of Nan province. Lane 1-15 were A01-A15, lane M: 1 kb DNA ladder, lane p is the positive control using *S. narbonensis* DNA as a template.

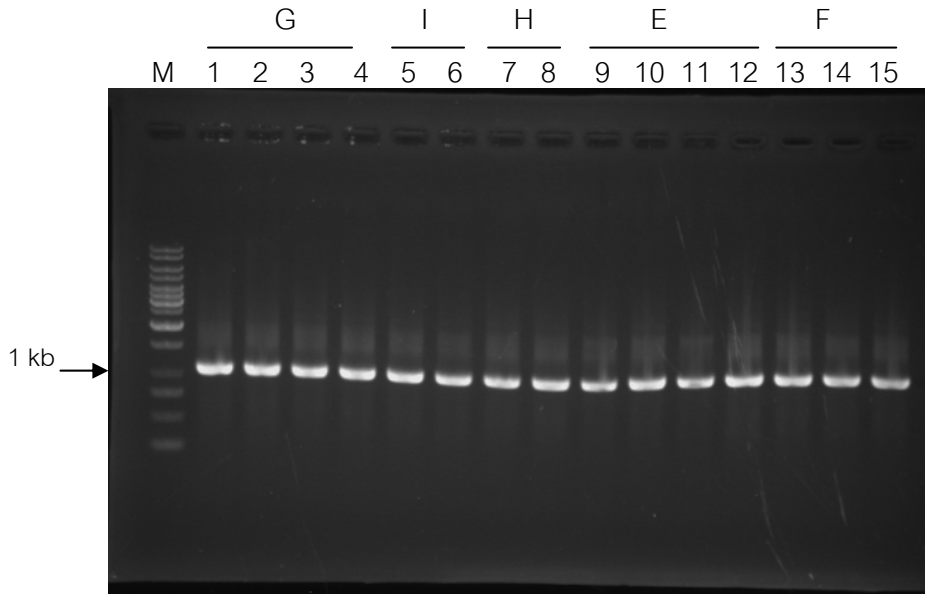


Figure 31 1% agarose gel elctrophoresis of PCR product amplified from nested PCR, first amplification product was used as a template. Soil DNA samples were from paddy field and mangrove forest. Lane 1 -15 were G01-04, I01, I02, H01, H02, E01-E04, and F01-03, respectively. M was 1 kb DNA ladder.

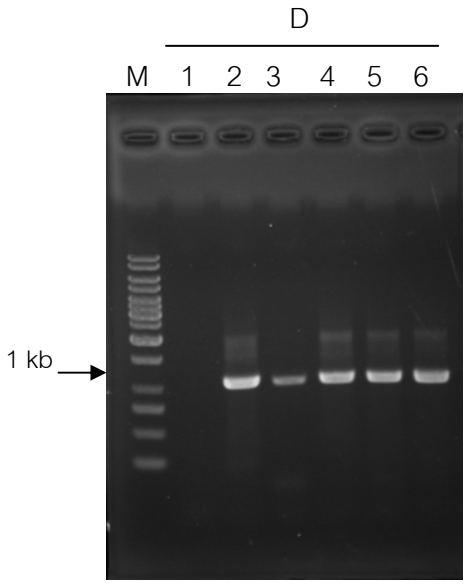


Figure 32 1% agarose gel elctrophoresis of PCR product amplified from nested PCR, first amplification product was used as a template. Soil DNA samples were from Khokeaw National Park. Lane 1-6 were D01-D06 respectively. M was 1 kb DNA ladder.

4.10 16S rDNA library construction

Single PCR products or pooled PCR samples (2-6 PCR products) were pooled according to soil pH range. The PCR products were purified by gel extraction (Qiagen) and amount of DNA were compared to the know concentration of DNA ladder. The purified fragments were ligated with TA cloning vector (RBC) for 4°C overnight and cloneed into *E. coli* competent cells. Positive clones were screened based on blue-white screening on alpha-complementation of Lac Z' resulting a white colony which contain insert. Table 48 showed pooled or single PCR product used in RFLP analysis.

Table 48 Single or pooled samples according to soil pH range

Location	Sample	Code	soil pH
Mountain	A01	SA01	5.09 – 5.89
forest	A05, A09, A10, and A12	PA02	3.58 – 3.97
(MT)	A07, A08, A11, A13, A14, and A15	PA03	4.04 - 4.94
	D01, D02, and D06	PD01	8.22 – 8.53
	D03	SD03	6.58
	D04	SD04	7.20
	D05	SD05	5.98
Paddy field	G01, G02, G03, and G04	PG01	4.43 – 5.29
(PD)	I01	SI01	8.51
	I02	SI02	7.93
	H01 and H02	PH01	4.79 – 5.20
Mangrove	E01	SE01	5.88
forest	E02 and E03	PE02	6.81 – 6.89
(MG)	E04	SE04	7.63
	F01, F02, and F03	PF01	7.13 – 7.25

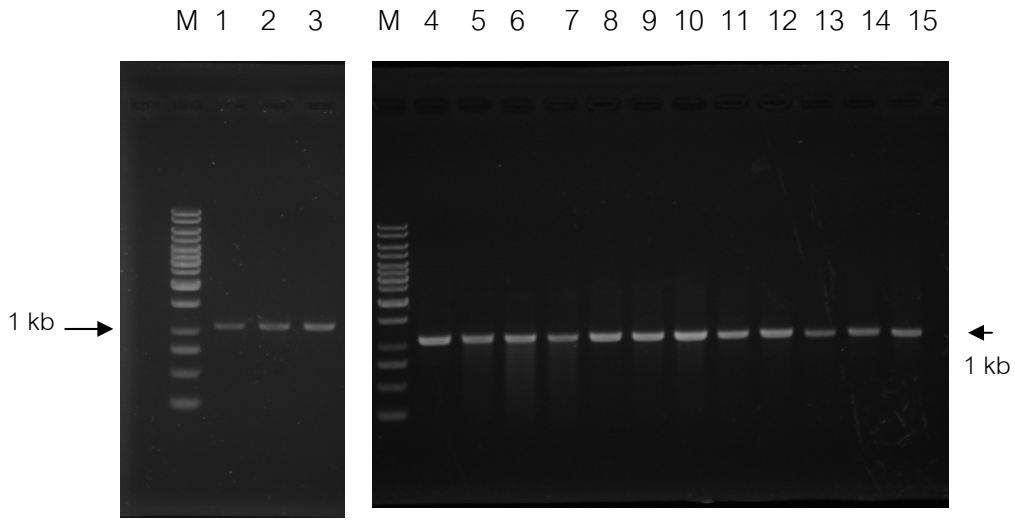


Figure 33 1% agarose gel elctrophoresis of purified pooled PCR products or single PCR product to be ligated with TA cloning vector. M: 1 kb DNA ladder, lane1: sample A01, lane2: pooled samples of A05, A09, A10, and A12 lane3: pooled samples of A07, A08, A11, A13, A14, and A15, lane 4: pooled samples of G01, G02, G03, and G04, lane 5: sample I01, lane6: sample I02, lane 7: pooled sample H01 and H02, lane 8: sample E01, lane 9: pooled sample E02 and E03, lane10: sample E04, lane11: pooled sample F01, F02, and F03, lane12: pooled sample D01, D02, and D06, lane13: sample D03, lane14: sample D04, and lane15: sample D05.

4.10.1 Screening of 16S rDNA clones

In the initial screening of 16S rDNA clones (blue-white screening), white *E. coli* colonies that contained correct DNA insertion were identified by direct amplification of the inserted DNA fragment with a stretpomyces 16S rDNA primer (StrepB and StrepF). To reduce experimental time cost, the PCR was carried out with boiled colony PCR as the DNA template. Figure 34 to 39 indicated that colonies with correct PCR product length (1,074 bp or about 1 kb) were easily differentiated from colonies containing plasmid without insert or incorrect insert.

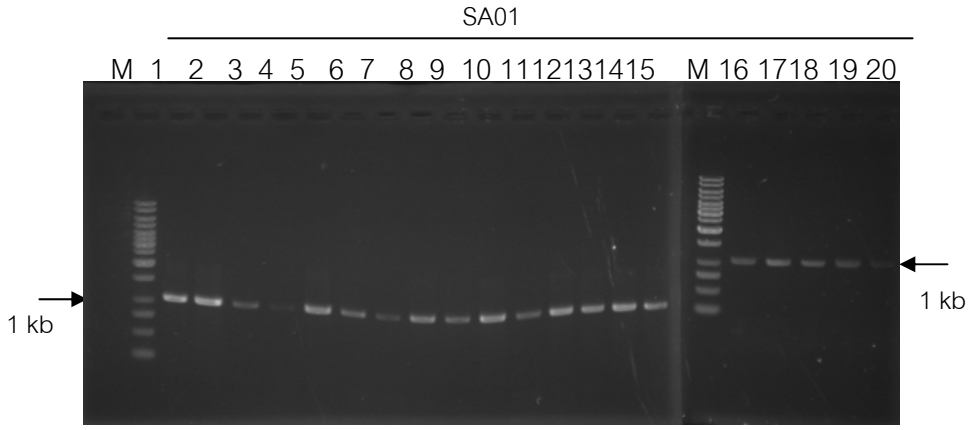


Figure 34 1 % agarose gel electropheresis of boiled colony PCR from SA01. Lane 1 – 20 were clone 1 – 20. Lane M: 1 kb DNA ladder.

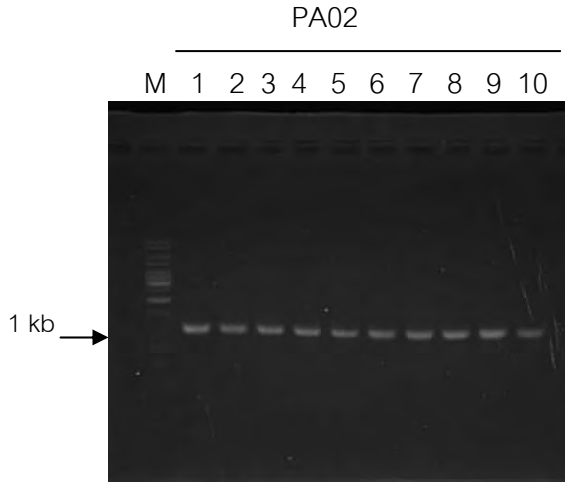


Figure 35 1 % agarose gel electropheresis of boiled colony PCR from PA02. Lane 1 – 10 were clone no. 1 – 10. Lane M: 1 kb DNA ladder.

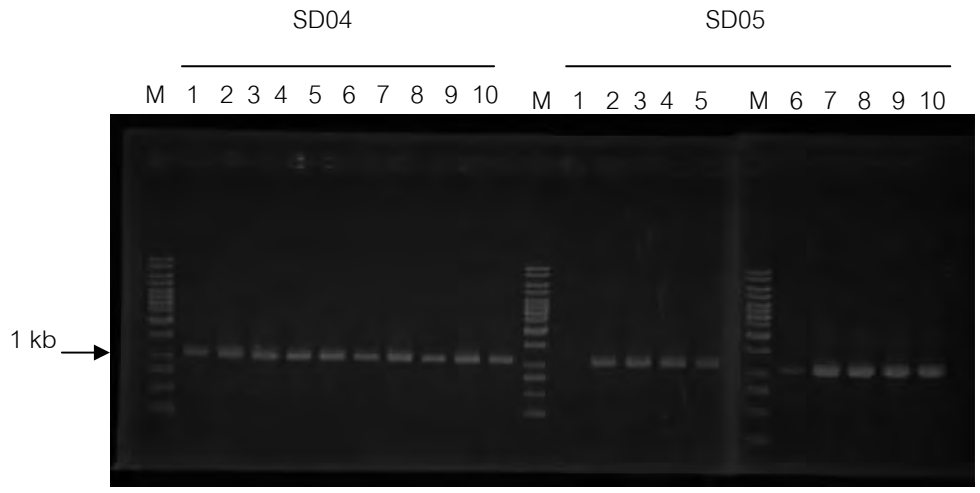


Figure 36 1 % agarose gel electropheresis of boiled colony PCR from SD04 and SD05. Lane 1 -10 were clone no. 1 – 10. Lane M: 1 kb DNA ladder.

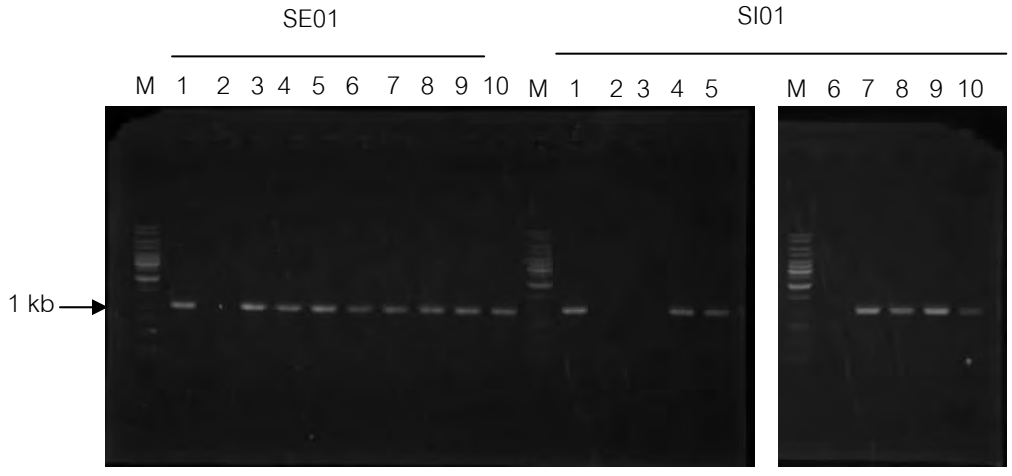


Figure 37 1 % agarose gel electropheresis of boiled colony PCR from SE01 and SI01. Lane 1 -10 were clone no. 1 – 10. Lane M: 1 kb DNA ladder.

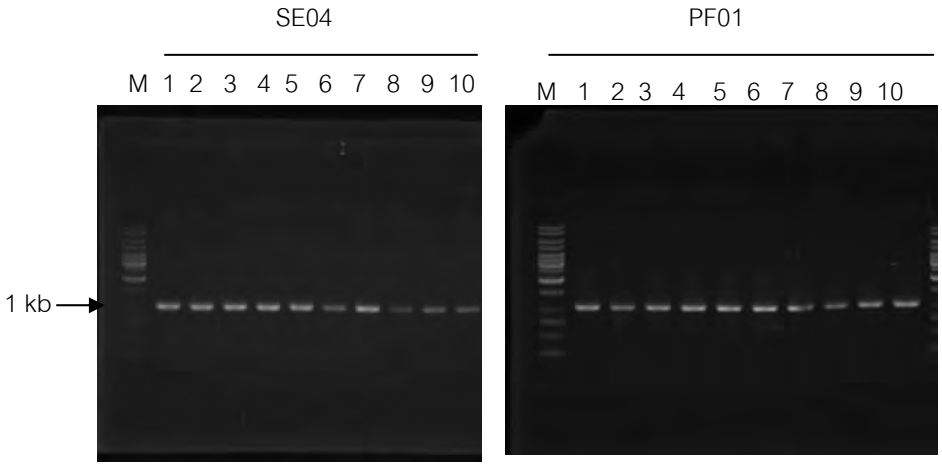


Figure 38 1 % agarose gel electropheresis of boiled colony PCR from SE04 and PF01. Lane 1 -10 were clone no. 1 – 10. Lane M: 1 kb DNA ladder.

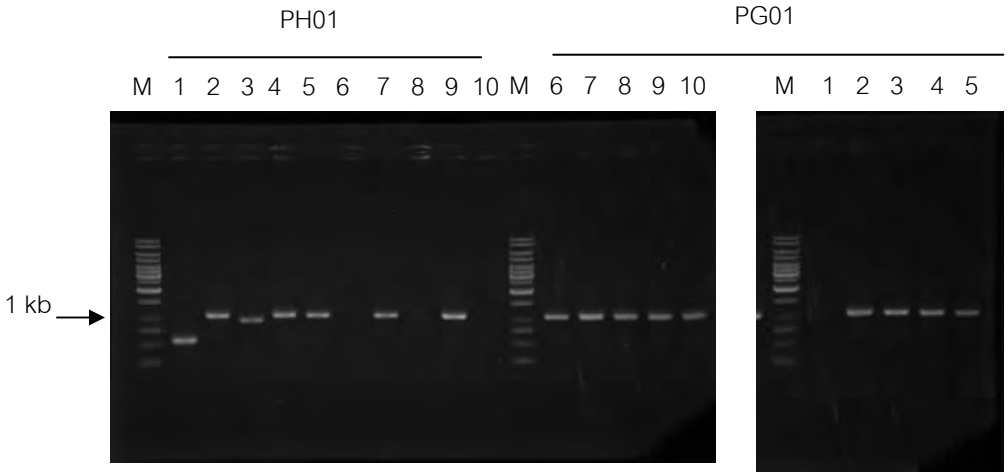


Figure 39 1 % agarose gel electropheresis of boiled colony PCR from PH01 and PG01. Lane 1 -10 were clone no. 1 – 10. Lane M: 1 kb DNA ladder.

4.11 Analysis of species diversity in a model bacterial community by RFLP

In order to test the approach from a computer simulated analysis which suggest that polymorphisms among restriction fragment lengths could be used to characterize *Streptomyces*, *Actinomyces* and other bacterial community, a model community containing three known *Streptomyces* (*S. venezuelae, S. narbonensis,* and *S. lividans*, *S. hygroscopicus*, and *S. rimosus*) were constructed. Figure 40 shown the PCR products about 1 kb from different *Streptomyces* species which were subsequenly digested with restriction enzyme (MspI) using 3% agarose gel in 1X TBE and electrophoresed at 80 Volts for 2.30 hr. compared to 2% agarose gel with the same condtion as shown in Figure 41 – 42.

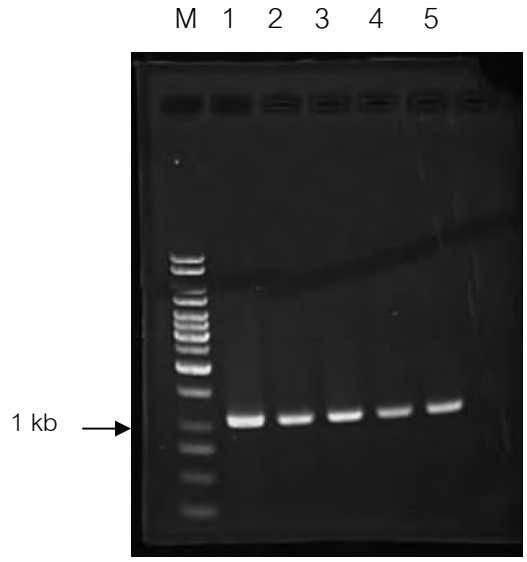


Figure 40 Boiled single colony PCR obtained directly from culture plate (NS media) using streptomyces specific primer (StrepB+StrepF). Lane M *1 kb DNA ladder,* lane 1: *S. venezuelae,* lane 2: *S. narbonensis,* lane 3: *S. lividans*, lane 4: *S. hygroscopicus*, and lane 5: *S. rimosus*.

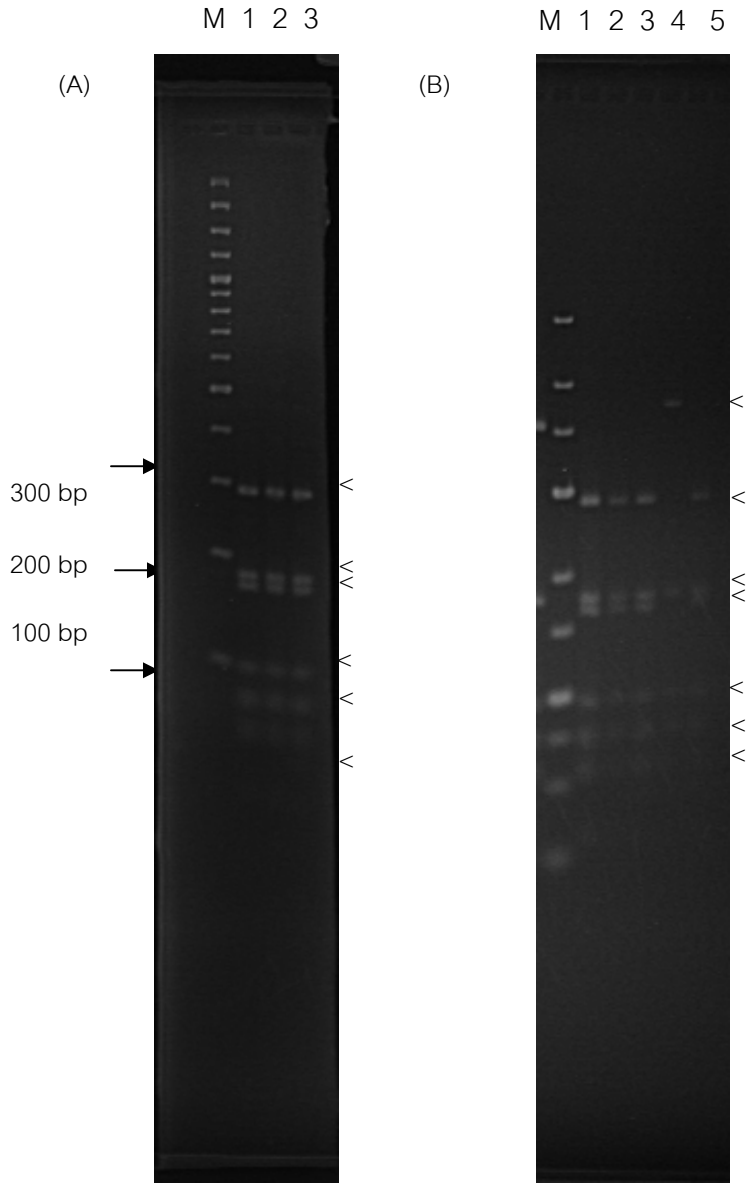


Figure 41 3% agarose gel electrophoresis of digested fragments using *Msp*I from *S. venezuelae* (1), *S. narbonensis* (2), *S. lividans* (3), *S. hygroscopicus* (4), and *S. rimosus* (5) in 1X TBE. Electropheresed was 80 Volt 2.30 hr. on 20 x 20 cm size of agarose. M: 100 bp lader. An > indicate a fragment obtain from electrophoresis. (A) was genomic DNA that obtained from extraction method (Kutchma, 1998) and (B) was boiled colony from single colony plated was used as DNA template.

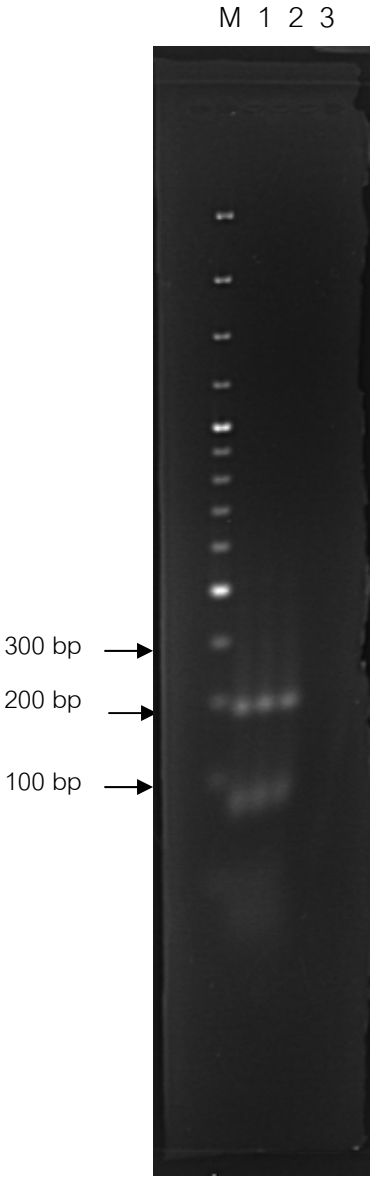


Figure 42 2% agarose gel electrophoresis of digested DNA fragment using *Msp*I from *S. venezuelae* (1), *S. narbonensis* (2) and *S. lividans* (3) in 1X TBE. Electropheresed was 30 Volt 20 hr. on 20 x 20 cm size of agarose gel. M: 100 bp lader.

4.12 Restriction Fragment analysis of 16S rDNA

10 – 17 µl of PCR product from 16S rDNA amplification were digested with *Msp*I (isoschizomer *Hpa*II) restriction enzyme with a high average number of restriction sites and phylogeneticaly related with bioactivity. Boiled colonies PCR amplification products obtained directly from 16S rDNA clone library. Resulting RFLP patern were shown in figure 43 - 46. The 16 OTUs obtained from 100 clones were shown in Figure 47. The summary of RFLP analysis were shown in Table 49.

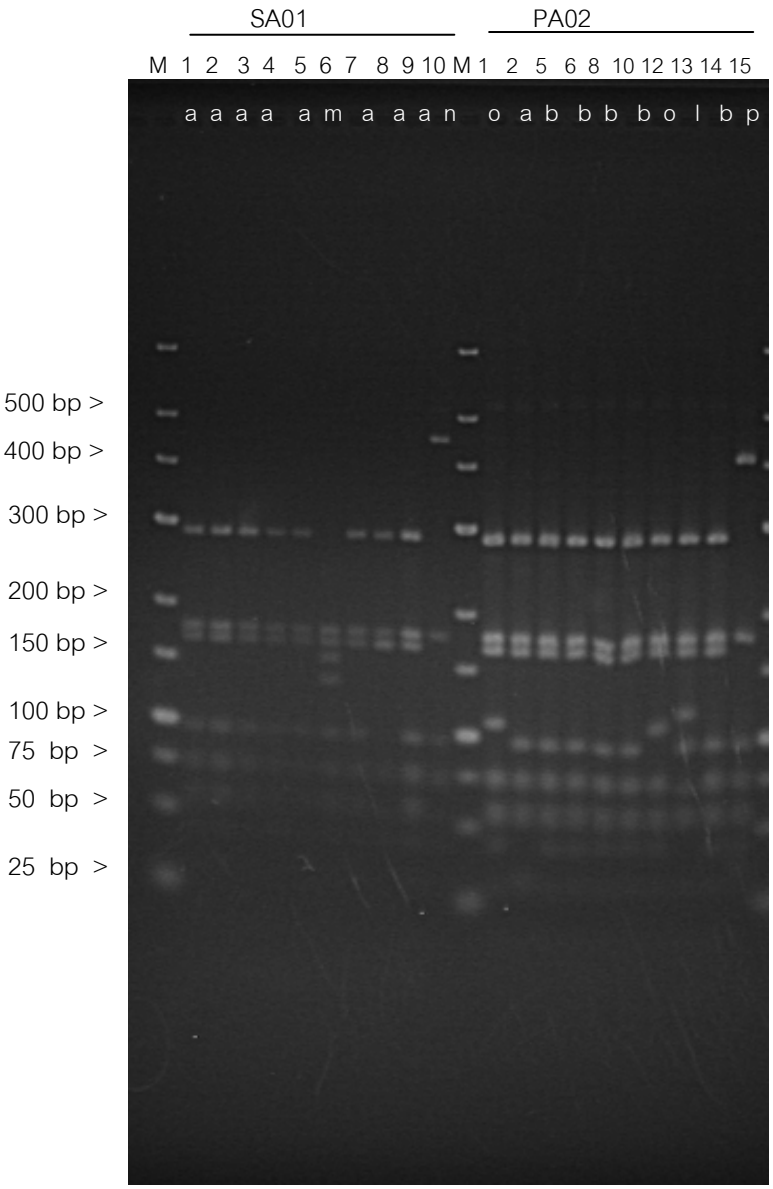


Figure 43 Restriction fragment length polymorphism patterns of 10 clones from 16S rDNA library (pooled or single PCR products from forest mountain sample in Nan province) after digestion by *Msp*I. Shown was a EtBr stained gel (3% agarose). Lane M represents Low Range DNA ladder, and numerical of each lane represent 10 different 16S rDNA clones. Lower case letters indicated RFLP type.

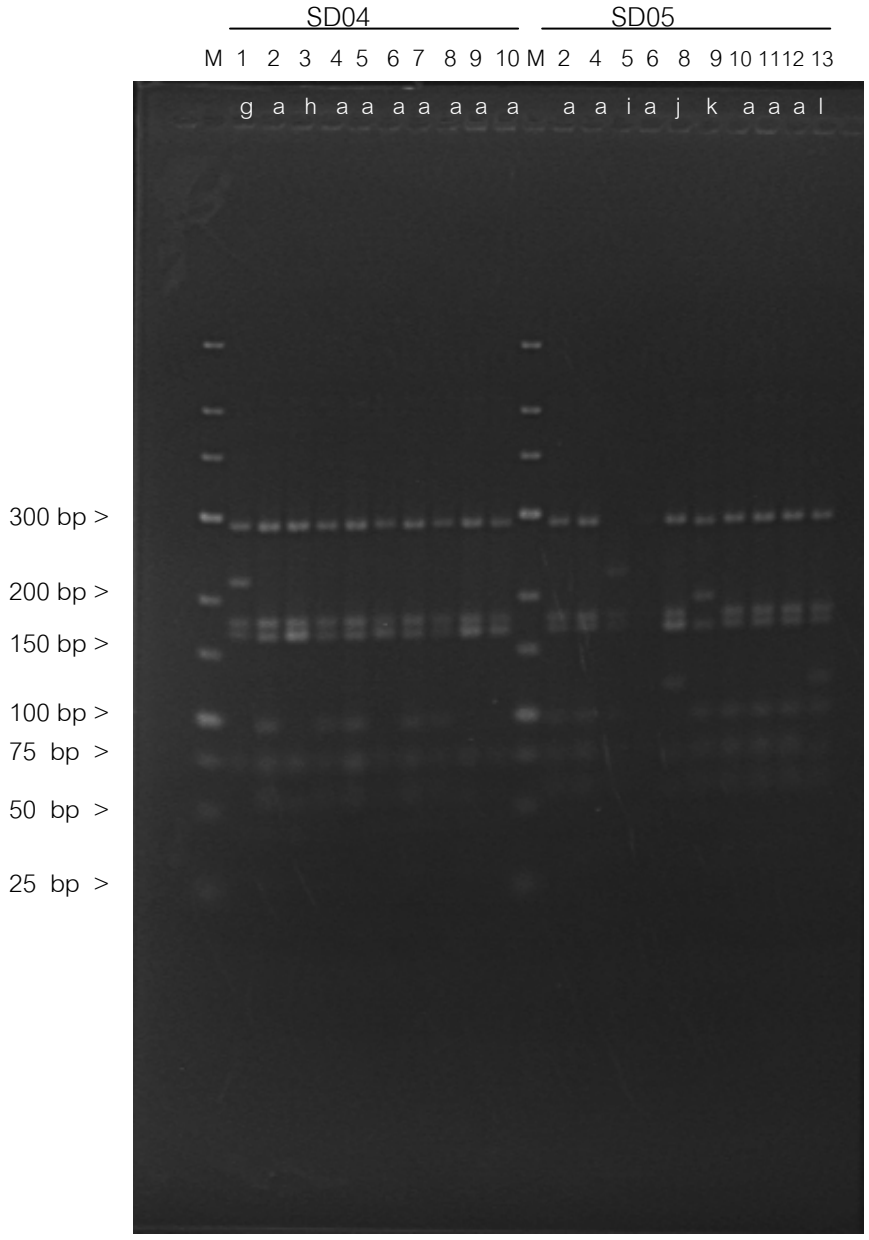


Figure 44 Restriction fragment length polymorphism patterns of 10 clones from 16S rDNA library (pooled or single PCR products from forest mountain sample in Chonburi province) after digestion by *Msp*I. Shown was a EtBr stained gel (3% agarose). Lane M represents Low Range DNA ladder, and numerical of each lane represent 10 different 16S rDNA clones. Lower case letters indicated RFLP type.

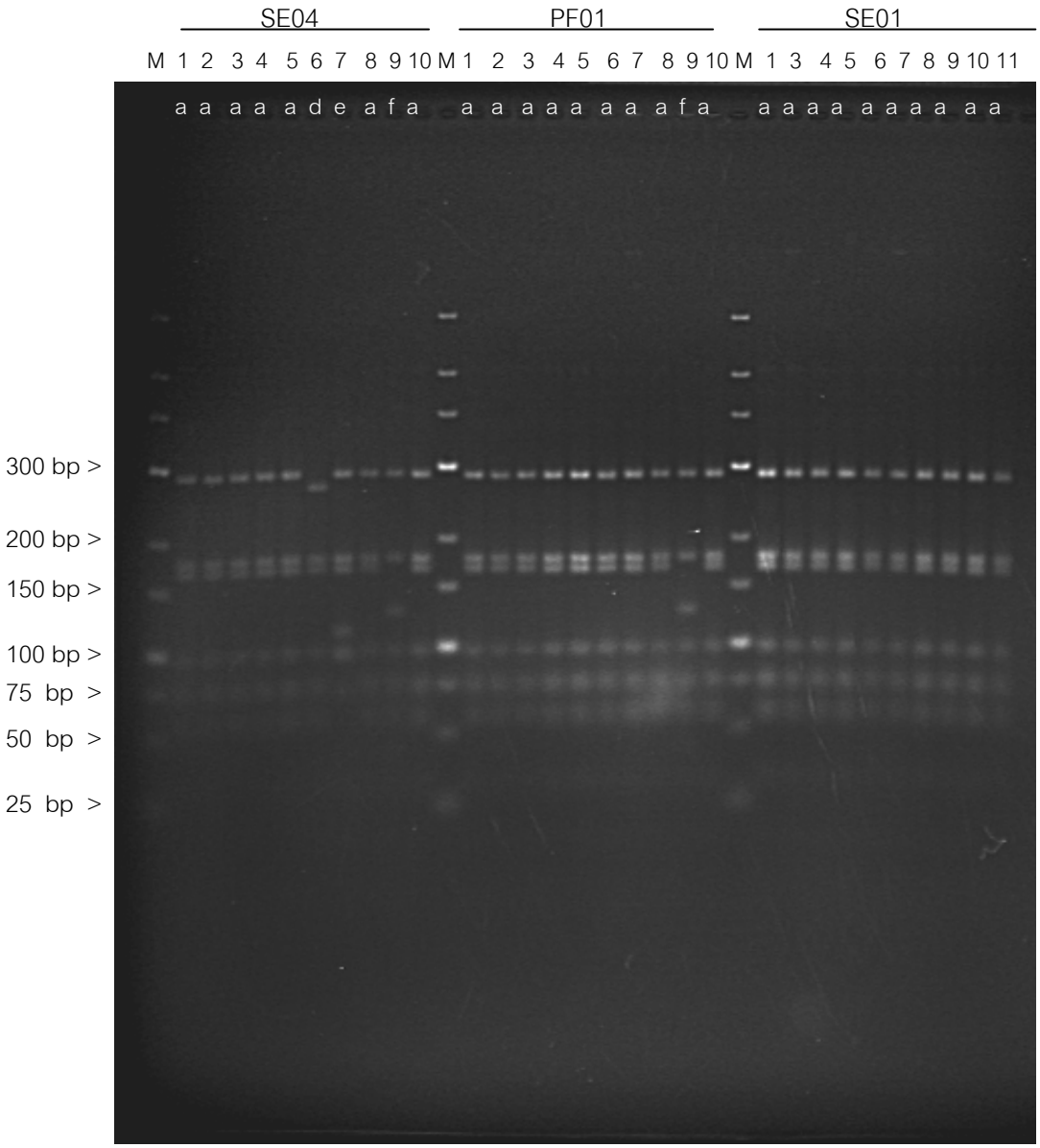


Figure 45 Restriction fragment length polymorphism patterns of 10 clones from 16S rDNA library (pooled or single PCR products from mangrove forest sample) after digestion by *Msp*I. Shown was a EtBr stained gel (3% agarose). Lane M represents Low Range DNA ladder, and numerical of each lane represent 10 different 16S rDNA clones. Lower case letters indicated RFLP type.

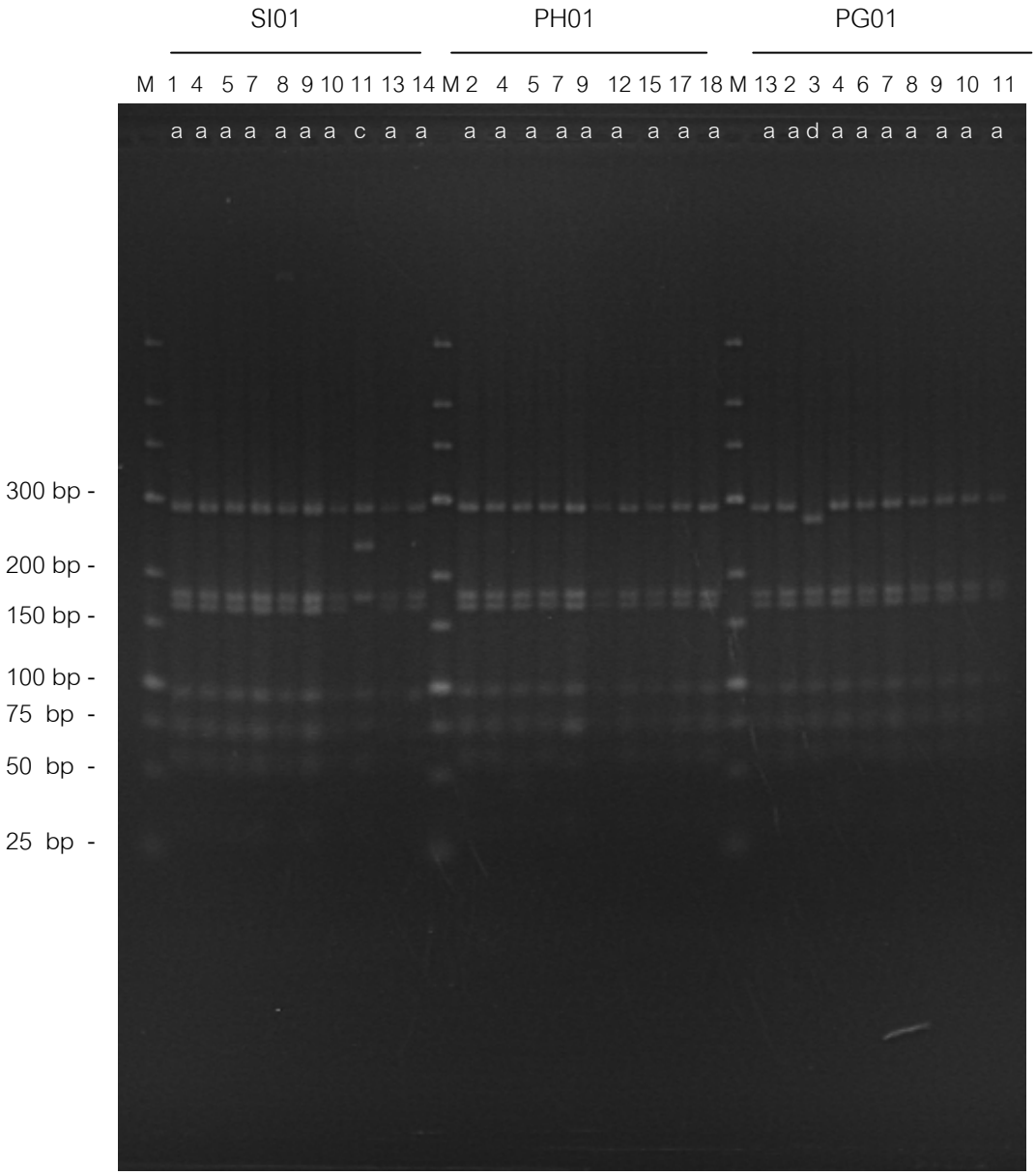


Figure 46 Restriction fragment length polymorphism patterns of 10 clones from 16S rDNA library (pooled or single PCR products from paddy field sample) after digestion by *Msp*I. Shown was a EtBr stained gel (3% agarose). Lane M represents Low Range DNA ladder, and numerical of each lane represent 10 different 16S rDNA clones. Lower case letters indicated RFLP type.

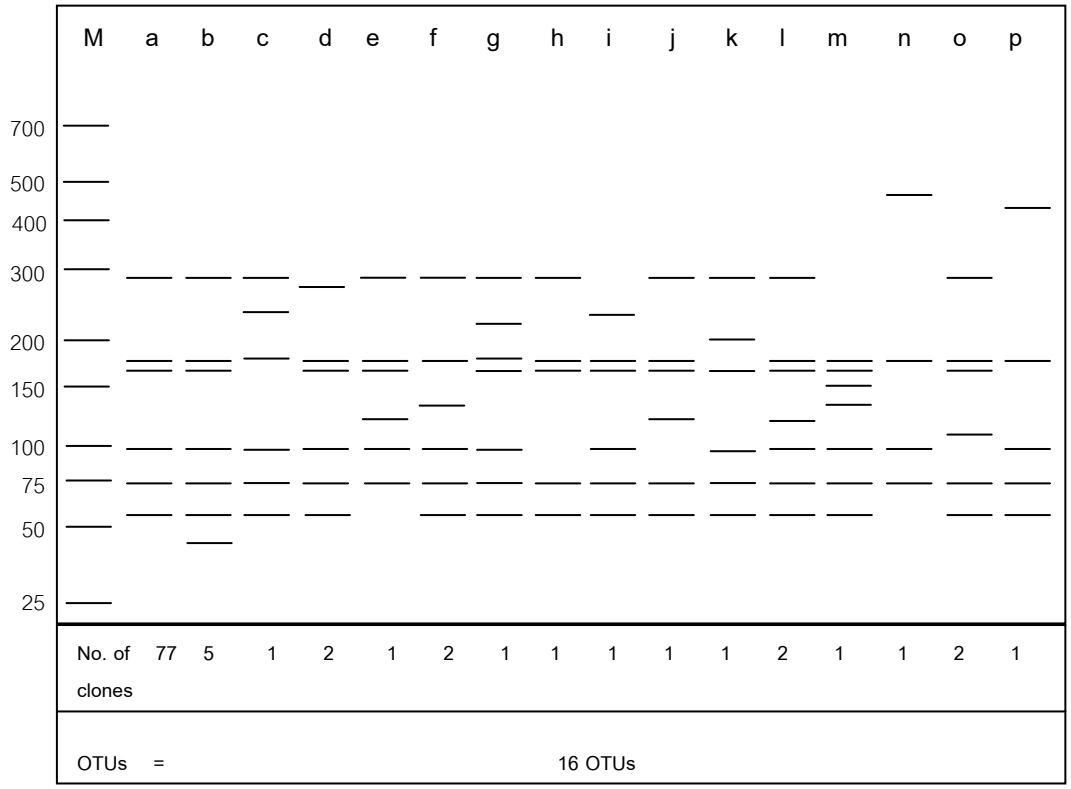


Figure 47 Scaled illustration summarized the restriction patterns of 16S rDNA digested with *Msp*I from a EtBr stained gel (3% agarose) representing 16 clones OTUs identified in 100 soil clone libraries. Each of these OTUs contained one or more representative clones indicated by lower case letters (lane a to p). M was low range DNA ladder (Fermentas) given in bp. The table at the bottom of the figure showed the numerical distribution of the soil clones and total OTUs detected.

Table 49 Summary of RFLP analysis

Location	Sample	No. of clones	Total no. of RFLP type	RFLP type to clone no. ratio	RFLP type	No. of restriction patterns digested with *Msp*I
Mountain	SA01	10			a (8), m (1), n (1)	3
forest	PA02	10	12	0.30	a (1), b (5), o (2), l (1), p (1)	5
(MT)	SD04	10			a (8), g (1), h (1)	3
	SD05	10			a (6), i (1), j (1), k (1), l (1)	4
Mangrove	SE04	10			a (7), d (1), e (1), f (1)	4
forest	PF01	10	4	0.13	a (9), f (1)	2
(MG)	SE01	10			a (10)	1
Paddy	SI01	10			a (9), c (1)	2
field	PH01	10	3	0.1	a (10)	1
(PD)	PG01	10			a (9), d (1)	2

CHAPTER V

DISCUSSION AND CONCLUSION

1. Restriction Fragment Length Polymorphism (RFLP) pattern in the 16S rDNA database

The ability of RFLP analysis to distinguish phylogenetic group of bacteria was theoretically evaluated through a computer simulation of the restriction patterns for 79 bacterial sequences including; 70 sequences in genus *Streptomyces*, 6 sequences from *Actinomyces*, and 3 sequences from outgroup bacteria (*E. coli*, *B. subtilis*, and *P. putida*). The sequences were retrieved from RDP database. A streptomyces specific primers from Rintala et al. (2001) was selected to anneal to 16S rDNA sequences in the RDP database. After digestion with thirty-three different restriction enzymes, the average cut site and pattern simulation were determined. From tetrameric (4-bp) restriction enzymes used, they gave 3-15 RFLP patterns (OTUs) with average number of restriction sites 2.00-17.00, where as hexameric (6-bp) restriction enzymes gave 1-5 patterns with average number of restriction sites 0.52-2.00. Therefore, hexameric restriction enzymes were omitted in the following analysis because of their insufficient RFLP data. Although, the *Aci*I (tetrameric restriction enzymes) gave highest average number of restriction sites (17.00), but yielded ambiguous results. It produced many near-coincident double or triple bands and some fragments too small to be resolved by the electrophoresis system. Therefore, *Aci*I was not selected. Some ambiguous results were caused by unexpected size fragments, most commonly those including end points of sequences (Moyer et al., 1996). The 1 kb 16S rDNA sequence yielded restriction fragments in the size range from 4 bp (lower limit of detection was able to detect in gel simulation) to 500 bp. In a DNA sequence, a 4-bp (equivalent to tetrameric) restriction enzyme recognition site would occur every 256 bases. Therefore, the use of a 4-bp theoretically would yield 4 restriction sites with a 1 kb gene fragment. Because of the moderately higher G+C contents of 16S rRNA genes [most bacterial 16S rDNAs have G+C components between 55 and 65% (Moyer et al., 1994)] the use of tetrameric

restriction enzymes such as *Hpa*II (isoshizomer of *Msp*I) and *Hae*III which recognizes GC sequence for *in silico* digestion yielded average number of restriction sites 9.6 and 10.5 from seventy *Streptomyces* 16S rDNA sequences (StrepBF region).

Some *Streptomyces* species could be assigned to a specific 16S rDNA genotype (inferred from unique RFLP pattern which equivalent to OTU), supporting the application of this analysis for identification. Informative characters (0 and 1) were scored from restriction pattern obtained from each of thirteen restriction endonulceases. Thirteen NJ trees (Figure 13) were constructed from the RFLP data sets from each restriction endonuclease *(Bfa*I, *Dpn*I, *BstU*I, *Hha*I, *Mse*I, *Nla*III, *Rsa*I, *Taq*I, *Alu*I, *Hae*III, *Mn*II, *Hpa*II, and *Aci*I), the distance matrix calculation using Nei-Li method from PAUP* program. The results showed that four tetrameric restriction enzymes (*Hha*I, *Rsa*I, *Alu*I, and *Hpa*II (isoschizomer of *Msp*I) could clearly rooting the out group species, supported the correct phylogenetic relationship from using 16S RFLP data. Moyer et al. (1996) reported that a combination of three tetrameric restriction enzymes (*Hha*I, *Rsa*I, and *BstU*I) gave good resolution based on their computer-simmulated groups and phylegeny of bacterial species based on 16S rDNA (1.5 kb). Furthermore, for the Gram-positive phylum, the used of tetrameric restriction enzymes *Mbo*I, *Hinf*I, *Taq*I, and *Hae*III gave highest percentage of successful phylogenetic affiliations based on RFLP analyzing 16S rDNA (1.5 kb) data. Normally, the choice of restriction enzymes was based on practical matters such as simplicity, commercial availability, and economy (Lanoot et al., 2005; Moyer et al.,1996). However, from the results, three tetrameric restriction enzymes (*Hha*I, *Alu*I, and *Hpa*II (isoshisomer of *Msp*I) were candidates for RFLP analysis of soil *Streptomyces*, and initially, the used enzyme *Msp*I (isoschizomer of *Hpa*II) for experiment was employed because it gave a high average number of restriction sites (10.5) and gave phylogenetically resolve tree for *Streptomyces*. The *Rsa*I was omitted because it gave low OTUs (3 OTUs) compared to *Hha*I, *Alu*I, and *Hpa*II (isoshizomer of *Msp*I), which gave 14, 8, and 13 OTUs, respectively.

A neighbor-joining tree constructed based on 16S rDNA sequence (StrepBF region) suggested that *Streptomyces* could be group into a major clade whereas *Actinomyces* were in different clade. Two *Streptomyces* species (*S. lactamdurans* and *S. tenebrarius*) were not separated from *Actinomyces* suggested that they were closely related to *Actinomyces*. The bioactivity randomly distributed as shown in figure 14.

However, many 16S rDNA sequences available in databases were not complete that lack the end points, therefore using eubacterial specific primer to anneal to the sequences about less than 1,500 bp would be excluded. The use of *Streptomyces* specific primer which annealed to 16S rDNA about 1,000 bp was sufficiently enough data to generate RFLP patterns by using appropriate restriction enzyme. Intraspecies diversity by RFLP analysis of biotechnologically important *Streptomyces* such as *S. hygroscopicus* was under construction.

2. Analysis of streptomyces diversity in a model community by RFLP

The computer-simulated analysis suggested that polymorphisms among restriction fragment lengths could be used to characterize bioactive producing *Streptomyces*. To test this approach, a model community containing three *Streptomyces* species (*S. venezuelae*, *S. narbonensis*, and *S. lividans*) was constructed. The 16S rDNA (strepBF region) was amplified by PCR. The PCR products were digested with *Msp*I and the products were separated with agarose gel electrophoresis. The predicted outcome based on a computer-simulated restriction analysis of sequences from RDP database indicated that the three bioactive producing *Streptomyces* species used should have the different RFLP patterns (Figure 16). The RFLP analysis of three bioactive producing *Streptomyces* species had the predicted patterns for the fragment above 100 bp suggested that the RFLP analysis by 3% agarose gel electrophoresis had sensitivity to detect polymorphism of fragment length > 50 bp compared to 2% agarose gel which could not give clear fragments patterns. However, limitation of agarose gel to differentiated small DNA fragments below 45 bp (Moyer et al., 1996) or near-coincident bands could be difficult in RFLP analysis.

3. Analysis of *Streptomyces* diversity in soil from 16S rDNA clones libraries

Nucleic acids were successfully extracted from soil samples with the need of agarose gel extraction purification for PCR amplification. The direct lysis method used in this study was helpful in extracting DNA from soil with little damage. The ratio of OD260/230 of soil DNA extract range from 0.5625 – 0.0186 indicated slightly humic acid contamination. However, by using BSA to prevent inhibition and less amount of DNA (1 µl) in 25 µl PCR reaction would overcome these contamination since the contaminant would be diluted below inhibition level. The 1.5 kb PCR products from the amplification of 16S rDNA using eubacterial specific primers (fD1 + rP2) were checked by agarose gel electrphoresis. Since some sample gave a faint band or produced no PCR product (A01, A02, A03, A04, A05, A06, and A13) the nested PCR which were used as a template for amplification of *Streptomyces* specific 16S rDNA primer (StrepB + StrepF) was carried on. The 1 kb PCR products were detected from soil samples. The samples which gave no PCR products (A02, A03, A04, A06 and D01) were excluded to further analysis which indicated that *Streptomyces* population could not be detected in those samples. A total of 100 clones (40 from MT, 30 from MG and 30 from PD) were chosen after they tested positive for alpha-complementation in Beta-galactosidase. The correct 16S rDNA inserts (1 kb) were digested with *Msp*I to generate RFLP data which equivalent to genotyping or OTUs (Moyer et al., 1994). The 1 kb 16S rDNA insert clones yielded restriction fragments in the size range from 50 bp (lower limit of detection) to 500 bp, 4-8 fragments detected.

The RFLP fingerprint analysis of single or pooled samples (according to pH range) was listed in table 48. At MT, 11 RFLP types were detected from 40 clones, At MG, and PD 4 and 3 RFLP types were detected from 30 clones respectively. The most abundant RFLP type (a type) constituted 58%, 87% and 93% (of total clone library of each location) at MT, MG, and PD respectively. Single-type clones, which occurred only once, were abundant at MT (20%) but were much less common at MG (7%) and PD (7%).

Thus, the 16S rDNA RFLP analysis should contribute to simple classification of natural soil *Streptomyces*. The distribution if 16S rDNAs clones within a library ultimately should approximate the relative distribution of soil bacteria in the habitat. However, the possibility of selection during the DNA extraction process exists, and card must be taken to achieve the highest possible level of efficiency in soil extraction and DNA recovery to avoid selection prior to PCR amplification of the cloned 16S rDNA.

Conclusions

RFLP analysis linked with DNA databases should be particularly helpful in selection of restriction enzyme used in study *Stretpomyces* diversity such as *Msp*I (*Hpa*II), *Hha*I, and *Alu*I, and studies requiring rapid examination of numerous clones from different soil samples. The number of OTUs and abundant of each OTUs were estimated. This approach was applied to the *Streptomyces* community in soil and showed that the *Streptomyces* community is dominated by 1 OUT (RFLP type a) and contained at least 16 OTUs, entities analogouse to *streptomyces* species. In comparison with computer-simulate RFLP which detect at least 9 OTUs from 70 *Stretpomyces* 16S rDNA strepBF region. Three OTUs (l, m, and f type) were found which corresponded to *S. lavendulae* (growth promotant producer, *Micromonospora olivasterospora* (antibacterial producer), and *Thermomonospora chromogena*, respectively. Rapid examination to estimate soil *Streptomyces* diversity by using *Msp*I (tetrameric restriction enzyme) to detect OTUs by an RFLP analysis of PCR-amplified 16S rDNA clone libraries was developed.

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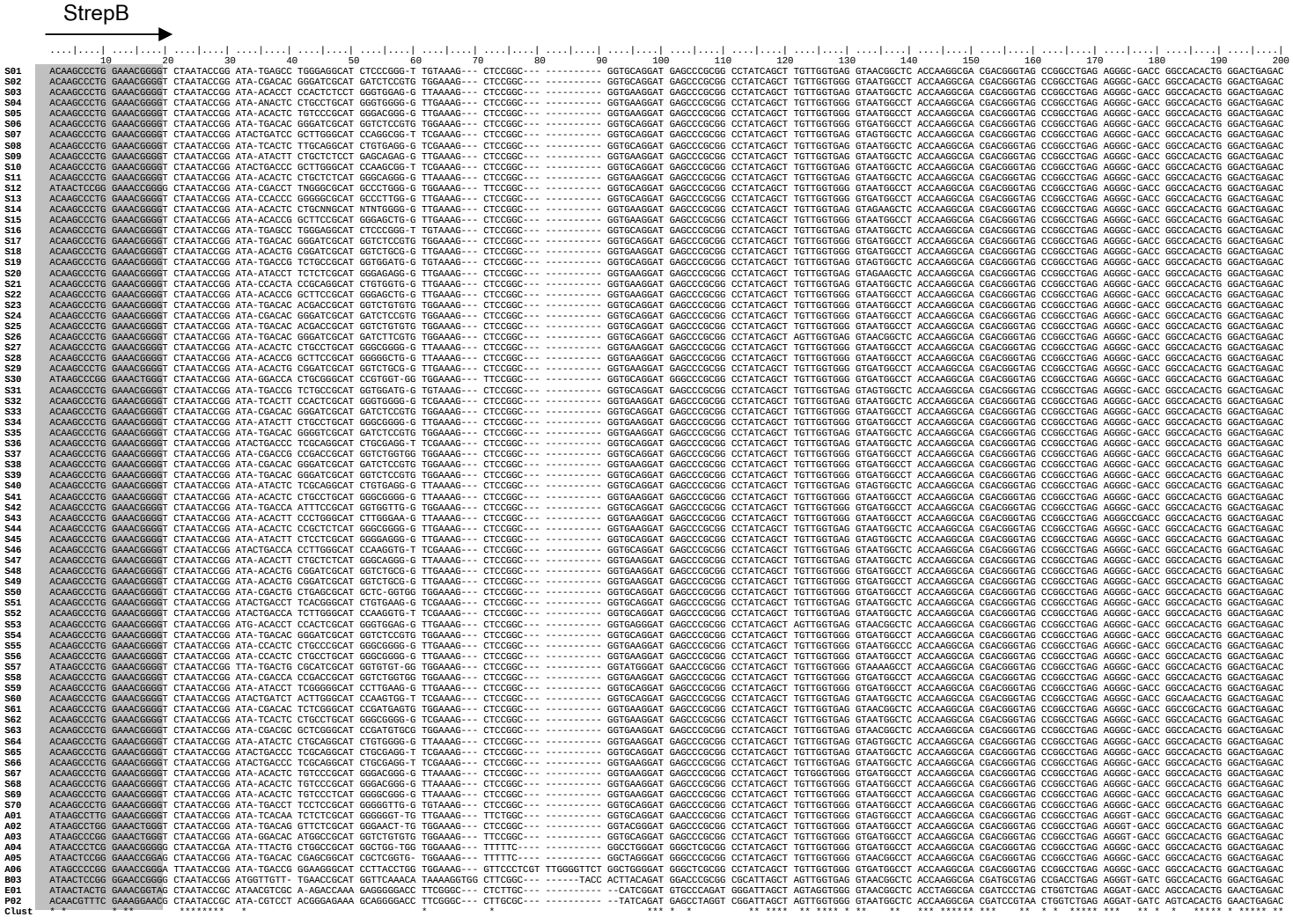
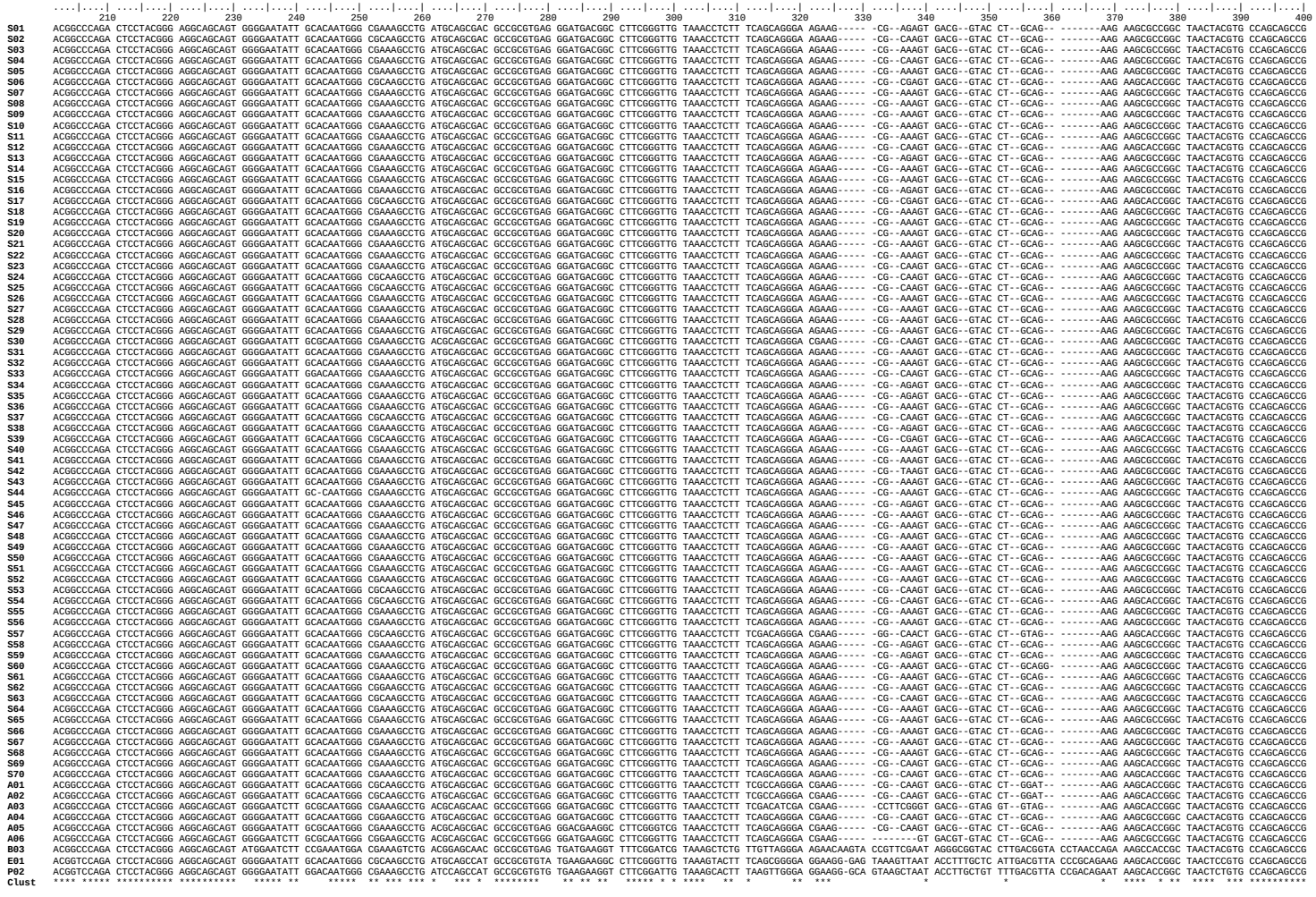
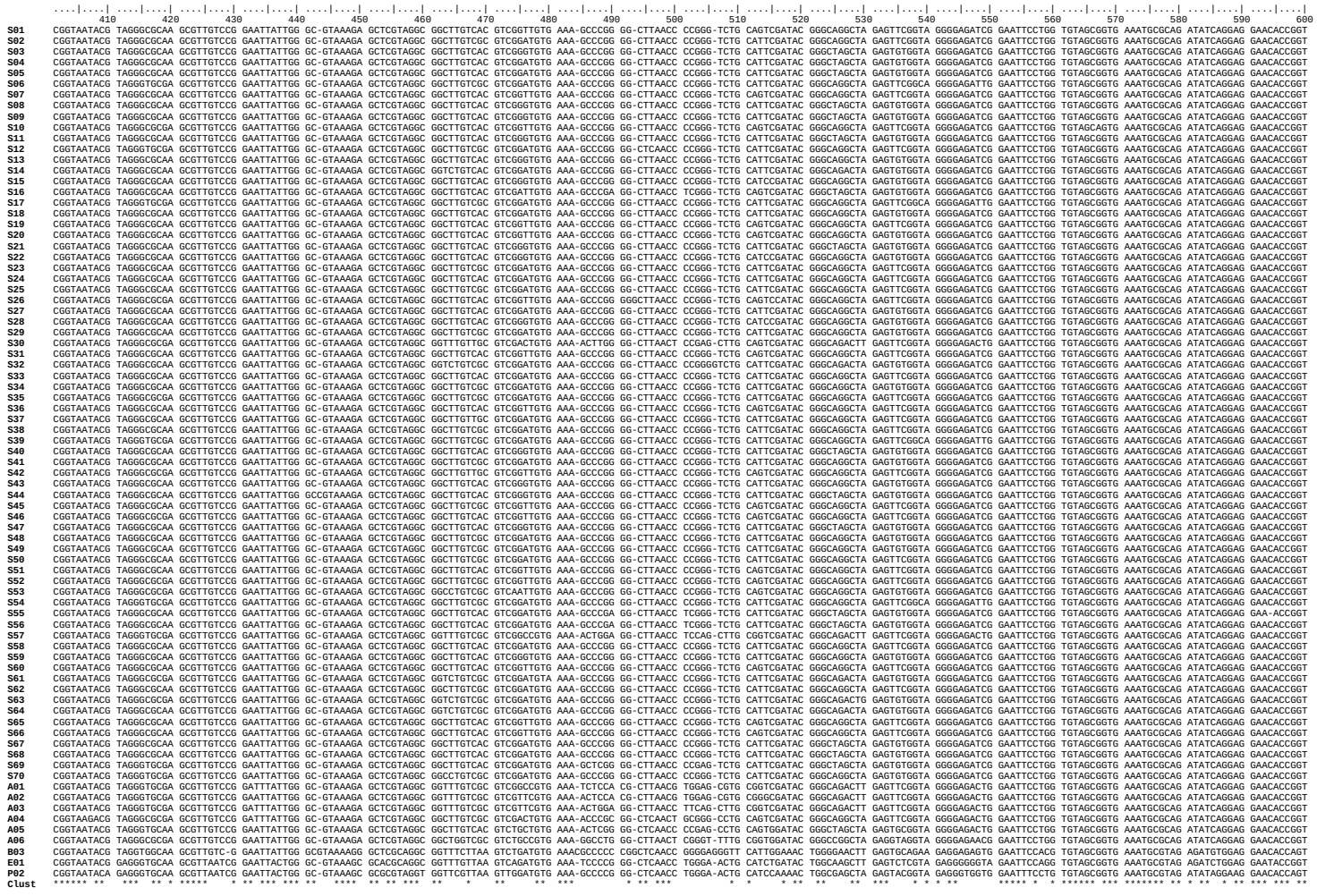
APPENDICES

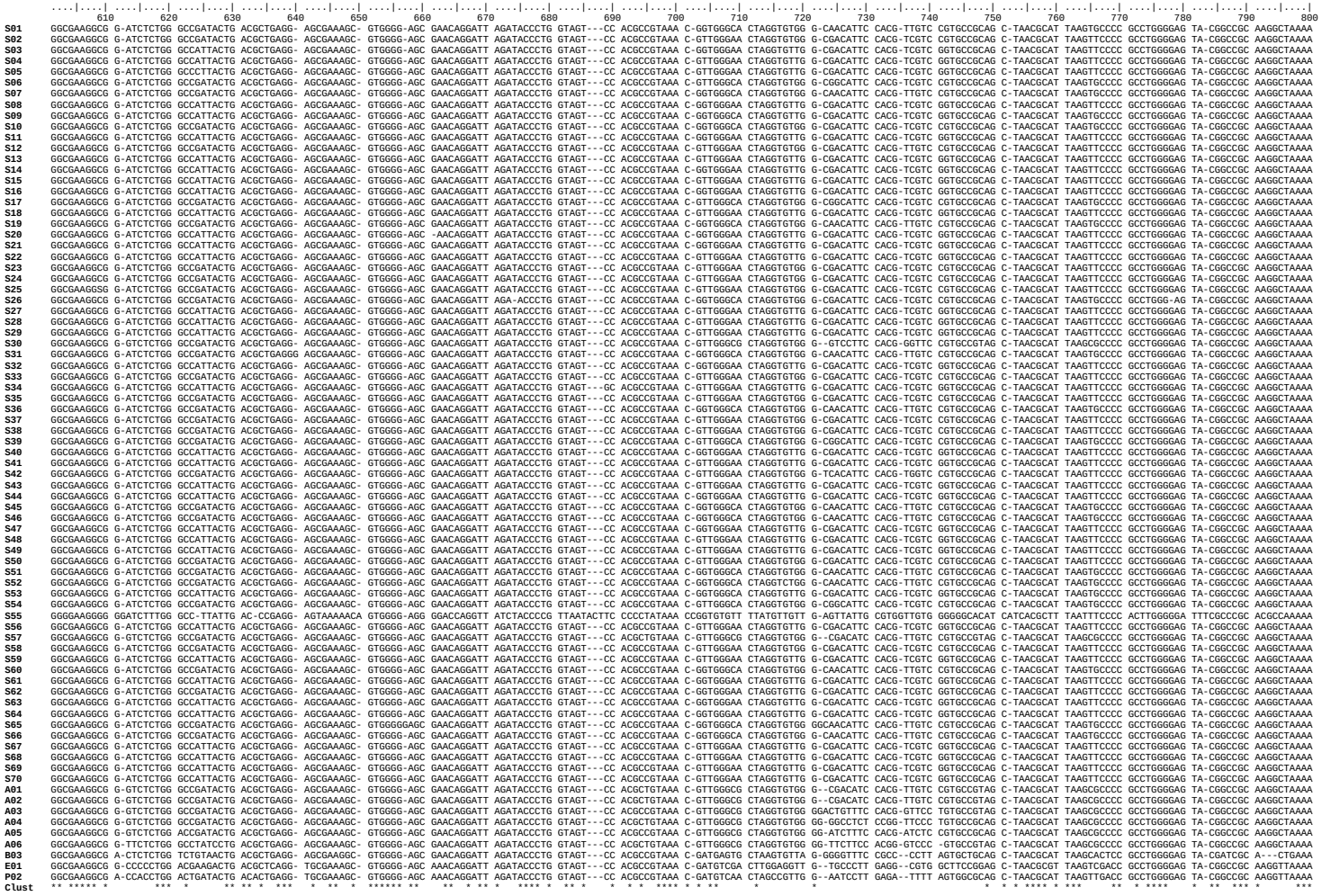
APPENDIX A

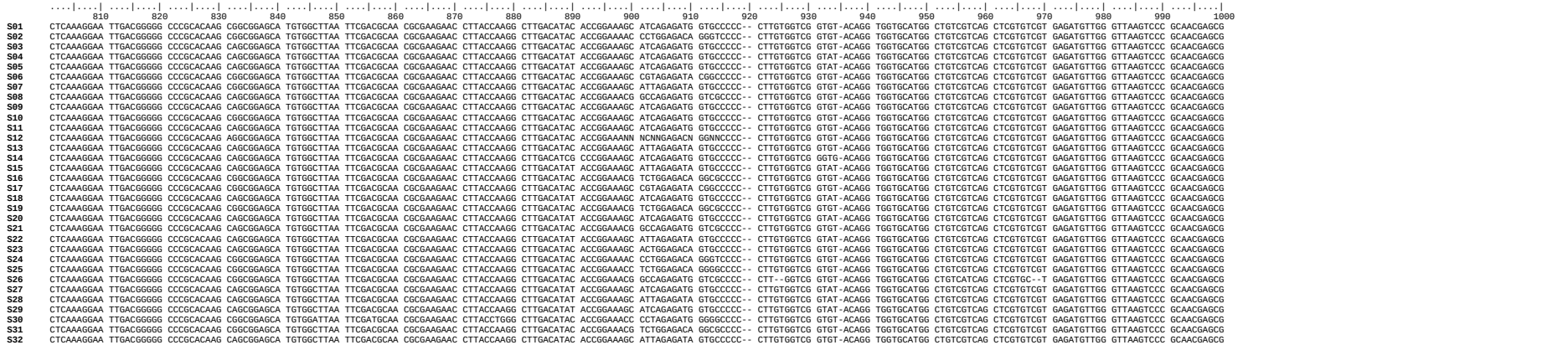
Location of Streptomyces specific primers (StrepB and StrepF)

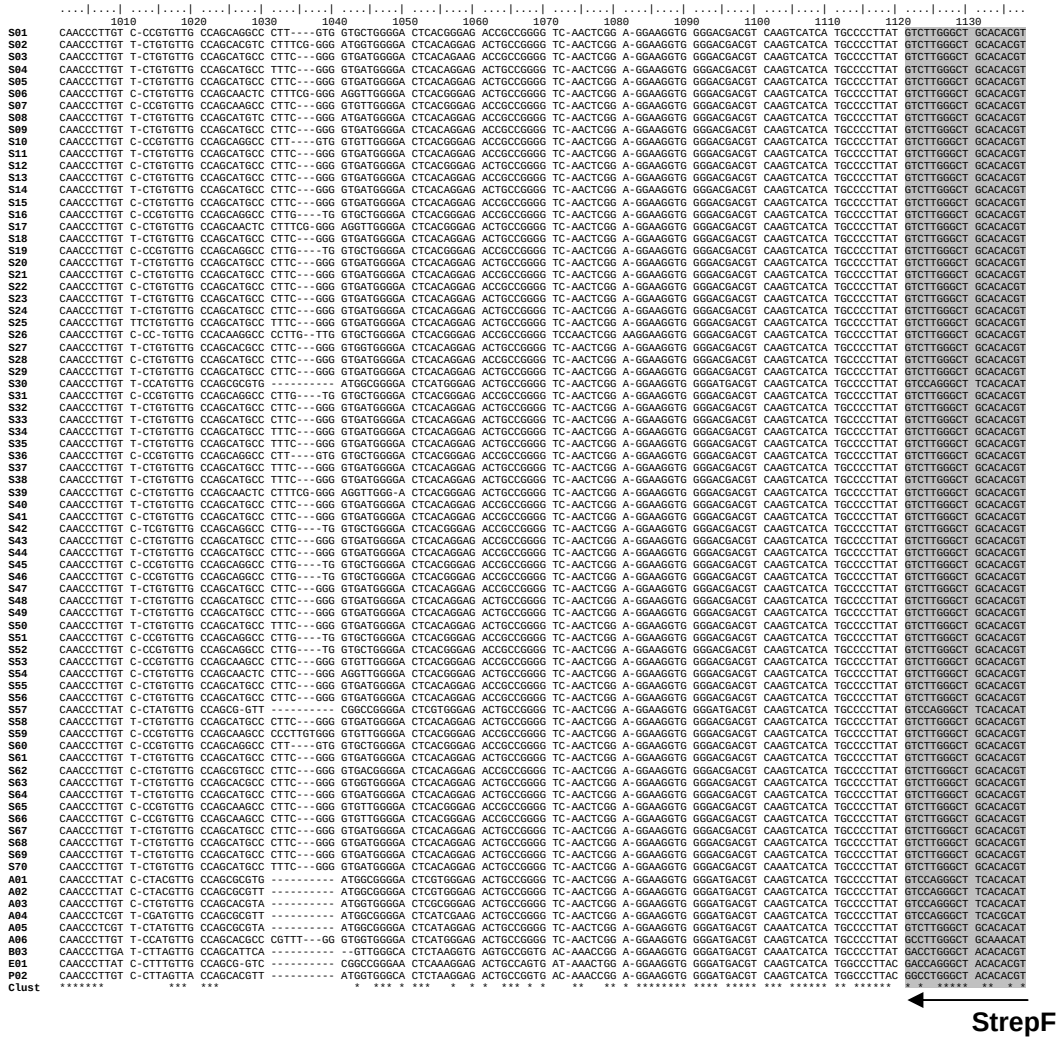











StrepF

APPENDIX B

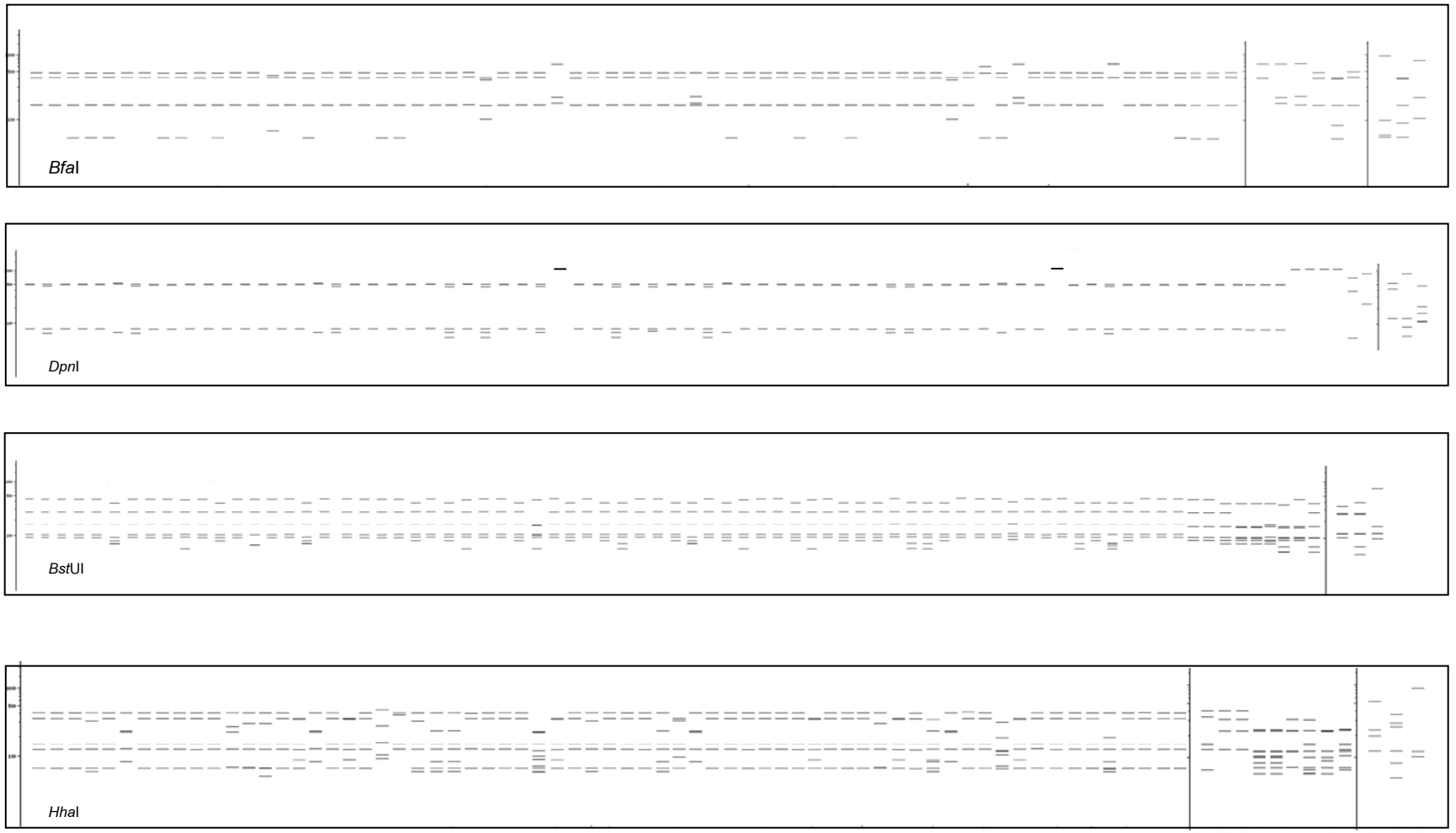
Phylogenetic tree reconstruction method using PAUP* 4.0 b 10

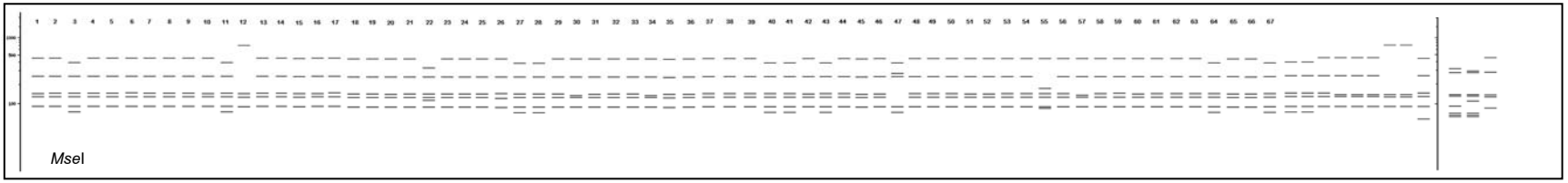
To construct the NJ tree from distance matrix (restriction fragment 0/1) or alignment file of DNA data. A reliable of the tree calculated by bootstrap value the tree by following procedure below.

1. Open program
2. The buffer window display show up.
3. Open file data matrix in form of nexsus or txt format from file menu
4. Select edit then select excecute from file menu
5. Select distant from Analysis menu to calculate pair wise distance.
6. Select NJ/UPGMA from Analysis then a pop up box for selection of clustering method. Select NJ. If Select Restriction site distance Nei-Li from distance options. Select Randomly, initial seed at Break ties then click OK.
7. A tree will show up in buffer display window.
8. Select Print NJ tree from Trees menu a pop up box. Plot type: Phylogram.
   - Rooting: define out group and add to the right box. If more than one out group make our group a monophyletic sister group to ingroup. Click OK.
   - Preview: select Save as PICT file and save file as .pct to the desire destination. Click done. Click done again.
9. Select Bootstrap/jackknife from Analysis menu. Random seed method: Bootstrap. Type of search: NJ/UPGMA Retain groups with frequency>50%. Click continue. Click OK.
10. Select Print Bootstrap concensus from Trees menu.
11. Rooting as describe Print NJ tree and then select preview for save file as describe above.

APPENDIX C

Computer simulation of RFLP of 79 microorganisms using 33 restriction enzymes

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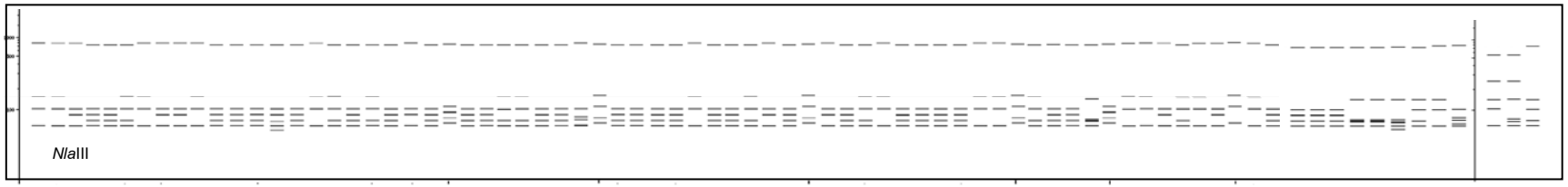
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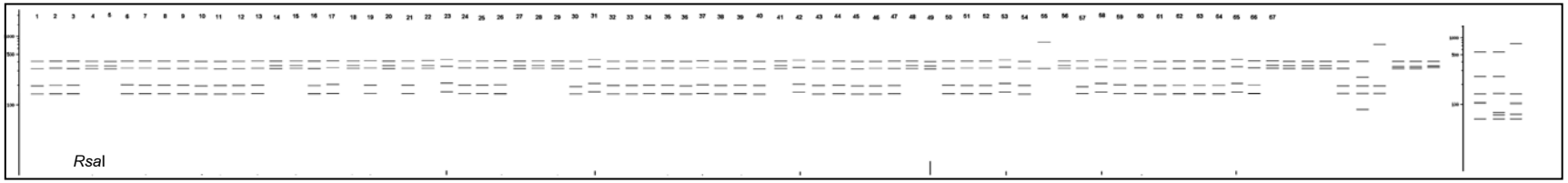
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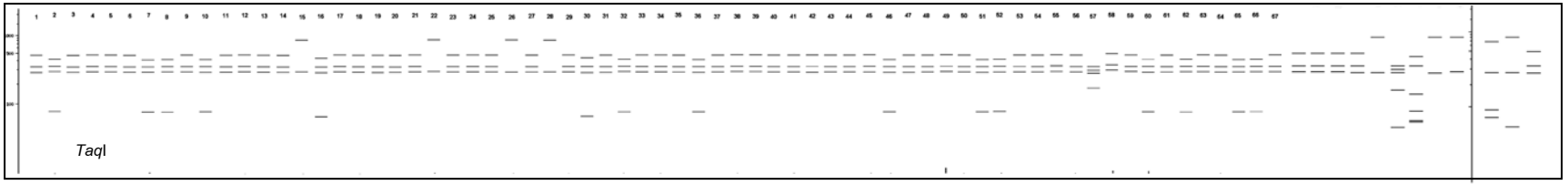
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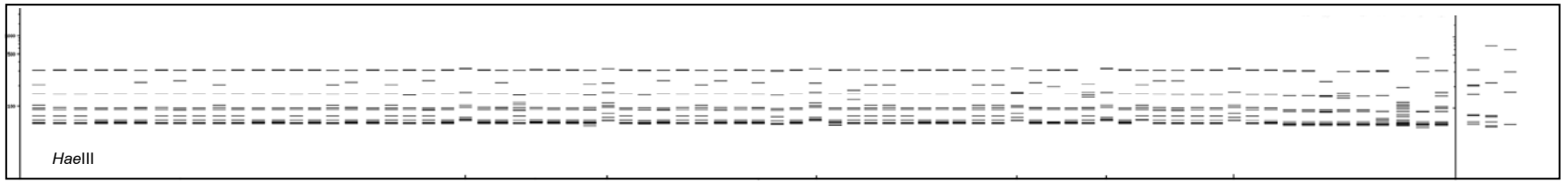
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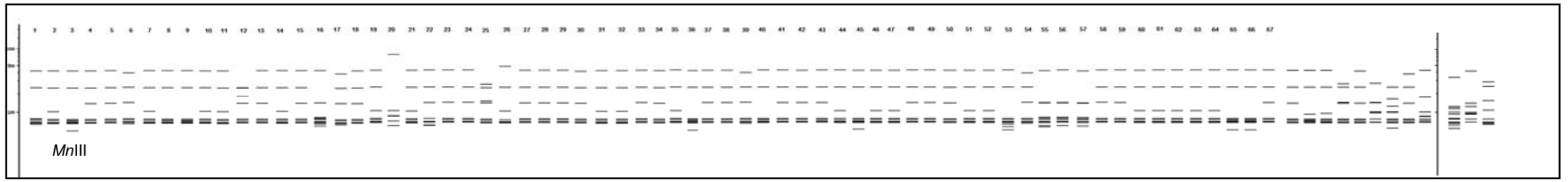
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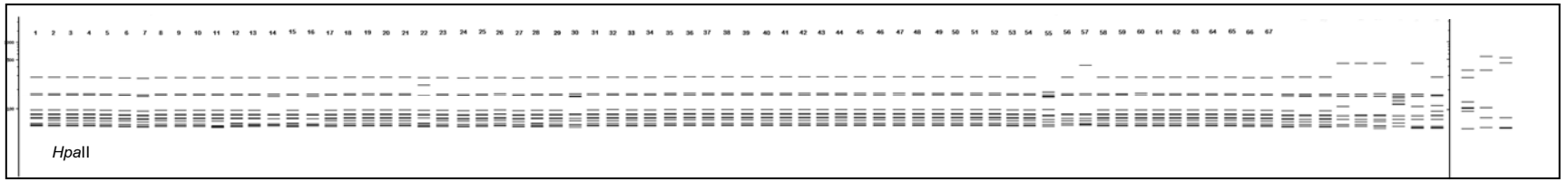
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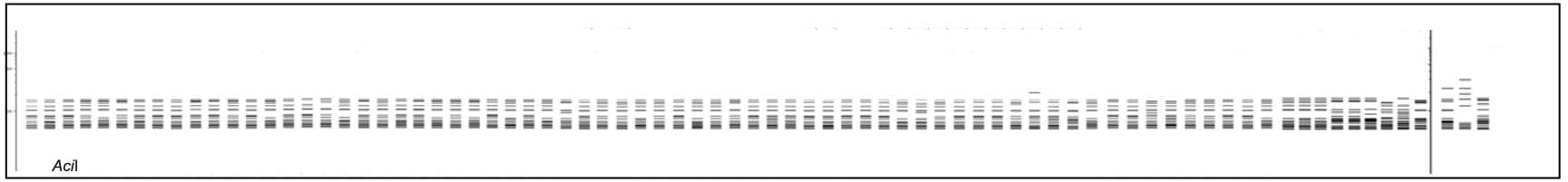
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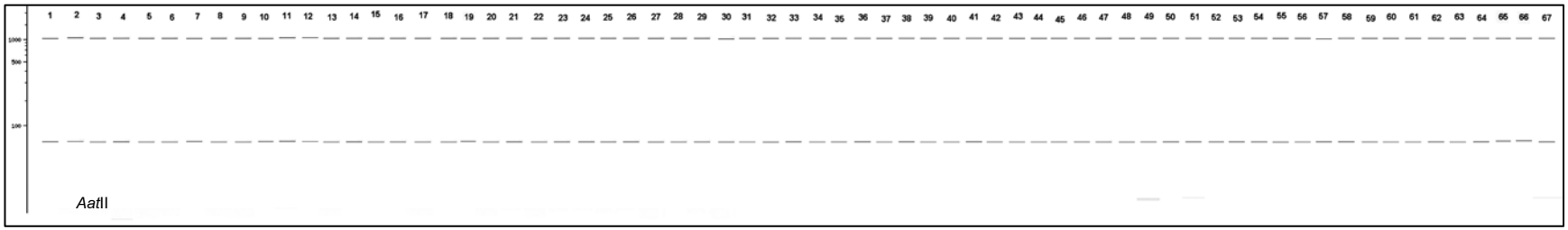
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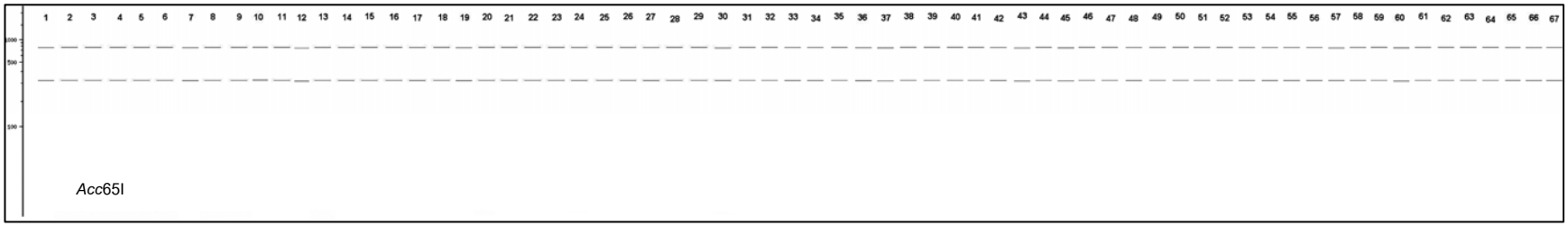
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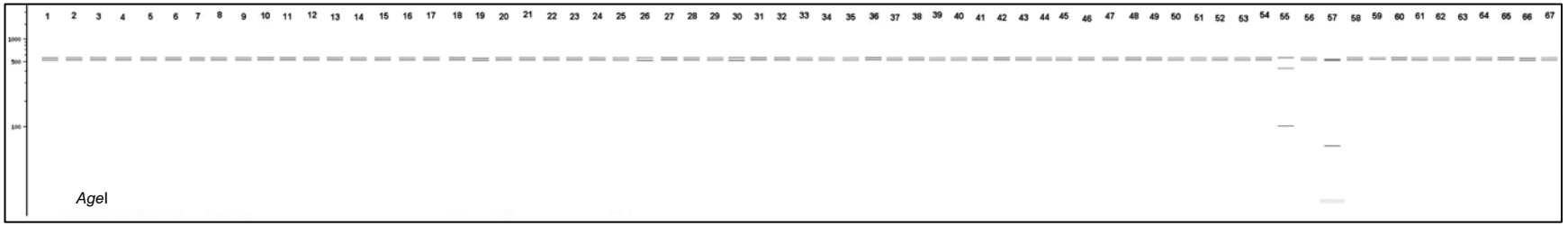
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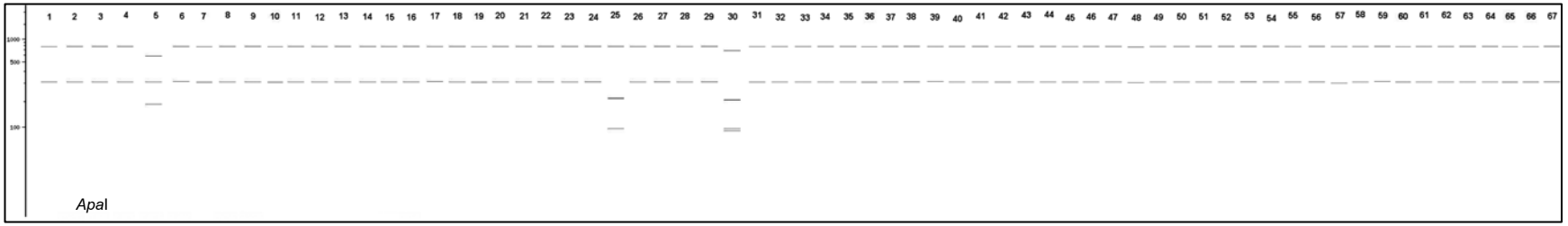
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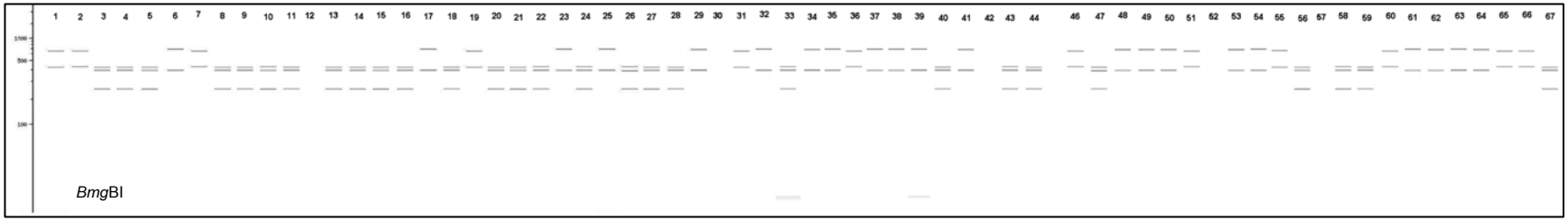
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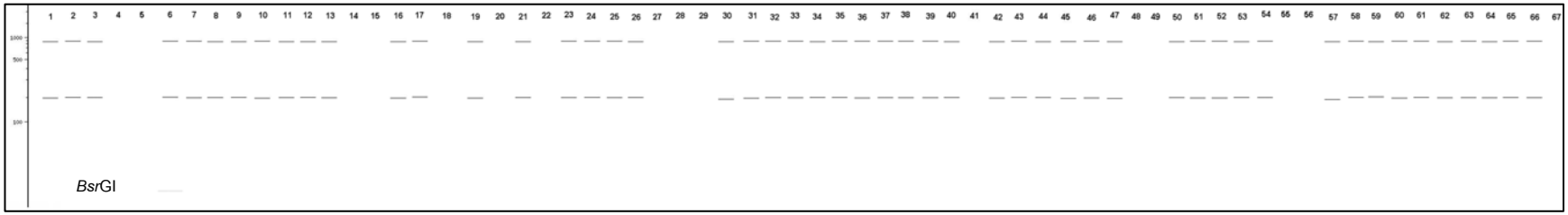
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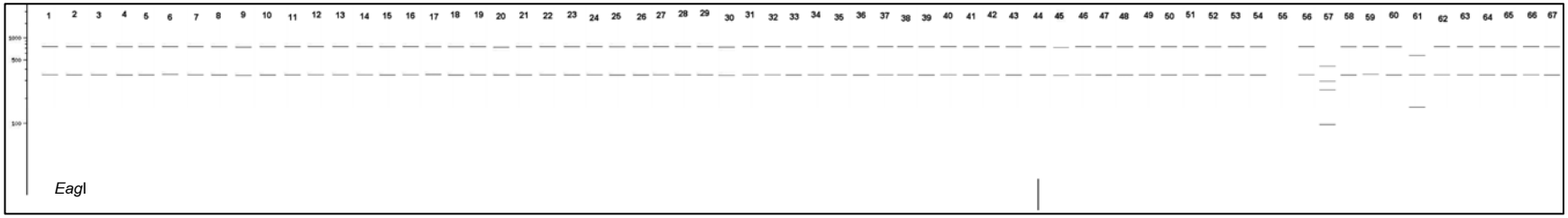
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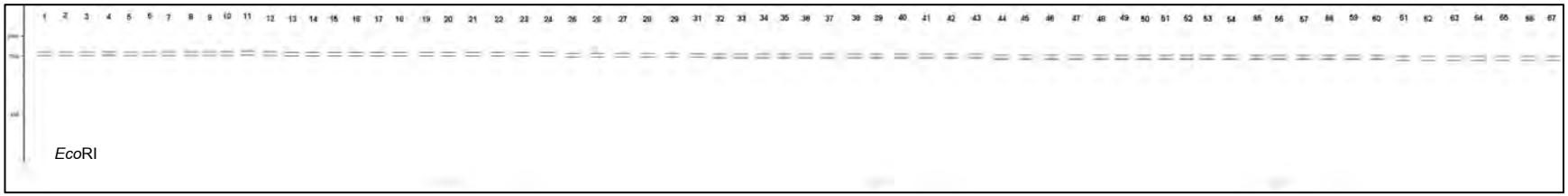
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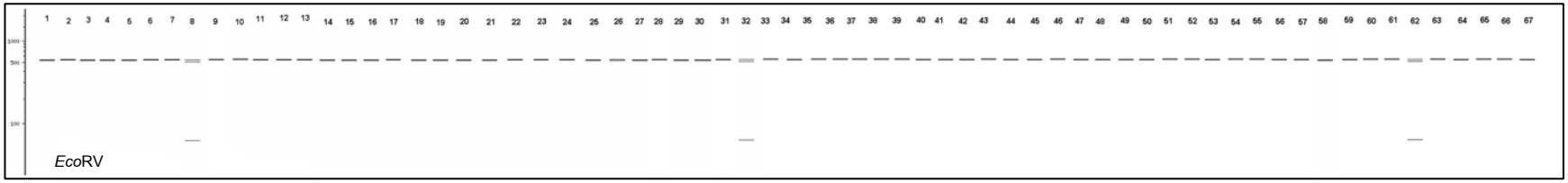
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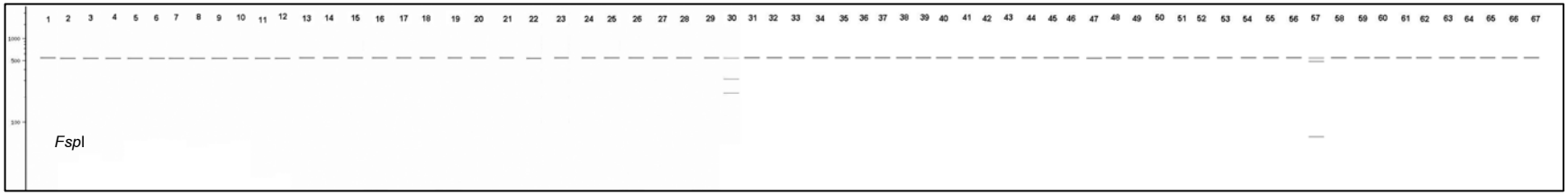
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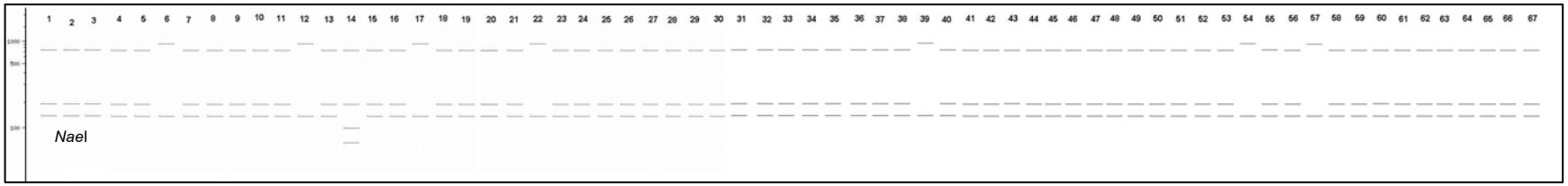
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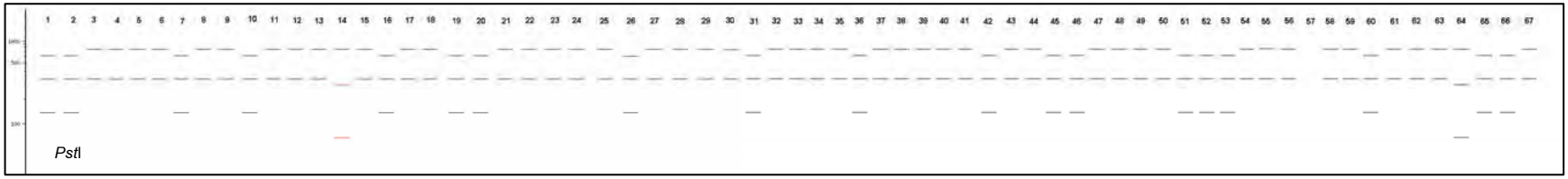
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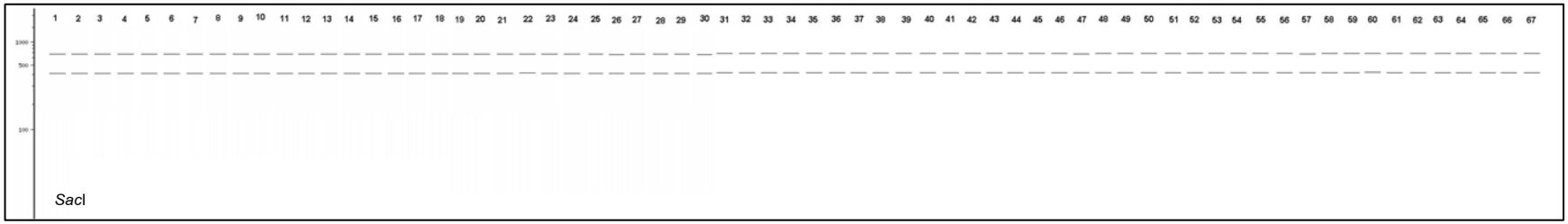
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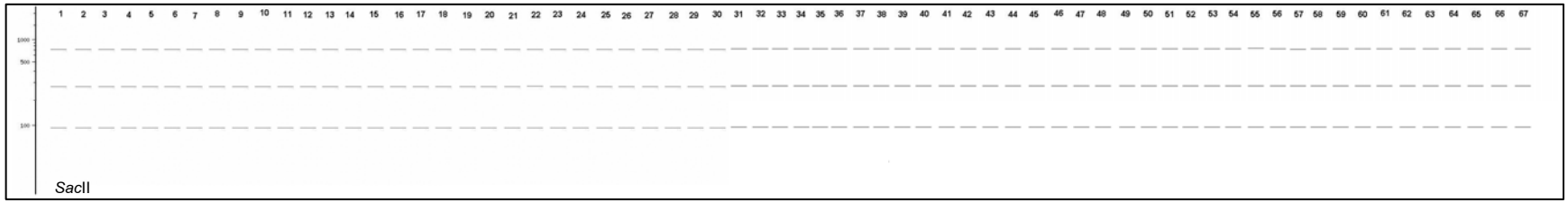
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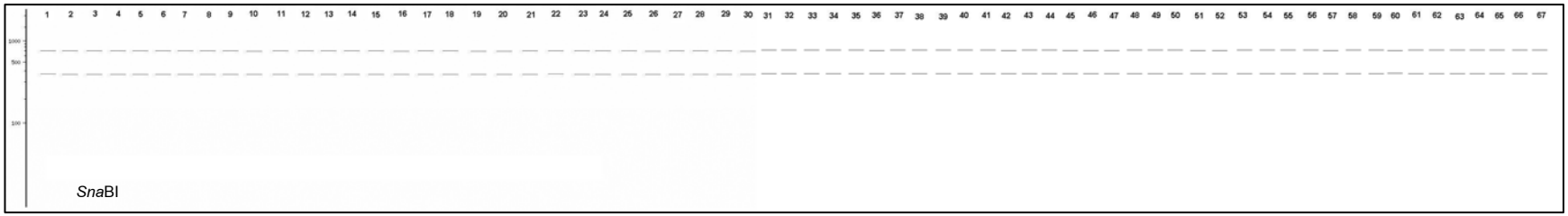
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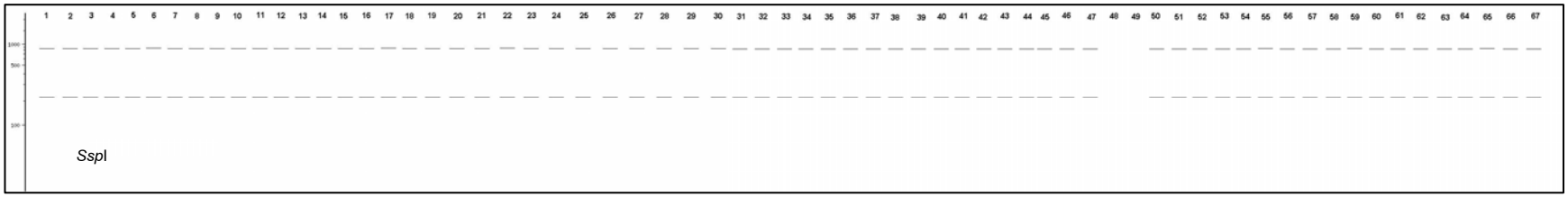
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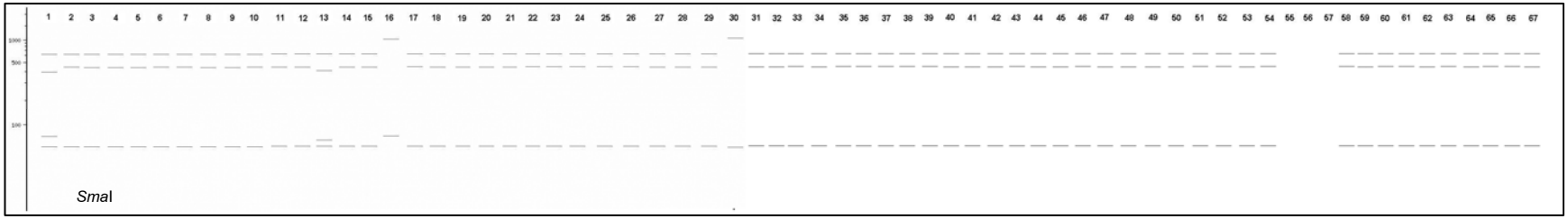
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BIOGRAHPY

Miss Sirinee Yodmuang was born on January, 19 1982 in Ubon Ratchathani proveince. She graduated with a Bachelor's Degree of Science (Biology), Second class honors on March 2005 from Faculty of Science, Khon Kaen University. She has been studying for her Master's Degree of Science at Department of Biochemistry, Faculty of Science, Chulalongkorn University since May 2005.