Screening of Soil Fungi from Plant Genetic Conservation Project Area, Rajjaprabha Dam, Suratthani Province which Produced Antimicrobial Substances

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ชื่อวิทยานิพนธ์	การคัดเลือกเชื้อราที่สร้างสารต้านจุลินทรีย์จากดินบริเวณโครงการพื้นที่ปกปักพันธุกรรมพืช เขื่อนรัชชประภา จังหวัดสุราษฎร์ธานี
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บทคัดย่อ

ทำการแยกเชื้อราจากดินบริเวณโครงการพื้นที่ปกปักพันธุกรรมพืช เขื่อนรัชชประภา จังหวัดสุราษฎร์ธานี ในเดือนมกราคม เมษายน มิถุนายน และสิงหาคม 2553 พบมีปริมาณเชื้อราอยู่ในช่วง $4.2x10^{4}$-$1.1x10^{6}$ CFU/g และคัดเลือกเชื้อราจำนวน 181 ไอโซเลทมาเพาะเลี้ยงในอาหารเหลวที่อุณหภูมิ $25^{\circ}C$ เป็นเวลา 3 สัปดาห์ กรองแยกน้ำเลี้ยงเชื้อและเส้นใย จากนั้นทำการสกัดสารด้วยเอธิลอะซีเตท หรือเฮกเซน ได้สารสกัดจำนวน 543 สาร ประกอบด้วยสารสกัดส่วนน้ำเลี้ยงเชื้อด้วยเอธิลอะซีเตท (BE) สารสกัดจากเส้นใยที่สกัดด้วยเอธิลอะซีเตท (CE) และสารสกัดจากเส้นใยด้วยเฮกเซน อย่างละ 181 สาร นำสารสกัดทั้งหมดมาทดสอบฤทธิ์ต้านจุลินทรีย์ด้วยวิธี colorimetric broth microdilution กับจุลินทรีย์ก่อโรคคนจำนวน 12 สายพันธุ์ ได้แก่ *Staphylococcus aureus* (SA) methicillin-resistant *S. aureus* (MRSA) *Escherichia coli* (EC) *Pseudomonas aeruginosa (*PA) multidrug-resistant *Acinetobacter baumannii* (AB005 และ AB007) *Candida albicans* (CA28 และ CA53) *Cryptococcus neoformans* (CN12 และ CN13) *Microsporum gypseum* (MG) และ *Penicillium marneffei* (PM) พบว่าสารสกัด 311 สาร (57.27%) จากเชื้อราดิน 145 ไอโซเลท

(80.11%) ยับยั้งเชื้อก่อโรคได้ 1 ถึง 12 สายพันธุ์ โดยแสดงฤทธิ์ต้านเชื้อ SA มากที่สุด (38.86%) รองลงมา คือ CN13 (33.71%), MRSA (24.91%), CN12 (19.44%), CA28 (14.10%), CA53 (12.62%), PM (7.52%), MG (7.14%), AB007 (2.66%), AB005 (1.71%), EC (1.11%) และ PA (0.37%) ตามลำดับ โดยมีค่าความเข้มข้นต่ำสุดที่ยับยั้งการเจริญของเชื้อ (minimum inhibitory concentration, MIC) และค่าความเข้มข้นต่ำสุดที่ฆ่าเชื้อแบคทีเรีย (minimum bactericidal concentration, MBC) หรือเชื้อรา (minimum fungicidal concentration, MFC) อยู่ในช่วง 1-200 และ 2->200 µg/ml ตามลำดับ ทำการจัดจำแนกเชื้อราที่มีฤทธิ์ต้านจุลินทรีย์สูง (ค่า MIC น้อยกว่า 10 µg/ml) และ/หรือมี NMR profile ที่น่าสนใจ จำนวน 34 ไอโซเลท ด้วยวิธีทางสัณฐานวิทยาและวิธีทางชีวโมเลกุลได้ 7 สกุล ประกอบด้วย *Absidia* (n=1), *Aspergillus* (n=11), *Fusarium* (n=6), *Paecilomyces* (n=3), *Penicillium* (n=9), *Myrothecium* (n=1) และ *Trichoderma* (n=3) นำสารสกัดส่วน CH จากเชื้อ *Trichoderma brevicompactum* RSPG27, *Aspergillus sclerotiorum* RSPG179, *Aspergillus unguis* RSPG204 และ *Aspergillus* section *Usti* RSPG206 ซึ่งแสดงฤทธิ์ต้านจุลินทรีย์ดีที่สุด ให้ค่า MIC อยู่ในช่วง 1-4 µg/ml ไปศึกษากลไกการออกฤทธิ์เบื้องต้นด้วยกล้องจุลทรรศน์อิเล็คตรอนชนิดส่องกราด (scanning electron microscope, SEM) พบว่าสารสกัดหยาบทำให้เซลล์จุลินทรีย์ถูกทำลายและเกิดการเปลี่ยนแปลงอย่างชัดเจน ผลการทดลองแสดงให้เห็นว่าเชื้อราที่แยกมาจากดิน ในพื้นที่ปกปักพันธุกรรมพืช เขื่อนรัชชประภาเป็นแหล่งของสารออกฤทธิ์ทางชีวภาพที่น่าสนใจ

Thesis Title Screening of soil fungi from Plant Genetic Conservation Project area, Rajjaprabha dam, Suratthani province which produced antimicrobial substances

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ABSTRACT

Soil fungi were isolated from soil samples collected from Plant Genetic Conservation Project area, Rajjaprabha Dam, Surat Thani province in January, April, June and August, 2010. Soil fungal densities were in the range of $4.2x10^4$-$1.1x10^6$ CFU/g. One hundred and eighty-one different morphotypes of soil fungi were selected and cultivated in broth medium at 25^oC for 3 weeks. Culture filtrate and fungal mycelium were separated and extracted by either ethyl acetate or hexane. Five hundred and forty-three extracts comprising 181 ethyl acetate extracts from culture broth (BE), 181 ethyl acetate extracts from fungal mycelia (CE) and 181 hexane extracts from fungal mycelia (CH) were obtained. All extracts were determined for their antimicrobial activities using a colorimetric broth microdilution method against twelve human pathogenic microorganisms including *Staphylococcus aureus* (SA), methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* (EC), *Pseudomonas aeruginosa (*PA), multidrug-resistant *Acinetobacter baumannii* (AB005 and AB007), *Candida albicans* (CA28 and CA53), *Cryptococcus neoformans* (CN12 and CN13), *Microsporum gypseum* (MG) and *Penicillium marneffei* (PM). The results demonstrated that 311 crude extracts (57.27%) from 145 isolates (80.11%) showed antimicrobial activity against one to twelve test microorganisms. The fungal crude extracts were highly active against SA (38.86%) followed by CN13 (33.71%), MRSA (24.91%), CN12 (19.44%), CA28 (14.10%), CA53 (12.62%), PM (7.52%), MG (7.14%), AB007 (2.66%), AB005 (1.71%), EC (1.11%) and PA (0.37%), respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC or MFC) values were varied in the range of 1-200 and 2->200 µg/ml. Thirty-four soil fungi showing strong activity (MIC < 10 µg/ml) and/or

interesting NMR profiles were identified based on morphological and molecular data into 7 genera including *Absidia* (n=1), *Aspergillus* (n=11), *Fusarium* (n=6), *Paecilomyces* (n=3), *Penicillium* (n=9), *Myrothecium* (n=1) and *Trichoderma* (n=3). Cell hexane extracts from *Trichoderma brevicompactum* RSPG27, *Aspergillus sclerotiorum* RSPG179, *Aspergillus unguis* RSPG204 and *Aspergillus* section *Usti* RSPG206 were the most active extracts with MIC values of 1-4 μg/ml. Electron microscopic observation of the treated cells with these strongly active extracts showed pronounced morphological changes consistent with cell damage. The results show that soil fungi isolated from Plant Genetic Conservation Project area, Rajjaprabha dam could be a potential source of antimicrobial substances.

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LIST OF ABBREVIATIONS AND SYMBOLS

CFU	=	Colony forming unit
TFU	=	Total fungal unit
kg	=	Kilogram
g	=	Gram
mg	=	Milligram
µg	=	Microgram
ng	=	Nanogram
ml	=	Milliliter
µl	=	Microliter
cm	=	Centimeter
mm	=	Millimeter
M	=	Molar
mM	=	Millimolar
rpm	=	Round per minute
pH	=	Potential of hydrogen ion
GANA	=	Glucose ammonium nitrate agar
TSM	=	*Trichoderma* selective media
NMR	=	Nuclear magnetic resonance

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Antibiotic resistance is a type of drug resistance where a microorganism is capable to survive exposure to an antibiotic. Nowadays, antibiotic resistance has become an important problem in the public health system worldwide. Infections caused by resistant microorganisms frequently fail to respond to conventional treatment, resulting in prolonged illness and greater risk of death (Odonkor and Addo, 2011). There is a need to find new antibiotics from various sources and natural products, which are still a worth promise (Newman and Cragg, 2007).

Filamentous fungi are well-known as the major producers of diverse bioactive natural products. Secondary metabolites produced by fungi usually exhibit toxicity or inhibitory effects on other organisms. Therefore, many fungal secondary metabolites have been developed for using in pharmaceutical, industry, agriculture and environment (Shwab and Keller, 2008). In addition, most of these compounds have been discovered from fungi inhabited in soil (Livermore, 2011).

Soil fungi play an important role in improving soil quality by recycling of organic matter and aggregation (Morton, 2005). There are many reports on soil fungi which produced bioactive substances. Cazar *et al*. (2005) extracted terreic acid and butyrolactone I from *Aspergillus terreus* that is active against *Erwinia carotovora*, a plant pathogenic bacterium. Xiao-Yan *et al*. (2006) found trichokonins from *Trichoderma koningii* inhibit Gram-positive bacteria and plant pathogenic fungi. Moreover, Petit *et al*. (2009) isolated *Penicillium* sp. producing a new compound

which has activities against *Candida albicans*, *Listeria monocytogenes* and *Bacillus cereus*. Although many soil fungi have been exploited, the potential ones can still be found from an undisturbed area. Thus, Rajjhaprabha Dam Plant Genetic Conservation Project area under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhron or RSPG area is a good place to look for soil fungi producing interesting antimicrobial substances.

1.2 Reviews of the literature

1.2.1 Soil in general (Subbarao, 1999)

Soil is the outer sheathing of the earth which comprises of loosely arranged layers of inorganic and organic matter in different stages of organization. It provides the physical support needed for the anchorage of the root system and serves as the source of air, water and nutrients which are necessary for plant growth. Each soil zone may contain various soil types and each type of soil is characterized by the presence of different horizons. The formations of soil horizons rely on climate, living organisms, parent rock material, topography and time.

1.2.2 Soil physicochemical environment (Standing and Killham, 2007)

1.2.2.1 Water

Water carries ions, nutrients, dissolved gases and heat, as well as microorganisms. Soil water potential is the sum of matrix, osmotic and pressure potentials, and is the key measure of the water activity in the soil.

1.2.2.2 Organic and inorganic matters

The reduced forms of inorganic chemicals such as Fe^{2+}, SO_3^-, S^0, NH_4^+ and NO_2^- are served as the chemical energy of chemoautotrophs. Organic matter does not provide only the energy source for heterotrophic soil microbes; it is also the main source of nutrients, as most of the N, P and S.

1.2.2.3 Temperature

Soil temperature is an important factor of activity and distribution of soil microorganisms. Temperature affects microbial physiology, nutrient, substrate diffusion and water activity. In addition, temperature involves rates of physical, chemical and physiological reactions in soil environment.

1.2.2.4 Light

Light stimulates plant seed germination, seedling establishment and growth. It also directly affects the microbial distribution and activity near soil surface or where light can reach and served as an energy source of photoautotrophic soil microorganisms.

1.2.2.5 Soil atmosphere and redox potential

The characteristics of soil atmosphere are strongly correlated to soil water regime. Soil water potential is critical to define the diffusive supply of oxygen. Oxygen tends to display a gradient of concentration from the surface to the margin of the rooting depth.

1.2.2.6 pH

Soil pH represents a major factor of microbial distribution and activity in soil. The pH of the soil is the product of a number of determinants and processes. First, it is evaluated by the parent material and the degree of mineral weathering since formation. Second, the biological processes of soil microbes are able to change the soil pH to varying degrees. Several soil microorganisms can tolerate pH condition that is unsuitable for them. For example, various fungi isolated from acid forest soils are not acidophiles but they are highly competitive under considerable acidity.

1.2.3 Soil microorganisms (Subbarao, 1999; Zuberer and Wollum II, 2005)

Soil organisms are both numerous and highly diverse. Several soil organisms are small and can be seen by using the magnification. The microorganisms inhabited in soil including bacteria, actinomycetes, fungi, algae, protozoa and virus

are mentioned as microflora. They are the key of the biological changes in this environment (Takahashi *et al*., 2008).

The dominant microorganism in all soils is fungi. Fungi are an abundant group in acid soil because acidic condition is not promoting the growth of bacteria and actinomycetes. The most common fungus isolated from soil is the mitosporic fungi. Yeasts can be found in soil but their numbers are low. In soil ecosystem, fungi play a very important role in the recycling of important chemical elements by degrading organic matter. Their hyphal networks grow through soils, helping to bind soil particles into aggregates. Some of the fungi are capable to grow symbiotically with plant roots resulting in increasing nutrient and water uptake. However, fungi also have a dark site as pathogen to animals, plants and microbes. Furthermore, they produce harmful metabolic byproducts known as mycotoxin (Morton, 2005).

1.2.4 Groups and roles of soil fungi

Soil fungi can be classified into three groups as follows:

1.2.4.1 Decomposers or saprophytic fungi (Morton, 2005; Hoorman, 2011)

Fungi transform dead plants and animals into fungal biomass, carbon dioxide (CO_2) and small molecules such as organic acids. Saprophytic fungi degrade complex substrates such as cellulose, hemicellulose, pectin and lignin in plant cell wall (Figure 1). Fungi are able to decompose the carbon ring structures in some hazardous compounds. They are also essential for immobilizing or holding nutrients in the soil. Many organic acids produced by fungi increase humus organic matter in soil that are resistant to decomposition and stay for a long time.

(A) **(B)**

Figure 1 An example of wood-decay fungi

(A) A closer view of brown-rooted wood

(B) Scanning electron micrograph of brown-rooted wood

Source: http://forestpathology.cfans.umn.edu/microbes.htm

1.2.4.2 Pathogens or parasites (Morton, 2005; Hoorman, 2011)

Pathogenic or parasitic fungi are fungi that form negative relationships with other living organisms. *Verticillium*, *Pythium*, *Rhizoctonia* and *Phytophthora* are examples of plants pathogenic fungi, a major cause of economic losses in agriculture whereas entomopathogenic and nematode-destroying fungi (Figure 2) are useful as bio-control agents.

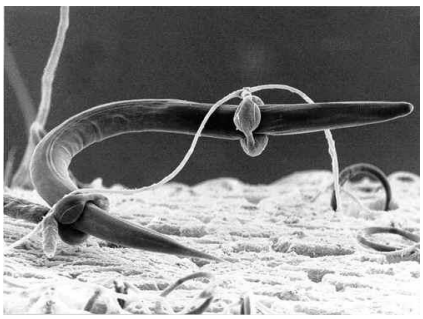

Figure 2 Nematode captured by the nematode-destroying fungus

Source: http://www.uoguelph.ca/~gbarron/N-D%20Fungi/n-dfungi.htm

1.2.4.3 Fungal symbionts: mycorrhizae, lichens and endophytes (Finlay, 2007; Hoorman, 2011)

Mycorrhiza is a symbiotic relationship between fungi and plant roots (Figure 3). A mycorrhizal network helps the plant roots be more proficient for collecting soil nutrients by increasing the surface area of plant roots and transporting nutrients back to the plant. Fungi produce enzymes such as protease and phosphatase to mineralize and release nitrogen and phosphorus that increase the exuberance of soil. Furthermore, fungal hyphae clump soil particles together and form strong macroaggregates, increasing water infiltration along with water holding capacity in soil. Nowadays, mycorrhizal products are commercially available to use for planting time.

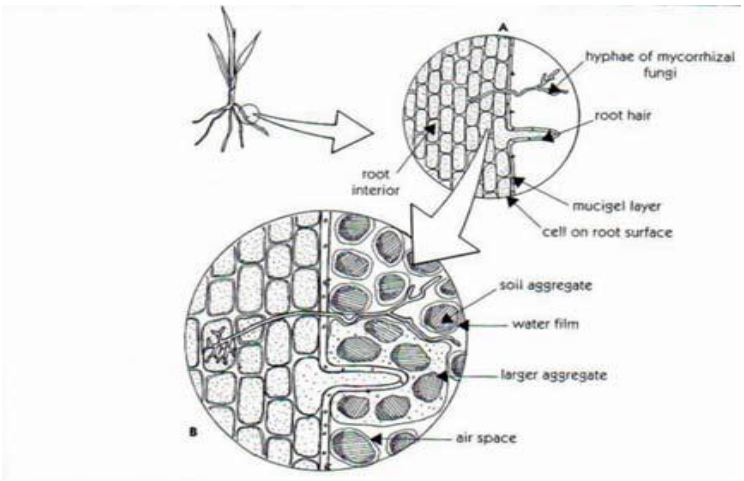

Figure 3 Mycorrhizal fungus and plant roots

Source: Hoorman, 2011

Fungi can form symbiotic relationship with photosynthetic partners like green algae or cyanobacteria. This relationship is called “lichen”. Most of fungi that form lichens are ascomycetes, however some basidiomycetes also have this ability.

Figure 4 Photograph of lichens on basalt

Source: http://commons.wikimedia.org/wiki/File:Lichen_squamulose.jpg

Endophytic fungi are widely present in the root of many plants. Some of them produce mycotoxins, which provide utilities on their host plant in resistance to feed on herbage or other types of environment stress.

Moreover, antagonism between soil microorganisms is also a common community interaction. As for fungi, they present antibiotic production that is helpful for their survival than disease development. Fungi producing antibiotic are mainly in ascomycetous genera such as *Aspergillus*, *Fusarium* and *Penicillium* (Morton, 2005).

1.2.5 Bioactive metabolites from soil fungi

In 1928, penicillin, the first antibiotic was incidentally discovered by Sir Alexander Fleming who isolated *Penicillium notatum*, which produced Gram-positive bacteria killing compound (Makut and Owolewa, 2011). This discovery was the starting of the attention of secondary metabolites produced by microorganisms (Taylor *et al*., 2003) and fungi became the interesting source of bioactive compounds since then.

Soil fungi are one of the most diverse groups of microorganisms. Many researchers have successfully discovered active or novel compounds from fungi-inhabiting in soil. Metabolites produced by soil fungi perform broad range biological activities such as anti-bacteria, anti-fungus, anti-malaria, anti-cancer and anti-oxidant

that are valuable for biotechnological applications (Petit *et al*., 2009; Antipova *et al*., 2011).

1.2.5.1 Antimicrobial substances

Antimicrobial substances or antibiotics are now referred to compound produced by microorganisms, or to a similar compound which inhibits other microorganisms at low concentration (Denyer *et al*., 2004). The most well-known antibiotics produced by fungi are penicillins, cephalosporins and fusidic acid. Antimicrobial agents from soil fungi displayed activity toward a wide variety of pathogenic microorganisms are shown in Table 1.

Table 1 Antibacterial, antifungal, antiprotozoal and antiviral substances produced by soil fungi

Compound	Fungal name	Test microorganisms	Reference
Penitricin	*Penicillium aculeatum*	*Pseudomonas aeruginosa*	Okuda *et al*., 1984
3,4-dimethoxyphenol 1,3,5-trimethoxybenzene	*Aspergillus fumigatus*	*Staphylococcus aureus* *Micrococcus luteus* *Candida albicans*	Furtado *et al*., 2002
Sporogen AO-1 *p*-Hydroxybenzoic acid	*Penicillium* sp.	*Phytophthora capsici* *Pythium ultimum* *Rhizoctonia solani*	Kang *et al*., 2003
6-n-pentyl-•-pyrone	*Trichoderma harzianum*	*Paecilomyces variotii* *Penicillium notatum* *Nematospora corylii* *Mucor miehei* *Bacillus brevis* *Bacillus subtilis* *Sarcina lutea* *Enterobacter dissolvens* *Armillaria mellea*	Tarus *et al*., 2003
Penicillic acid	*Aspergillus sclerotiorum*	*Phytophthora* spp.	Kang and Kim, 2004
Butyrolactone I Terreic acid Butyrolactone 4', 4"-diacetate 3'-(3-Methylbutyl)-butyrolactone II Lovastatin Lovastatin acetate	*Aspergillus terreus*	*B. brevis* *B. subtilis* *M. luteus* *E. dissolvens* *Ervinia carotovora* *P. syringae* *M. miehei* *P. variotii* *P. notatum* *Nematospora coryli* *Botrytis cinerea*	Cazar *et al*., 2005

Table 1 (cont.) Antibacterial, antifungal, antiprotozoal and antiviral substances produced by soil fungi

Compound	Fungal name	Test microorganisms	Reference
Quinoxiline bicyclic octadepsipeptides UK-63598 Monocillin IV	*Penicillium* sp.	Human immunodeficiency virus (HIV)	Jayasuriya *et al*., 2005
Trichokonins	*Trichoderma koningii*	*S. aureus* *B. subtilis* *Streptococcus faecalis* *Clavibacter michiganensis* *Saccharomyces cerevisiae* *C. albicans* *Fusarium oxysporum* *B. cinerea* *R. solani* *Valsa mali* *Verticillium dahlia* *Curvularia lunata* *Bipolaris sorokiniana* *Colletotrichum lagenarium*	Xiao-Yan *et al*., 2006
Sterile culture filtrate	*Penicillium aurantiogriseum* *Penicillium viridicatum*	*B. subtilis*	Khaddor *et al*., 2007
Sclerotiorin Isochromophilone VI Pencolide Penicillic acid	*Penicillium sclerotiorum* *Penicillium simplicissimum*	*Escherichia coli* *S. aureus* *Salmonella typhimurium* *Streptococcus pyogenes* *Listeria monocytogenes*	Takahashi *et al*., 2008
Trichothecenes	*Fusarium* sp.	*Paracoccidioides brasiliensis*	Campos *et al*., 2009
Citrinin Penicillic acid Unknown	*Penicillium citrinum* *Penicillium aurantiogriseum* *Penicillium viridicatum* *Penicillium waksmanii*	*C. albicans* *B. subtilis* *S. aureus* *Salmonella typhi* *E. coli*	Gharaei-Fathabad *et al*., 2009
Xanthepinone	*Phoma medicaginis*	*Magnaporthe grisea* *Phytophthora infestans* *B. cinerea*	Liermann *et al*., 2009
Mollicelline K Mollicelline L Mollicelline M Molliceline B Molliceline C Molliceline E Molliceline J	*Chaetomium brasiliense*	*Plasmodium falciparum* *Mycobacterium tuberculosis* *C. albicans*	Khumkomkhet *et al*., 2009

Table 1 (cont.) Antibacterial, antifungal, antiprotozoal and antiviral substances produced by soil fungi

Compound	Fungal name	Test microorganisms	Reference
Crude cell free supernatant	*Aspergillus* sp. *Scopulariopsis* sp. *Curvularia* sp. *Phoma* sp. *Lasiodiplodia theobromae* *Fusarium* sp. *Acremonium* sp. *Aureobasidium pullulans*	*M. luteus* *E. coli* *P. aeruginosa* *S. aureus* *B. subtilis* *Klebsiella planticola* *C. albicans*	Kumar *et al*., 2010
Cell free culture filtrates	*Chaetomium globosum* *Fusarium oxysporum* *Aspergillus biplane* *Cochliobolus lanatus* *Emericella nidulans*	*S. aureus* Methicillin-resistant *S. aureus* (MRSA) *Aspergillus niger*	Sheikh, 2010
Sclerotiorin Sclerotioramine	*Penicillium citreonigrum*	*Staphylococcus epidermidis* *Candida* strains	Wang *et al*., 2010
Dihydroxymethyl pyranone	*Aspergillus candidus*	*E. coli* *Klebsiella pneumoniae* *Proteus vulgaris* *P. aeruginosa* *S. aureus* *S. pyrogenes* *C. albicans* *Cryptococcus humicola* *Geotrichum candidum* *Microsporum canis* *Syncephalastrum racemosum* *Penicillium marneffei* *Aspergillus flavus* *Aspergillus fumigatus*	Elaasser *et al*., 2011
Intracellular and extracellular methanolic extracts	*Aspergillus niger* *Aspergillus flavus* var.*columinaris*	*E. coli* *P. vulgaris* *S. typhimurium* *Shigella dysenteriae* *S. aureus* *Enterococcus faecalis* *B. cereus* *C. albicans*	Fawzy *et al*., 2011

1.2.5.2 Anticancer or cytotoxic metabolites

Anticancer or cytotoxic agents are a group of bioactive metabolites that can be found from soil fungi. In the past 50 years, number of anticancer drugs based upon fungal metabolites was successfully discovered (Qureshi *et al*., 2011) and

several species of soil fungi have been reported on the production of cytotoxic compounds (Table 2).

Table 2 Anticancer or cytotoxic metabolites isolated from soil fungi

Compound	Fungal name	Test cell line/test organism	Reference
Fungal crude extract	*Fusarium acuminatum* *Fusarium avenaceum* *Fusarium culmorum* *Papulospora* sp.	Human skin fibroblasts (GM3349)	Abbas *et al*., 1984
Rubratoxin B	*Penicillium purpurogenum*	tsFT210 Human fibrosarcoma cell (HT1080)	Wang *et al*., 2007
Hamavellone A Hamavellone B 87-250904-F1 Emodin	*Hamigera avellanea*	Human breast adenocarcinoma cell line (MCF-7) Human oral cavity carcinoma cell line (KB) Human small cell lung carcinoma cell line (NCI-H187)	Isaka *et al*., 2008
Mollicelline K Mollicelline L Mollicelline M Mollicelline N Mollicelline B Mollicelline C Mollicelline E Mollicelline F Mollicelline H Mollicelline J	*Chaetomium brasiliense*	KB Lymphoma cell line (BC1) NCI-H187 Cholangiocarcinoma	Khumkomkhet *et al*., 2009
Rugulosone	*Emericella rugulosa*	KB BC1 NCI-H187	Moosophon *et al*., 2009
Dihydroxymethyl pyranone	*Aspergillus candidus*	Human epithelial cell (HEp-2) Liver hepatocellular cell (HepG2)	Elaasser *et al*., 2011
Intracellular and extracellular methanolic extracts	*Aspergillus niger* *Aspergillus flavus* var.*columinaris*	HepG2	Fawzy *et al*., 2011
L-asparaginase	*Fusarium equiseti*	Data not shown	Hosamani and Kaliwal, 2011
Fusarisetin A	*Fusarium* sp.	Human breast cancer cell line MDA-MB-231	Jang *et al*., 2011
Fudecadione A Fulvic acid	*Penicillium* sp.	MCF-7 KB NCI-H187	Pittayakhajonwut *et al*., 2011

Table 2 (cont.) Anticancer or cytotoxic metabolites isolated from soil fungi

Compound	Fungal name	Test cell line/test organism	Reference
Culture filtrate	*Aspergillus niger* *Penicillium citrinum* *Penicillium purpurrescens* *Penicillium rugulosum* *Penicillium* sp.	Brine shrimp	Qureshi *et al*., 2011
Polyketide	*Exophiala pisciphila*	Human lung adenocarcinoma epithelial cell line (A-549) Hela cell Pancreatic adenocarci-noma cell line (PANC-28) Human hepatoma cell line (BEL-7402)	Wang *et al*., 2011
Cell free supernatant	*Fusarium solani* *Emericella nidulans*	Human epithelial colorectal adenocarcinoma cell line (Caco-2)	Mohamed, 2012

1.2.5.3 Antioxidative agents

Free radicals are toxic molecules that have one or more unpaired electron. They are a cause of many diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and aging because they can break DNA, protein, lipids and carbohydrates within tissue (Meghashi *et al*., 2010). Superoxide (O_2-), peroxyl (ROO·), alkoxyl (RO·), hydroxyl (HO·), and nitric oxide (NO·) are examples of oxygen-centered free radicals, known as reactive oxygen species (ROS). Antioxidants are molecules which protect the cells via reducing the oxidative stress (Arora and Chandra, 2011b). Soil fungi are a potential source of antioxidative molecules as shown in Table 3.

Table 3 Antioxidant potential of soil fungi

Compound	Fungal name	Test free radicals/activity	Reference
Data not shown	Arbuscular mycorrhizal fungi	Exhibited higher enzymatic activities related to the removal of reactive oxygen species	Goicoechea *et al*., 2005
Fungal culture	*Aspergillus* sp.	DPPH ion	Arora and Chandra, 2010
Culture filtrate	*Aspergillus fumigatus*	DPPH ion Ferric ion Nitric ion	Arora and Chandra, 2011a
Ethyl acetate extracellular fungal extract	*Penicillium citrinum* *Penicillium granulatum* *Penicillium wentii*	DPPH ion Ferric ion Nitric ion	Arora and Chandra, 2011b
Dihydroxymethyl pyranone	*Aspergillus candidus*	DPPH ion	Elaasser *et al*., 2011
L-asparaginase	*Penicillium* sp.	DPPH ion	Soniyamby *et al*., 2011

1.2.6 Applications of molecular methods in the fungal identification

Historically, the classification of fungi was based on a combination between microscopy and culture-based techniques (Wengenack and Binnicker, 2009). However, traditional identification methods relied on morphological features are limited, mainly due to the difficulty in phenotypical character differentiation especially at the species level (Ropars *et al*., 2012). Moreover, the traditional methods for fungal identification through the microscopic examination and visual observation depend on different personal experience of the taxonomists. In recent years, molecular approaches are gainful analytical tools for the identification and characterization of fungi (Plaza *et al*., 2003). Molecular techniques have offered greater specificity and sensitivity for identifying the microorganisms at diverse taxonomic levels (Sette *et al*., 2006).

1.2.6.1 DNA extraction (Somma, 2004)

DNA extraction is the first step in most molecular biological studies. The DNA extraction protocol has to provide a high throughput of samples, yield, purity, reproducibility and scalability of the biomolecules as well as speed, accuracy and reliability (Tan and Yiap, 2009). Quality and purity of nucleic acids are some of

the most critical factors for PCR analysis. Suitable extraction method should be selected for obtaining highly purified nucleic acids free from contaminants. Examples of contaminants that could affect PCR amplification are listed in Table 4. The DNA extraction requires cell lysis, inactivation of cellular nucleases and separation of target DNA from cell debris. The common lysis procedures comprise of the mechanical disruption (e.g. grinding, hypotonic lysis), chemical treatment (e.g. detergent lysis, chaotropic agents, thiol reduction) and enzymatic digestion (e.g. proteinase K). Solvent extraction is used to remove contaminants from DNA. Phenol-chloroform is frequently used for proteins eliminating. Isopropanol or ethanol is commonly used to precipitate and concentrate nucleic acids. To heighten amount of target DNA, an inert carrier (e.g. glycogen) can be added to the mixture for enhancing the efficiency of precipitation. Other precipitation methods of nucleic acid consist of selective precipitation by salting out or changes in pH.

Table 4 Examples of the PCR process inhibitors

Inhibitor	Inhibiting concentration
SDS	> 0.005%
Phenol	> 0.2%
Ethanol	> 1%
Isopropanol	> 1%
Sodium acetate	> 5 mM
Sodium chloride	> 25 mM
EDTA	> 0.5mM
Hemoglobin	> 1mg/ml
Heparin	> 0.15 i.u./ml
Urea	> 20 mM
Reaction mixture	> 15%

Source: Somma, 2004

1.2.6.2 The nuclear ribosomal DNA (Edel, 1998)

The fungal nuclear ribosomal DNA is organized as an rDNA unit, which is tandemly repeated. One unit comprises three rRNA regions: the small nuclear rRNA (18S), the 5.8S rRNA (5.8S) and the large rRNA genes (28S). In one unit, the genes are separated by the internal transcribed spacer (ITS1 and ITS2) and two rDNA units are separated by the intergenic spacer (IGS) (Figure 5). The last rRNA gene (5S) may or may not be included in the repeated unit, relying on the

fungal taxa. The 18S rDNA evolves relatively slowly and is useful for comparing between high taxonomic levels. The non-coding regions (ITS and IGS) evolve faster and are useful for comparing fungal species within a genus or strains. Some regions of 28S rDNA are also variable between species. Ribosomal DNA sequences are generally used for the taxonomic and phylogenetic studies because they are found universally in living cells. These sequences consist of variable and conserved regions, permitting the comparison and identification of organisms at different taxonomic levels.

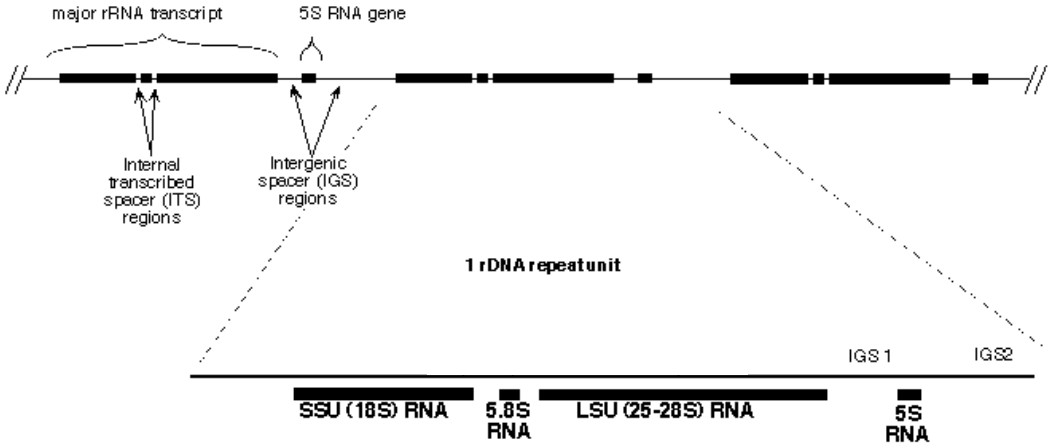

Figure 5 Schematic of the nuclear ribosomal DNA in fungi

Source: http://biology.duke.edu/fungi/mycolab/primers.htm

1.2.6.3 Molecular methods in soil microbiology

The general principle of most molecular techniques is based on the electrophoresis separation of PCR products amplified from DNA or RNA (Sichler *et al*., 2007). Several approaches using for molecular identification in soil community include:

1.2.6.3.1 DNA hybridization

DNA hybridization with a labeled probe is used to detect the presence of complementary target sequence in a complex of nucleic acid molecules (Wetmur, 1991). Double stranded DNA is denatured to single stranded DNA by the increased temperature or pH. The denatured DNAs are separated by agarose gel electrophoresis

and transferred to either a nitrocellulose or nylon membrane. The membrane-bound DNA is then hybridized with labeled probes homologous to target gene (Olive and Bean, 1999).

1.2.6.3.2 Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Sichler *et al*., 2007)

Principle of these techniques is the separation of DNA fragments having the same length but different nucleotide sequence by electrophoresis in a gradient of increasing denaturing strength, which is set up either via chemical (denaturants e.g. urea and formamide-DGGE) or physical way (temperature-TGGE). The fragments will move according to their melting behavior under the different denaturing conditions. A fingerprint comprising of bands at different migration distance will appear in the polyacrylamide gel.

1.2.6.3.3 Polymerase chain reaction (PCR) (Spiegelman *et al*., 2005)

PCR is a way to amplify nucleic acid from whole-cell extracts or total community DNA from an environment sample. DNA is exposed to a thermostable polymerase and provided repetitive cycles of template strand denaturation, oligonucleotide primer annealing and polymerization of the template-primer duplex. This process offered the exponential amplification of the template DNA. More details of PCR are described in 1.2.6.4.

1.2.6.3.4 Restriction length polymorphism (RFLP) (Rasmussen, 2012)

The first step of this technique is amplification of an interesting gene. The amplified fragments are then cut with restriction enzymes resulting in the formation of restriction fragments of different sizes. An electrophoresis is used to separate the treated DNA fragment.

1.2.6.3.5 Terminal restriction length polymorphism (T-RFLP) (Schütte *et al*., 2008)

T-RFLP is also relied on PCR amplification of a target gene using primers labeled with a fluorescent dye. The PCR product is digested with restriction

enzymes that have four base-pair recognition sizes. After the restriction reaction, the mixture of fragments is separated by electrophoresis and determined the different sizes of terminal fragments using an automated DNA sequencer.

1.2.6.3.6 Single-strand conformational polymorphism analysis (SSCP)

SSCP is a method to separate the same size of single-stranded DNA (denatured double-stranded DNA) based on the different conformations formed which depended on their sequences by non-denaturing polyacrylamide gel electrophoresis (PAGE) (Sichler *et al*., 2007). The motility of DNA in a gel matrix is partially determined by its shape (Spiegelman *et al*., 2005).

1.2.6.3.7 Ribosomal intergenic spacer analysis (RISA) (Spiegelman *et al*., 2005)

RISA is a PCR-based method that amplifies the prokaryotic ribosomal intergenic spacer (IGS) region. The IGS region possesses a high degree of sequence variability. This technique creates a community profile based on the species-specific length polymorphisms in this region and can be used to distinguish between different strains and closely-related species.

1.2.6.3.8 Pulse-field gel electrophoresis (PFGE) (Nassonova, 2008)

PFGE is a technique for separation of high molecular weight DNA ranging from 10 kb to 10 Mb using agarose gel electrophoresis with an electric field that alternates in two directions. This technique greatly promotes the structural and functional analysis of genome in various organisms.

1.2.6.4 Polymerase chain reaction (PCR)

1.2.6.4.1 Overview of PCR methods (Edel, 1998)

The polymerase chain reaction (PCR) is a potent method with widespread applications in the molecular biology. Since its development in 1985, the specificity, sensitivity and speed of this technology have led to the development of many methods for a broad range of molecular biological research areas. This reaction permits *in vitro* amplification of specific DNA fragments from complex DNA

samples. Any nucleic acid sequence can be cloned, analysed or modified. Fungal studies will continue to progress with PCR and new methods are regularly reported.

1.2.6.4.2 The standard principle of PCR (Edel, 1998)

The standard PCR method requires a DNA template containing the target region and two oligonucleotide primers flanking this region. A thermostable DNA polymerase (*Taq* polymerase) is used for the amplification. The PCR procedure consists of three steps which are determined by temperature condition:

DNA denaturation: In this step, the hydrogen bonds between complementary bases are broken down by high temperature (90-95°C). The double-stranded DNA subsequently becomes single-stranded DNA.

DNA annealing: The reaction temperature is decreased to 50-65°C allowing the target specific oligonucleotide primers anneal to the 5'end of the two single-stranded templates.

DNA extension: The polymerase binds to the primer-template hybrid and begins DNA formation at 72 °C.

This sequence of three steps corresponds to one cycle of PCR. In the second cycle, the newly synthesized DNA strands are separated from the original strands by the denaturation and each strand acts again as template in the next step. Therefore, *n* cycles of PCR allow a 2^n-fold amplification of the target DNA (Figure 6).

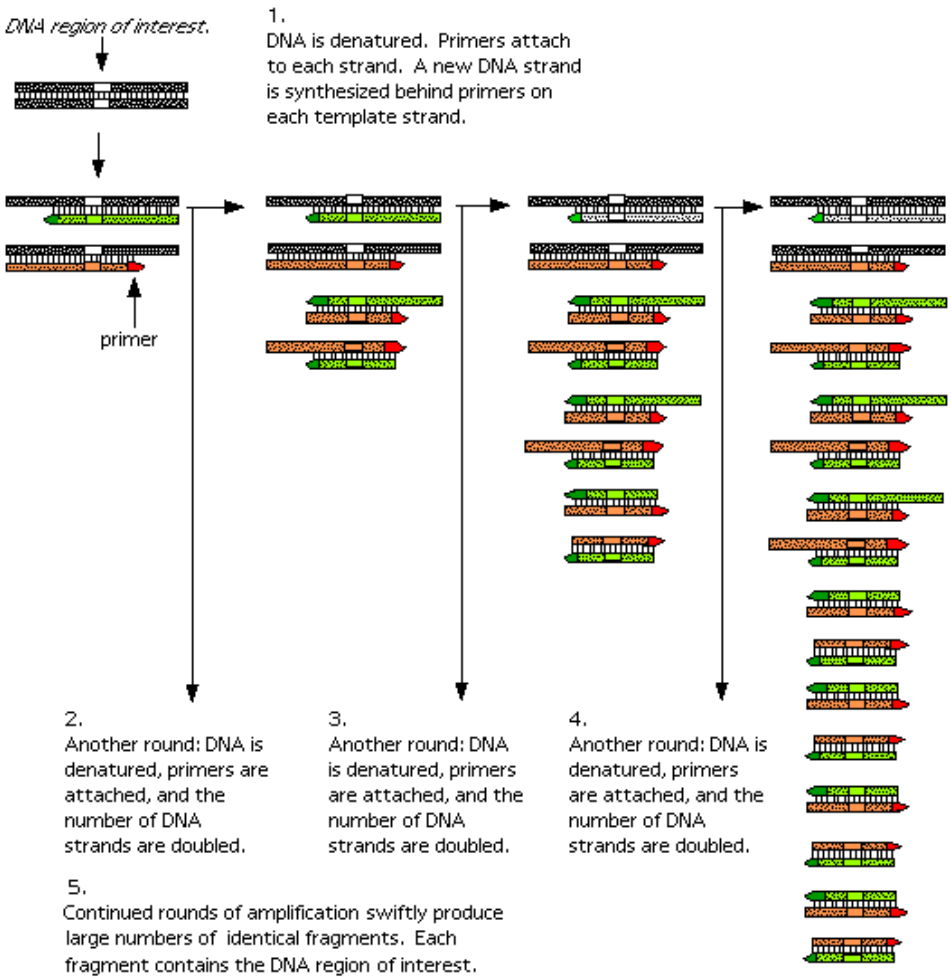

Figure 6 Schematic of PCR amplification

Source: http://www.accessexcellence.org/RC/VL/GG/polymerase.php

1.2.6.4.3 PCR reaction components and conditions

The template DNA, oligonucleotide primers, DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) are mixed in PCR buffer containing magnesium ions ($MgCl_2$). The final volume of the reaction mixture ranges from 25-100 µl (Edel, 1998). Standard conditions for the concentration of the different components are given in Table 5.

Table 5 Standard conditions for PCR amplification: concentration of the different PCR components

Component	Concentration
DNA template	10-100 ng
Amplification buffer	1/10 final volume (buffer is supplied 10X concentrated with the *Taq* polymerase
$MgCl_2$	0.5-5 mM (typically 1.5 mM)
dNTPs	20-200 µM each of dATP, dCTP, dGTP and dTTP
Primer 1 (forward)	0.1-0.5 µM
Primer 2 (reverse)	0.1-0.5 µM
Taq polymerase	0.5-2.5 units
Sterile deionized water	To final volume
Final volume	25-100 µl

Source: Edel, 1998

DNA template (Grunenwald, 2003): The concentration and quality of DNA templates are relate to the successful rate of the PCR amplification. The DNA template should be less damage and high purity. The higher concentration of DNA templates are recommended for long PCR (>5 kb).

PCR buffer (Grunenwald, 2003): A buffer system of 10 mM Tris-HCl (pH 8.3–8.4 at 20–25°C) and 50 mM KCl is normally used in standard PCR amplifications using *Taq* DNA polymerase. These standard buffer systems are commercially available and have been shown to produce satisfactory PCR amplifications in most cases.

$MgCl_2$ (Grunenwald, 2003): The magnesium concentration may affect all of the followings: primer annealing, DNA strand denaturation temperatures of both templates and PCR product, product specificity, formation of primer-dimer, DNA polymerase activity and fidelity. Insufficient magnesium can decrease yield of the PCR products whereas excess magnesium results in accumulation of nonspecific products.

dNTPs (Grunenwald, 2003): The lower concentration of dNTPs, the greater specificity and fidelity of PCR amplifications. On the other hand, the high concentration of dNTPs could induce the error rate of DNA polymerase.

Primer (Innis and Gelfand, 1990): High primer concentrations promote mispriming and increase both of nonspecific products and primer-dimer, while low concentration affects to the PCR yield.

DNA polymerase (Innis and Gelfand, 1990): The high concentration of enzyme causes the accumulation of nonspecific products, whereas low concentration could give an insufficient amount of desired PCR product.

Additive reagents: Several additive reagents are added into the PCR reaction to increase specificity and yield when all else fails (Roux, 1995; Lorenz, 2012). The list and function of the additive reagents frequently used for PCR are shown in Table 6.

1.2.6.5 Basic methods for the detection of PCR products

1.2.6.5.1 Direct visualization using ethidium bromide (Jenkins, 1994)

The most common and easiest method used for detecting PCR is ethidium bromide staining. Ethidium bromide is a fluorescent dye that intercalates between the staked bases of DNA. DNA products are visualized and can be photographed under UV light at 260 nm. Identification of the PCR product is based on the occurrence of a DNA band of the prospective length. Sizing of the DNA bands is achieved by running the PCR products compared with DNA markers. The major disadvantage of ethidium bromide detection is the dye only detect bands that contain ~5 ng or more of DNA. Another disadvantage is this method can lead to faulty interpretations if the nonspecific bands are excess or very close to the same size as the desired DNA product due to all of the DNA products (both specific and nonspecific) will be stained.

1.2.6.5.2 Incorporation of radioactive and nonradioactive labels (Jenkins, 1994)

Incorporation of specific labels is done with the radioactive or non-radioactive labels directly added into the amplified products. Incorporation of specific labels decreases the amount of one or more non-labeled deoxynucleotide triphosphates (dNTPs) and increases a corresponding labeled dNTP. ^{32}P and ^{35}S are usually used as radioactive labels, whereas biotin and digoxigenin are used as non-

radioactive labels. Detection of PCR products labeled with radioisotopes is achieved by autoradiography on dried gels or membrane which the DNA products have been transferred. The detection of PCR products labeled with biotin or dioxigenin requires the transfer of the DNA products to membrane, then visualized by the treatment of the membrane with a streptavidin-enzyme conjugate and a chromogen substrate appropriate for the specific enzyme. The sensitivity of this method is greater than ethidium bromide staining. They can detect bands that contain <1 ng of DNA. The major disadvantage of this method is that the visualization of both specific and nonspecific PCR products. The number of nonspecific bands can be seen greater than ethidium bromide staining. However, direct labeling of PCR products is not generally employed.

1.2.6.5.3 Detection of specific PCR products using a DNA probe (Jenkins, 1994)

The use of DNA probe is recommended for identification of a specific PCR product. DNA probe hybridizes to a region of the DNA located internally between the two PCR primers. Only the specific amplified product will be detected. The DNA probe can be either labeled with radioisotopes or non-radioactive markers. The use of labeled DNA probes increases sensitivity and accuracy over ethidium bromide staining; thus they are widely used for the detection of PCR products.

Table 6 Additive reagents used in PCR amplification

Additive reagents	Concentration	Function	Referrence
Gelatin Bovine serum albumin Nonionic detergent e.g. Tween-20, Triton X-100	0.01% 10-100 µg/ml 0.05-1%	Stabilize DNA polymerase	Gelfand, 1989
Dimethyl sulfoxide (DMSO) Formamide Glycerol Polyethylene glycol	1-10% 1.25-10% 5-20% 5-15%	Affect the Tm of primers, the thermal activity profile of *Taq* DNA polymerase, as well as the degree of product strand separation	Gelfand, 1989
Tetramethylammonium chloride (TMAC)	1-3 M	Eliminate nonspecific priming	Uggozoli and Wallace, 1992
$(NH_4)_2SO_4$	15-30 mM	Increase the ionic strength of the reaction mixture, altering the denaturation and annealing temperatures of DNA, and also affect polymerase activity	Grunenwald, 2003
N,*N*,*N*-trimethyglycine (betaine)	1-3 M	Increase the thermostability of DNA polymerases, as well as to alter DNA stability such that GC-rich regions melt at temperatures more similar to AT-rich regions	Mytelka and Chamberlin, 1996

Source: Grunenwald, 2003

1.2.7 Molecular phylogenetic analyses

1.2.7.1 DNA sequencing

DNA sequence analysis has become the most useful method for inferring phylogenetic relationships between organisms. Bruns *et al*. (1990) explained the benefits of DNA sequencing for phylogenetic analysis as "the large number of characters compared can substantially increase the resolving power". In general, the DNA cloning and direct sequencing are employed for sequencing of PCR products. Direct sequencing is widely used for the phylogenetic studies more than DNA cloning because this technique generates consensus sequence of each nucleotide and minimizes possible errors from misincorporation (Takamatsu, 1998).

1.2.7.2 DNA sequences alignment

The sequence alignment is a way of arranging the sequences of DNA for identifying the similar region between the sequences. This step could show the evolutionary relationships among the taxa (Mount, 2004). Alignment can be done automatically through the softwares such as Clustal X/W and can be subsequently refined manually.

1.2.7.3 Phylogenetic tree reconstruction

1.2.7.3.1 Method of analysis

1.2.7.3.1.1 Distance criterion (Harrison and Langdale, 2006)

Distance methods (e.g. neighbor-joining, UPGMA) construct trees by calculating pairwise distances between sequences that are most similar. This method considers the current states of characters without regarding to the evolutionary of history. They are simplicity and speedy but only one tree is obtained. The distance tree is not possible to examine conflicting tree topologies. Although, distance methods are frequently useful for creating an initial tree, they should be used for final trees with discretion.

Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) (Hall, 2001)

UPGMA is an example of clustering method. The program searches the pair of taxa with the fewest distance between them and sets the branching between them as half of the distance. It then joins the two taxa into a cluster and redraws the matrix with the distance from the cluster to each of the remaining taxa. Since the cluster serves as a substitute for two taxa, the number of entries in the matrix is reduced by one. That process is repeated on the new matrix and repeated until the matrix contains a single entry. The set of matrices is then used to build up the tree by starting at the root and moving out to the first two nodes represented by the last two clusters.

Neighbor joining (Hall, 2001)

Neighbor joining (NJ) is similar to UPGMA in a distance matrix manipulation but it directly calculates distances to internal nodes without creating clusters. NJ uses net divergence of each taxon from all other taxa to calculate a corrected distance matrix. NJ then finds the pair of taxa with the lowest corrected distance and computes the distance from each of those taxa to the node that joins them. A new matrix is then created in which the new node is substituted for those two taxa. NJ does not assume that all taxa are equidistant from a root.

1.2.7.3.1.2 Character-based criterion

Maximum parsimony (Harrison and Langdale, 2006)

Maximum parsimony constructed trees based on assumption about ancestral relationships as well as current data. Groups establish on the basis of shared characters and the fewest mutations of characters are taken to be the most parsimonious. With multiple characters, different groupings may be equally possible or parsimonious, and thus multiple trees are generated. The exhaustive enumeration, branch and bound or heuristic search are employed to find the most parsimonious tree (MPTs). A majority rule consensus tree shows nodes that are stable in half to all of the most parsimonious trees and the percentage of trees in which a given topology exists is shown on the branches.

Maximum likelihood (Harrison and Langdale, 2006)

Maximum likelihood analysis calculates the probability of every individual nucleotide substitution, giving a specific model of sequence evolution. The analysis starts with a specified tree derived from the input dataset (for example a NJ tree) and swaps the branches on the starting tree until the tree with the highest likelihood score is obtained. This score is a function both of the tree topology and the branch lengths (number of character state changes). Likelihood analysis allows an absolute examination of the ascription made about sequence evolution but it is time-consuming for constructing the tree.

1.2.7.3.2 Tree evaluation

Bootstrapping analysis (Brinkman and Leipe, 2001)

Bootstrapping analysis is performed to verify the support for the branches of phylogenetic tree. Positions are re-sampled randomly from the alignment to obtain multiple subsamples (bootstrap replicates) of the original alignment. The results are exhibited as percentage of bootstrap support. Bootstrapping works with distance, parsimony and likelihood methods.

Consistency index (CI) (Lipscomb, 1998)

Consistency index is a relative amount of homoplasy within the tree. CI is calculated as the number of steps expected given the number of character states in the data, divided by the actual number of steps multiplied by 100.

$$CI = \frac{\text{total character state changes expected given the data set}}{\text{actual number of steps on the tree}} \times 100$$

Retention index (RI) (Lipscomb, 1998)

The retention index measures the amount of synapomorphy expected from a data set that is retained as synapomorphy on a tree.

$$RI = \frac{\text{maximum number of steps on tree - number of state changes on the tree}}{\text{maximum number of steps on tree - number of state changes in the data}} \times 100$$

Rescaled consistency index (RC) (Farris, 1989)

Rescaled consistency index observes homoplasy on scale from minimum possible (0) to maximum possible homoplasy (1). The formula for RC is:

$$RC = CI \times RI$$

Homoplasy index (HI) (Sang, 1995)

Homoplasy index describes the level of homoplasy of the characters. The formula for HI is:

$$HI = 1 - CI$$

1.2.8 Rajjaprabha dam

Rajjaprabha dam is constructed on Khlong Saeng, at Ban Chiew Larn, Ban Ta Khun district, Suratthani province. Its main purposes consist of power generation, irrigation, flood control, fishery enhancement and environment protection. After the project completion, the Chiew Larn Dam conferring by His majesty the King to be called as "Rajjaprabha" Dam, which means the "Light of the Kingdom" (http://www.ieahydro.org/reports/Annex_VIII_CaseStudy0701_ChiewLarn_Thailand.pdf). In 2005, Electricity generating authority of Thailand (EGAT) requested to a Thai monarch King's permission to participate in the Plant Genetic Conservation Project area under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhron (RSPG) and allocated area of twelve dams under their control for RSPG project. Rajjaprabha dam has RSPG total area of 1,418 Rai. The topography of this area is a high limestone mountain range and tropical rainforest (Figure 7). The characteristic of soil from RSPG area has been studied by Yongchalermchai *et al*. (2011). In upper land, the landform is denudation surface from erosion and its topology is undulating to hilly. The parent material is residuum from sandstone. For the low land, the area is flat to nearly flat. The landforms varied from natural levee to alluvial plain and the parent material derived from transported materials as riverine alluvium. In general, the soils in RSPG area are somewhat well drained to well drained with rapid permeability and rapid to slow run off. Soil texture varied from sandy loam to sandy clay loam. Soil reaction was very strongly acid to slightly acid.

Organic matter content, amount of available phosphorus and percent of base saturation were low to very high. Total nitrogen and exchangeable magnesium were very low to medium. Available sulphur was low and available calcium and potassium were very low to high, exchangeable sodium was very low whereas cation exchange capacity was low to medium.

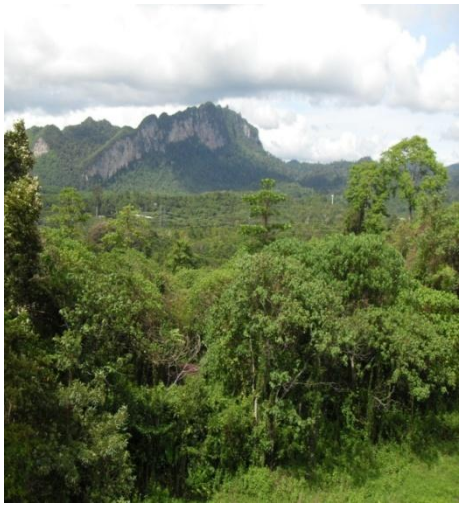

Figure 7 Photograph of Plant Genetic Conservation Project area under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhron at Rajjaprabha dam

1.3 Objectives of this study

1) To follow the objectives of Plant Genetic Conservation Project Under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG)

2) To search for potential antimicrobial producing fungi in soils from RSPG area.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Collecting sites

Soil samples were collected from five survey trails of Plant Genetic Conservation Project area Under The Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG), Rajjaprabha dam, Suratthani province.

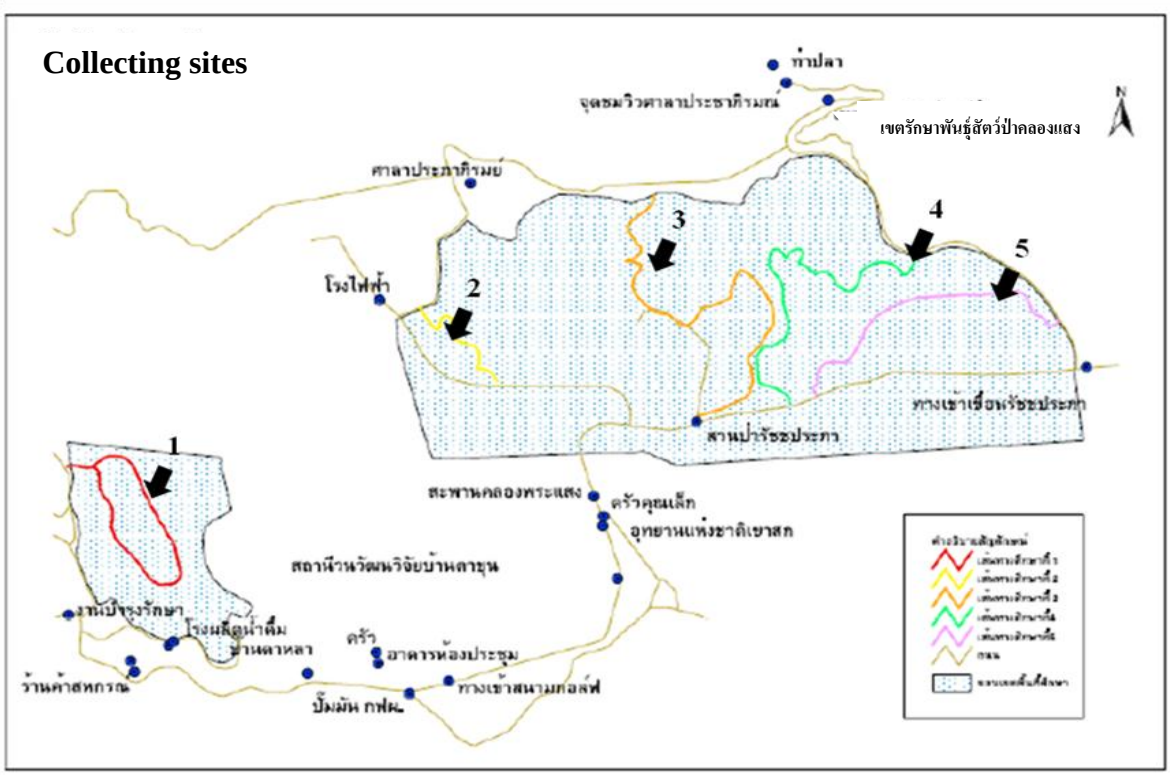

Figure 8 Map of Rajjaprabha dam showing the five survey trails

2.1.2 Test microorganisms

Bacteria

- *Staphylococcus aureus* ATCC 25923 (SA)
- Methicillin-resistant *Staphylococcus aureus* SK1 clinical isolate (MRSA SK-1)
- *Escherichia coli* ATCC 25922 (EC)
- *Pseudomonas aeruginosa* ATCC 27853 (PA)
- Multidrug-resistant *Acinetobacter baumannii* NPRC AB005 clinical isolate (AB005)
- Multidrug-resistant *Acinetobacter baumannii* NPRC AB007 clinical isolate (AB007)

Yeasts

- *Candida albicans* ATCC 90028 (CA28)
- *Candida albicans* NCPF 3153 (CA53)
- *Cryptococcus neoformans* ATCC 90112 (CN12), flucytosine-susceptible strain
- *Cryptococcus neoformans* ATCC 90113 (CN13), flucytosine-resistant strain

Filamentous fungi

- *Microsporum gypseum* SH-MU4 clinical isolate (MG)
- *Penicillium marneffei* clinical isolate (PM)

2.1.3 Chemicals

- Lacto phenol cotton blue
- Normal saline solution (0.85% NaCl) (Appendix)
- Ethanol (commercial grade)
- Glycerol (Fluka)
- McFarland Standard (Appendix)
- Dimethyl sulfoxide (DMSO) (Merck)
- D-glucose (BDH Prolabo)
- Phosphate buffer saline (PBS) pH 7 (Appendix)
- 1.8% resazurin (Appendix)

2.1.4 Media

- Rose-bengal chloramphenicol agar (RBC) (Merck)
- Potato dextrose agar (PDA) (Appendix)
- Potato dextrose broth (PDB) (Appendix)
- Mueller-Hinton broth (MHB) (Difco)
- Sabouraud dextrose agar (SDA) (Difco)
- Sabouraud dextrose broth (SDB) (Difco)
- Nutrient agar (NA) (Difco)
- Nutrient broth (NB) (Difco)
- Granulated agar (Difco)

2.1.5 Antibiotics

- Vancomycin (Sigma Chemical Co., USA)
- Gentamicin (NIDA Pharma Inc., Thailand)
- Amphotericin B (Bristol-Mayer Squibb Co., USA)
- Miconazole (Sigma Chemical Co., USA)
- Colistin (Atlantic Laboratories Co., Ltd Thailand)

2.1.6 Chemicals for molecular identification

- 0.5 M NaOH (Appendix)
- Tris-HCl pH 8 (Appendix)
- 50 mM $MgCl_2$ (iNtRON Biotechnology, Inc.)
- 10x PCR buffer (iNtRON Biotechnology, Inc.)
- 10 mM dNTPs mix (iNtRON Biotechnology, Inc.)
- *i*-Taq DNA polymerase (iNtRON Biotechnology, Inc.)
- Nanopure water
- Agarose (Research organics)
- TAE buffer (Appendix)

  (Tris Acetate EDTA buffer, pH 8)
- 6X Loading dye (Appendix)
- Lamda DNA/Eco911 (BstEII) Marker (Fermentas)
- Ethidium bromide (Fluka)
- Liquid N_2

- CTAB lysis buffer (Appendix)
- 5 M NaCl (Appendix)
- Chloroform: Isoamyl alcohol (24:1)
- 7.5 M Ammonium acetate
- 70% ethanol
- DNeasy® Plant Mini Kit (Qiagen)
- NucleoSpin® Plant II (Machery-Nagel)
- 4 M *N*,*N*,*N*-trimethyglycine (betaine) (Sigma-Aldrich)
- Sea sand (Sigma-Aldrich)

2.1.7 Instruments

- Stereozoom microscope (Olympus SZ-PT)
- Light microscope (Olympus CX31)
- Hemacytometer
- Microtube (Eppendorf)
- Pipette tips (Axygen)
- Automatic pipette (Eppendorf)
- Multi channel automatic pipette (Eppendorf)
- Hot plate stirrer (Thermolyne)
- Biosafety cabinet class II
- Autoclave (Tomy)
- 25°C Incubator (Gallenkamp)

- 35°C Incubator (Brandt)
- Incubator shaker
- Hot air oven (Binder)
- Freezer -20°C (Sanyo)
- Freezer -80°C (New Brunswick Scientific)
- Electronic balance (Sartorius)
- PCR thermal cycler (Bio-Rad)
- Centrifuge (Eppendorf 5417R)
- Gel electrophoresis machine (Bio-Rad)
- Gel documentation (Model Syngene Gene Genius)
- Heat block (Labnet)
- Lyophilizer (LABCONCO)

2.2 Methods

2.2.1 Collection of soil fungi

Soil samples were collected four times in January, April, June and August 2010 from five trails of RSPG areas, Rajjaprabha dam, Suratthani province, five samples per trail per collection. A total of 100 soil samples were obtained. Each soil sample was collected from 10-15 cm in depth from the soil surface. The samples were kept in sterile plastic bag and brought back to the Mycology laboratory, Prince of Songkla University for isolation.

2.2.2 Isolation and enumeration of soil fungi

Soil fungi were isolated from soil samples by the dilution plate method. Each sample from the first collection was individually isolated. For the second to forth collections, five samples in each trail were pooled as one sample. Each 10 g of soil sample was added to 90 ml sterile 0.85% NaCl solution (1:10 dilution). The sample suspensions were shaken in incubator shaker at room temperature (RT) for 10 minutes and left unshaken for 20 minutes. Then, 1 ml of the soil suspension was serially diluted in 9 ml 0.85% NaCl from 10^{-2}-10^{-6} and spread on rose-bengal chloramphenicol agar (RBC). Each dilution was spread in duplicate. Plates were incubated at RT for 3-5 days. Fungal colonies were counted (Figure 9) and calculated for fungal density in each sample. The fungal colonies with different morphologies were isolated by the hyphal tip isolation method onto potato dextrose agar (PDA) under stereozoom microscope. The pure cultures were transferred to PDA slant and maintained at RT until use.

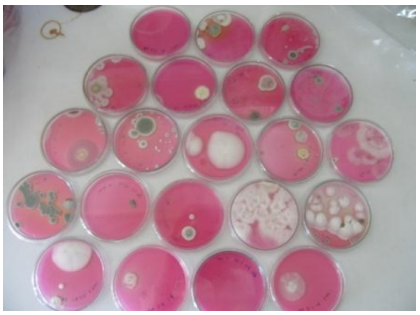

Figure 9 Soil fungal colonies on rose-bengal chloramphenicol agar

2.2.3 Cultivation of soil fungi in broth medium for chemical extraction

Soil fungi were selected from different morphologies to cultivate in potato dextrose broth (PDB). Selected soil fungi were cultured on PDA for 3-5 days or until their colonies were approximately 2 cm at RT. Then, the margin of colonies was cut into the size of 0.5 x 0.5 cm^2 with sterile surgical blade. Five pieces of mycelial agar plugs were inoculated into 300 ml PDB in 500 ml Erlenmeyer flask and incubated at RT for 3 weeks under stationary conditions (Phongpaichit *et al*., 2006).

Fermentation broth was separated into culture filtrate and fungal mycelium by filtration. The culture filtrate was extracted 3 times with 300 ml ethyl acetate (EtOAc) in separating funnel. Broth ethyl acetate was evaporated at 40-45ºC using a rotary vacuum evaporator to obtain the broth EtOAc extract (BE). The fungal mycelium was soaked in methanol (MeOH) for 2-3 days. The aqueous MeOH layer was concentrated by evaporation and water was added. The mixture was extracted with hexane 3 times and evaporated to dryness to give the cell hexane extract (CH). The aqueous layer was extracted 3 times with 300 ml EtOAc and evaporated using rotary vacuum evaporator to obtain the cell EtOAc extracts (CE) (Figure 10).

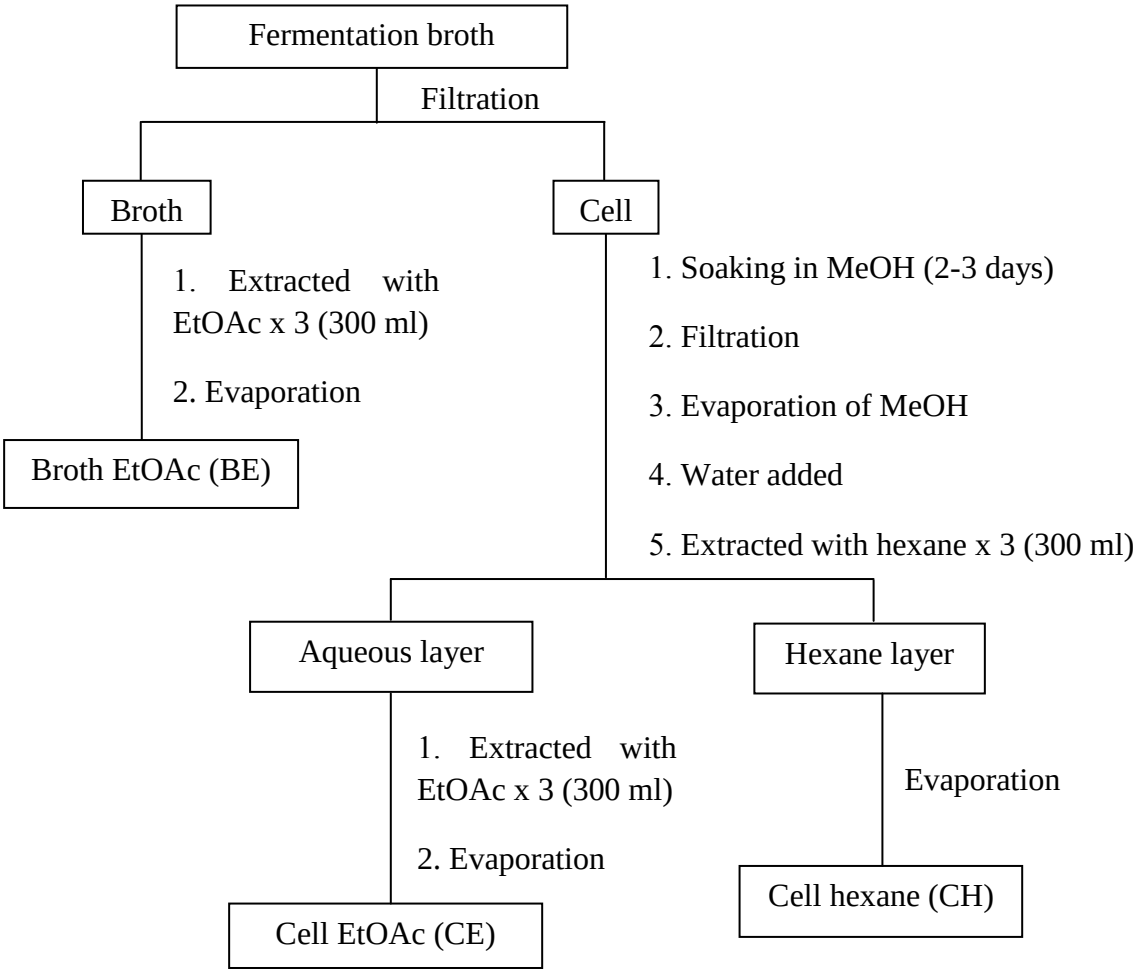

Figure 10 Chemical extractions of secondary metabolites produced by soil fungi

2.2.4 Preliminary antimicrobial activity testing at a concentration of 200 µg/ml

2.2.4.1 Crude extract preparation

Crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions (100 mg/ml) and kept at -20^{o}C. Stock solutions were diluted again with DMSO to obtain the concentration of 10 mg/ml and used as working solution.

2.2.4.2 Standard drug preparation

Vancomycin, gentamicin and colistin were dissolved in sterile distilled water (DW) to obtain stock solution of 16 mg/ml. Amphotericin B was dissolved in sterile DW at 10 mg/ml. DMSO was used to prepare a stock solution of miconazole at the concentration of 3.2 mg/ml.

2.2.4.3 Inoculum preparation

Bacteria were streaked on nutrient agar (NA) and incubated at 35°C for 18-24 hours. *C. albicans* and *C. neoformans* were cultured on Sabouraud dextrose agar (SDA). *C. albicans* was incubated at 35°C, 18-24 hours while *C. neoformans* was incubated at 25°C, 48 hours. Three to five single colonies were inoculated into nutrient broth (NB) for bacteria and Sabouraud dextrose broth (SDB) for yeasts. Bacterial and yeast inocula were then shaken at 150 rpm, 35°C in incubator shaker for 3-5 hours. After incubation, sterile normal saline (NSS) was used to adjust the turbidity of inocula. Bacterial inocula were adjusted to 0.5 McFarland standard (MF) and diluted 1:200 (~7.5×10^{5} CFU/ml) with Mueller-Hinton broth (MHB). Yeast inocula were adjusted to 2.0 MF and diluted 1:20 (~3×10^{7}) using SDB. Filamentous fungi were grown on SDA at 25°C until they produced spore. Sterile glass beads were added to fungal culture plate. Plates were shaken gently and 2 ml of sterile NSS were added. Spore suspensions were adjusted to $8x10^{3}$ conidia/ml with SDB using hemacytometer.

2.2.4.4 Antibacterial activity testing (modification of CLSI M07-A9, CLSI, 2012)

Crude extract working solutions (10 mg/ml) were diluted 1:25 with MHB to obtain the concentration of 400 µg/ml. Triplicate 50 µl of crude extracts were placed into sterile microtiter plates and 50 µl of bacterial inocula were added. Therefore, the final concentrations of crude extracts were 200 µg/ml. Plates were incubated at 35°C for 15 hours, and then 20 µl of 0.09% resazurin was added into each well. Plates were further incubated for 3 hours at 35°C for complete incubation (adapted from Sarker *et al*., 2007). Standard drugs were used as a positive control including vancomycin (10 µg/ml) for *S. aureus* and MRSA, gentamicin (10 µg/ml) for *E. coli* and *P. aeruginosa* and colistin (1,000 µg/ml) for *A. baumannii*.

2.2.4.5 Antifungal activity testing against yeasts (modification of Liu *et al*., 2007; CLSI M27-A3, CLSI, 2008a)

The antifungal activities of crude extracts against yeasts were investigated in a similar way to bacteria but SDB was used as culture medium. Tested microtiter plates were incubated at 35°C, 15 hours for *C. albicans* and 25°C, 24 hours for *C. neoformans*, and then 20 µl of 0.09% resazurin were added into each well. Plates were examined after further incubation for 3 hours for *C. albicans* and 24 hours for *C. neoformans*. Amphotericin B (10 µg/ml) was used as a positive control.

Interpretation: If a blue or purple color occurred indicating that crude extract can inhibit the growth of test microorganisms (positive result). A pink or colorless indicated that test microorganisms were survived or no inhibition (negative result).

2.2.4.6 Antifungal activity testing against filamentous fungi (modification of CLSI M38-A2, CLSI, 2008b)

Filamentous fungi were tested in a similar way to yeasts. Microtiter plates were incubated at 25°C for 7 days. The growth of fungal mycelium was observed everyday under stereo-zoom microscope. No growth of mycelium was reported as a positive result.

Active crude extracts were further evaluated for their minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs).

2.2.5 Determination of MIC, MBC and MFC

The MICs of crude extracts were determined in a similar way to the preliminary test but active crude extracts were diluted in triplicate using the serial dilution method. The final concentrations of crude extracts were 0.25-128 µg/ml. The lowest crude extracts concentration that inhibited the growth of tested microorganisms was recorded as the MIC.

The streaking method was used to determine the MBCs and MFCs. Wells of MIC and higher concentrations were streaked on NA for bacteria or SDA for yeasts and filamentous fungi. The lowest concentration that killed tested microorganisms was reported as the MBC or MFC.

The MICs values were categorized into 3 levels.

Strong activity	:	MIC $\leq$ 8 µg/ml
Moderate activity	:	MIC = 16-64 µg/ml
Weak activity	:	MIC = 128-200 µg/ml

2.2.6 Study on the possible mechanisms of action of the strongly active fungal crude extracts by scanning electron microscopy (SEM)

The strongly active crude extracts were selected to study the possible mechanisms of action by SEM. Test microorganisms were streaked on NA for bacteria or SDA for yeasts. Bacteria and *C. albicans* were incubated at 35°C for 18-24 hours. *C. neoformans* was incubated at 25°C for 48 hours. Three to five single colonies were transferred to 100 ml of MHB (bacteria) or SDB (yeasts). Culture broth was incubated at different times and temperatures depending on test microorganisms. Culture broth was centrifuged at 5000 rpm, 5 minutes and supernatant was discarded. Broth medium (10 ml) was added into a tube and mixed well. One ml of cells suspension and 1 ml of crude extract at 8 times their MIC (8xMIC) in broth medium

were added to obtain the final concentrations of 4xMIC. After incubation, culture broth was centrifuged at 5000 rpm for 5 minutes and washed 3 times with phosphate buffer saline (PBS) pH 7.

Fungal mycelial plugs (1 x 1 cm^2) from the margin of actively growing colony were cut and added into 4xMIC crude extract in SDB. Cells were incubated 3 days at 25°C and washed with PBS pH 7.

Treated cells were kept at 4°C and sent to Scientific Equipment Center, Prince of Songkla University for SEM study.

In the step of cell fixation, treated cells were fixed with 2.5% glutaraldehyde in PBS for 1-2 hours. Cells were washed with PBS and immersed in 1% OsO_4 for 1-2 hours. Cells were washed again with DW and dehydrated with alcohol series (50%, 70%, 80%, 90% and 100%). Cells were dried by critical point drying (CPD) method. Samples were mounted on a stub and gold-coated before observation with SEM.

2.2.7 Identification of soil fungi

Soil fungi presenting strong antimicrobial activity or interesting NMR profile were selected for identification.

2.2.7.1 Morphological identification

Macroscopic morphology

Soil fungi were cultured on PDA at RT for 3 weeks. The morphological characters such as color of colony, growth rate, colonial morphology and pigment production were observed and recorded. Isolates that do not produce any reproductive structures after 1 month of incubation were recorded as mycelia sterilia (Santos *et al*., 2013).

Microscopic morphology

Samples were stained with lactophenol cotton blue. Microscopic characters including sporulation, conidial shape, type of mycelia and other important characters were used for identification according to the keys e.g. Barnett and Hunter (1998) and Samson *et al*. (2004).

2.2.7.2 Molecular identification

Selected soil fungi were grown in 50 ml PDB and incubated at RT for 3-7 days in shaking incubator. Fermentation broth was filtrated with sterile gauze and mycelia were washed several times with sterile DW (~60°C). Fungal mycelia were placed on sterile tissue towel to absorb water, and then freeze-dried using lyophilizer.

2.2.7.2.1 Genomic DNA extraction

CTAB method

Freeze-dried fungal mycelia were transferred to sterile mortar and liquid nitrogen was added. Mycelia were ground into a fine powder and placed into sterile 1.5 ml microtube. Five hundred µl of CTAB lysis buffer (O'Donnell *et al*., 1997) was added, and then the microtube was incubated at 70°C for 45 minutes. 5M NaCl (140 µl) and 10% CTAB (50 µl) were added and the microtube was incubated at 65°C for 10 minutes. The microtube was centrifuged at 12,000 rpm, 4°C for 20 minutes and the lysate was transferred to a new tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added and inverted gently, then centrifuged at 12,000 rpm, 4°C for 10 minutes. An aqueous phase (upper) was transferred to a new tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm, 4°C for 10 minutes. Upper liquid phase was transferred to a new tube after that a half volume of 7.5 M ammonium acetate and a half volume of cold isopropanol were added. Mixture was kept at -20°C for at least 30 minutes and centrifuged at a maximum speed (14,000 rpm), at 4°C for 10 minutes. DNA pellet was washed twice with cold 75% ethanol and air dried. DNA pellet was resuspended with 30-50 µl sterile nanopure water. The DNA was stored at 4°C or -20°C until used.

Quick and dirty method (Wang *et al*., 1993)

Freeze-dried fungal mycelia were ground with sterile sea sand and 0.5 M NaOH (300-500 µl) in a microtube until no large pieces was observed. The microtube was centrifuged at 12,000 rpm, 25°C for 10 minutes. The supernatant was transferred to a new tube and diluted 1:10 with Tris-HCl pH 8. The DNA was kept at 4°C or -20°C until used.

2.2.7.2.2 DNA amplification

ITS1-5.8S-ITS2 and large subunit (28S) rDNA were amplified using universal and fungal specific primers (Table 7 and Figure 9). The PCR mixtures (Table 8) were amplified in a Bio-Rad Thermal Cycler. The PCR profiles are shown in Table 9.

Table 7 Universal and fungal specific primers used for DNA amplification and DNA sequencing

rDNA region	Primers	Direction	Sequence 5'-3'	References
ITS1-5.8S-ITS2	ITS1	Forward	TCCGTAGGTGAACCTGCGG	White *et al*., 1990
	ITS1F	Forward	CTTGGTCATTTAGAGGAAGTAA	
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	
	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	
LSU	LROR	Forward	ACCCGCTGAACTTAAGC	Bunyard *et al*., 1994 Landvik, 1996
	LR7	Reverse	TACTACCACCAAGATCT	
	JS1	Forward	CGCTGAACTTAAGCATAT	
	JS8	Reverse	CATCCATTTTCAGGGCTA	
SSU	NS5	Forward	AACTTAAAGGAATTGACGGAAG	White *et al*., 1990

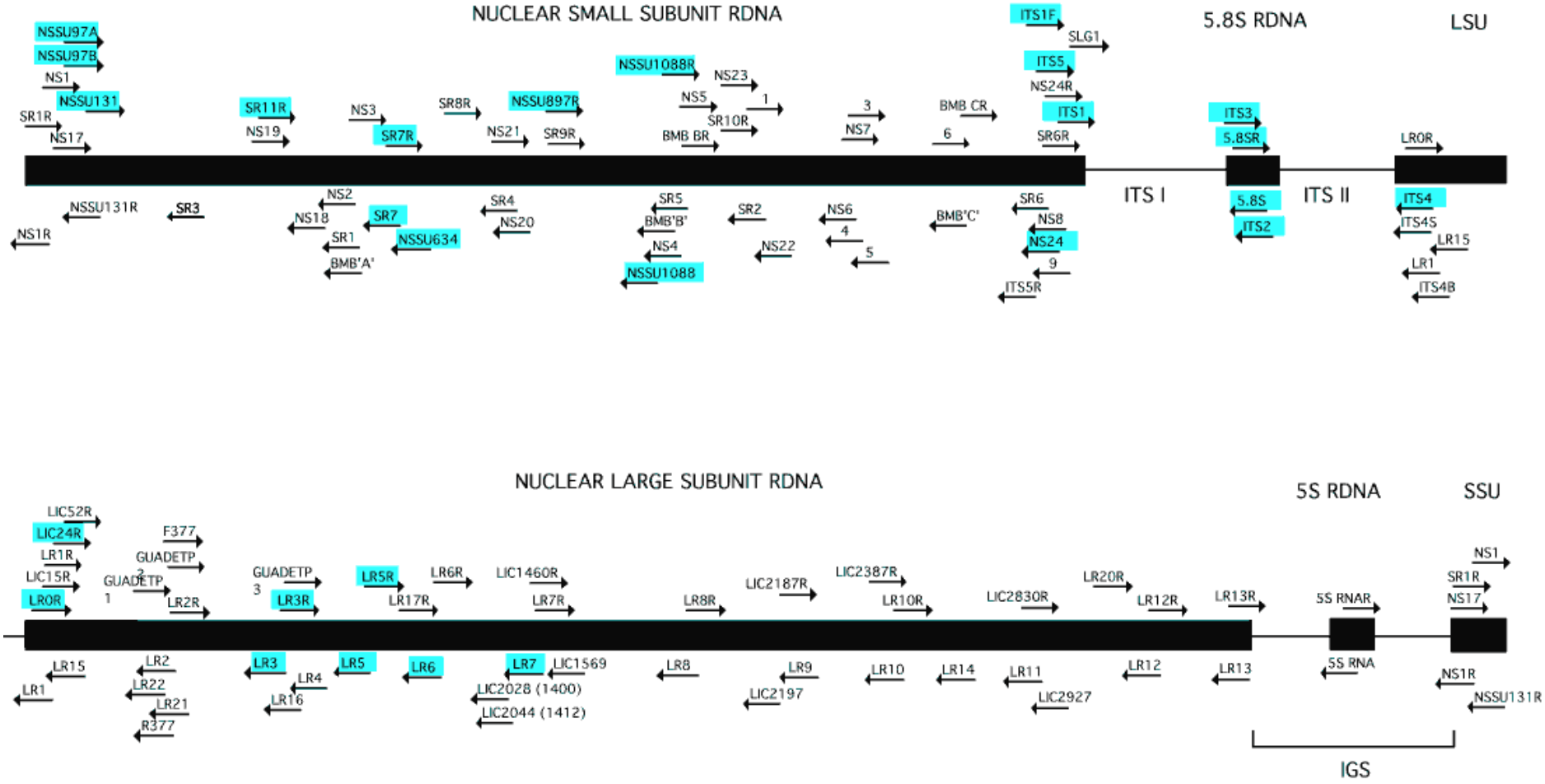

Figure 11 Universal and fungal specific primers map for small subunit (SSU), internal transcribed spacer (ITS) and large subunit (LSU) ribosomal DNA regions**.**

Source: http://www.lutzonilab.net/primers/page244.shtml

Table 8 PCR mixtures for DNA amplification

PCR mixtures	Stock concentration	Used volume (µl)	Final concentration of 50 µl
Master mix 1			
Nanopure water		35.8	
PCR buffer	10X	5	1X
$MgCl_2$	25 mM	5	2.5 mM
dNTPs	10 mM	1	0.2 mM
Forward primer	10 µg/ml	1	0.2 µg/ml
Reverse primer	10 µg/ml	1	0.2 µg/ml
Taq polymerase	5 units/µl	0.2	0.02 units/µl
DNA template	100-500 ng	1	2-10 ng
Master mix 2			
Nanopure water		30.8	
Betaine	4 M	5	0.4 M
PCR buffer	10X	5	1X
$MgCl_2$	25 mM	5	2.5 mM
dNTPs	10 mM	1	0.2 mM
Forward primer	10 µg/ml	1	0.2 µg/ml
Reverse primer	10 µg/ml	1	0.2 µg/ml
Taq polymerase	5 units/µl	0.2	0.02 units/µl
DNA template	100-500 ng	1	2-10 ng

Table 9 PCR profiles for DNA amplification

Steps (°C/min) / Primers	Preheat	Denaturation	Annealing	Extension	Cycle	Final extension
ITS1F/ITS4	94/2.30	94/0.35	53/0.30	72/1.30	40	72/10
ITS1/ITS4 ITS5/ITS4	94/2	94/1	55/1	72/2	35	72/10
NS5/ITS4 ITS5/LR7 LROR/LR7 JS1/JS8	95/2	95/1	55/1.30	72/2.30	34	72/10

2.2.8.2.3 DNA determination

The quantity and quality of genomic DNA and PCR product were examined and roughly estimated by observing the intensity of the DNA band in 1 % agarose gel electrophoresis after stained with ethidium bromide (final concentration 2.5×10^{-5} mg/ml) for 15-20 minutes. The tinted gel was monitored under UV light transilluminator and photographed with gel documentation.

2.2.8.2.4 DNA sequencing, DNA sequences alignment and phylogenetic tree reconstruction

PCR products were purified and directly sequenced by the Macrogen Inc., Korea. The consensus sequence of each isolate was assembled by BioEdit v7.1.7 (Hall, 2012) and compared with NCBI database by BLAST (Basic Local Alignment Tool) program on NCBI website (http://www.ncbi.nlm.nih.gov). The selected fungal sequences and related sequences obtained from NCBI database were aligned in ClustalW (Thompson *et al*., 1994). Phylogenetic trees were created using maximum parsimony analysis by PAUP*v4.0b10 (Swofford, 2002). The heuristic searches with the default options method were used for construct parsimonious trees and the best

tree was estimated by the Kishino-Hasegawa (KH) test (Kishino and Hasegawa, 1989). The assessment of confidence for each clade was performed by bootstrapping values with the default parameters. The fungal DNA sequences were submitted to NCBI database.

CHAPTER 3

RESULTS

3.1 Number of isolated soil fungi

Soil samples were collected four times (25 samples/time) in January, April, June and August, 2010. For the first collection, 5 to 6 samples per trail were collected and each individual sample was isolated. It was found that the fungal density of each sample is in the same range and the fungal morphotypes are similar. Therefore, the samples from each trail of the 2nd to the 4th collections were pooled before isolation. Six hundred and twenty-nine isolates of soil fungi were obtained. Soil samples had fungal density in the range of $4.2x10^4$-$1.1x10^6$ CFU/g (Table 10).

3.2 Fungal species identified by morphology

From 629 isolates of soil fungi, 398 isolates (63.28%) can be identified by their morphology into 27 genera (Table 10). *Penicillium* is the most frequent isolated genus (21.94%) followed by *Aspergillus* (18.60%), *Trichoderma* (4.77%), *Gongronella* (3.66%), *Fusarium* and *Mucor* (2.07%), *Cunninghamella* (1.91%) and other genera (0.16-1.59%). Two hundred and thirty-one isolates (36.88%) did not produce any reproductive structures and were grouped as unidentified fungi including mycelia sterilia, dematiaceous fungi, and one mucorales species (Table 11 and Figure 12).

Table 10 Number of fungal isolates and fungal density in soil samples

Sample/time	Total no. of soil fungi (CFU/g)					No. of isolated soil fungi (isolates)
	1st trail	**2nd trail**	**3rd trail**	**4th trail**	**5th trail**	
1st collection (January 2010)						240
1	5.2×10^5	3.6×10^5	5.2×10^5	1.0×10^5	1.0×10^5	
2	3.4×10^5	5.1×10^5	3.6×10^5	4.4×10^5	3.3×10^5	
3	1.1×10^5	3.5×10^5	3.4×10^5	4.7×10^5	3.9×10^5	
4	5.6×10^5	1.2×10^5	ND	8.7×10^4	6.2×10^5	
5	4.1×10^5	6.5×10^4	ND	9.0×10^4	3.4×10^5	
6	5.6×10^5	ND	ND	ND	3.6×10^5	
2nd collection* (April 2010)	4.2×10^4	1.6×10^5	4.7×10^4	1.1×10^5	8.3×10^4	125
3rd collection* (June 2010)	8.0×10^5	3.5×10^5	3.7×10^5	5.3×10^5	5.6×10^4	144
4th collection* (August 2010)	4.6×10^5	6.9×10^5	5.9×10^5	1.1×10^6	5.8×10^5	120
Total						629

*pooled samples

ND = Not done

Table 11 Number of fungal genera isolated from soil samples

No.	Fungal genera	No. of fungal isolates					Percentages of isolation
		1st*	2nd	3rd	4th	Total	
1	*Absidia*	1	1	3		5	0.79
2	*Acremonium*			3		3	0.48
3	*Aspergillus*	34	33	27	23	117	18.60
4	*Aureobasidium*			3		3	0.48
5	*Botryoderma*	1				1	0.16
6	*Cladosporium*	1	4	3		8	1.27
7	*Colletotrichum*		3			3	0.48
8	*Cunninghamella*	7	2	2	1	12	1.91
9	*Curvularia*				1	1	0.16
10	*Exophiala*				1	1	0.16
11	*Fusarium*	5		5	3	13	2.07
12	*Gliocladium*			2		2	0.32
13	*Gongronella*	13	5	5		23	3.66
14	*Humicola*			1		1	0.16
15	*Leptosphaeria*	1				1	0.16
16	*Mucor*	3	2	4	4	13	2.07
17	*Neotestutina*				2	2	0.32
18	*Paecilomyces*			5	5	10	1.59
19	***Penicillium***	**74**	**18**	**25**	**21**	**138**	**21.94**
20	*Pestalotiopsis*				1	1	0.16
21	*Phoma*				2	2	0.32
22	*Pseudotorula*	1				1	0.16
23	*Scedosporium*			1		1	0.16
24	*Sepedonium*	1				1	0.16
25	*Trichoderma*	5	3	13	9	30	4.77
26	*Verticillium*		1	2		3	0.48
27	*Xylaria*	1				1	0.16
28	Unidentified fungi	100	53	40	39	232	36.88
	Total	248	125	144	112	629	100

*Collection time: 1st collection (January 2010), 2nd (April 2010), 3rd (June 2010), 4th (August 2010)

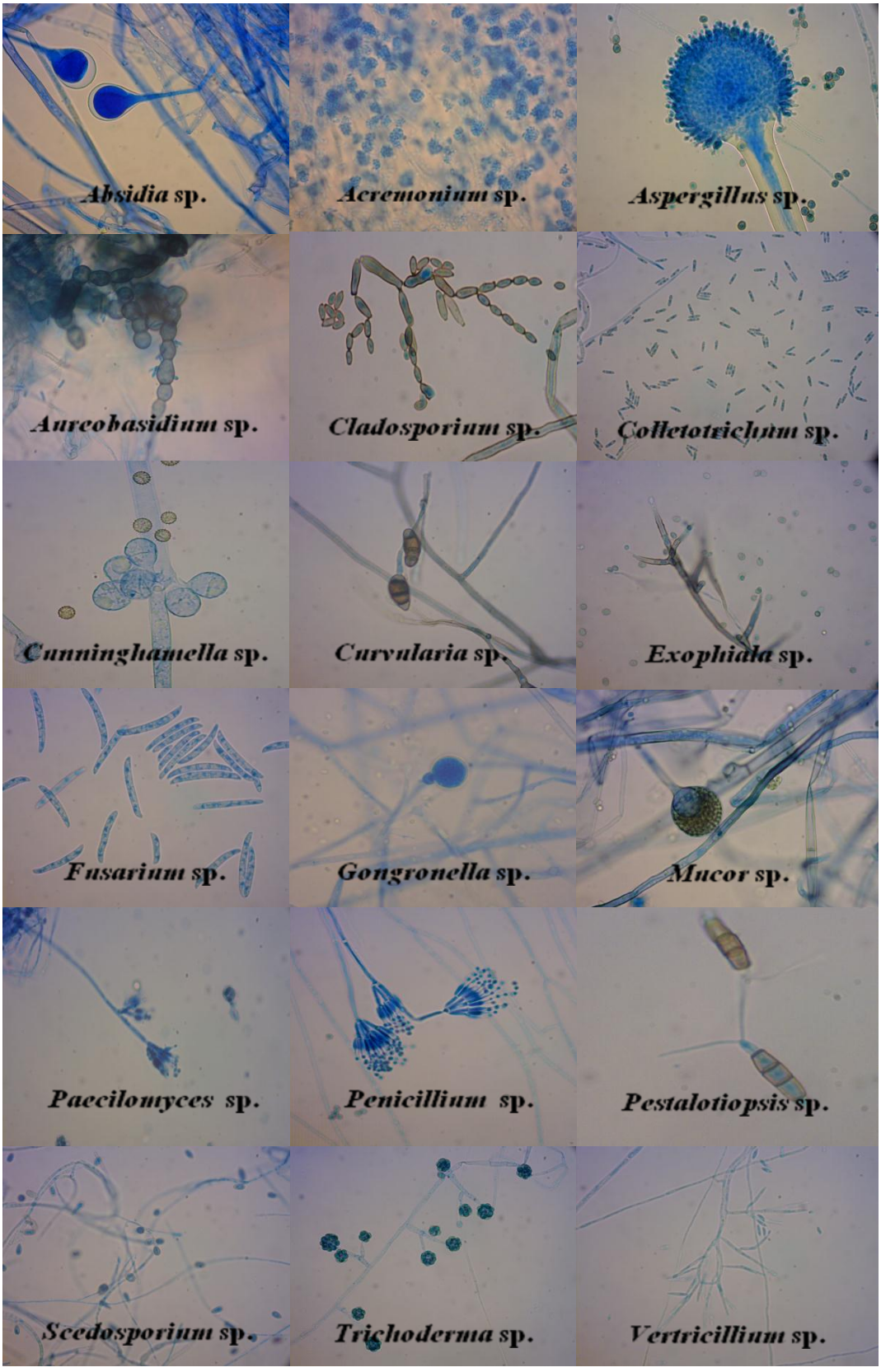

Figure 12 Microscopic morphologies of representative isolated soil fungi (40X)

3.3 Antimicrobial activity of fungal crude extracts

3.3.1 Preliminary test at a concentration 200 µg/ml

Three crude extracts (BE, CE and CH) were obtained from each isolate. A total of 543 crude extracts from 181 selected soil fungi were first screened for their antimicrobial activity against twelve human pathogens at a concentration of 200 µg/ml by a colorimetric broth microdilution test. The results in Table 12 showed that 311 crude extracts (57.27%) presented activity toward at least one test strain. Fungal crude extracts displayed the most activity against *S. aureus* ATCC 25923 (38.86%) followed by *C. neoformans* ATCC 90113 (33.71%), MRSA SK-1 (24.91%), *C. neoformans* ATCC 90112 (19.44%), *C. albicans* ATCC 90028 (14.10%), *C. albicans* NCPF 3153 (12.62%), *P. marneffei* clinical isolate (7.52%), *M. gypseum* SH-MU4 (7.14%), *A. baumannii* NPRC AB007 (2.66%), *A. baumannii* NPRC AB005 (1.71%), *E. coli* ATCC 25922 (1.11%) and *P. aeruginosa* ATCC 27853 (0.37%).

One hundred and forty-five out of 181 selected soil fungi (80.11%) had activity against one or more strains of test microbes with *S. aureus* ATCC 25923 was the most susceptible strain (60.77%) followed by *C. neoformans* ATCC 90113 (51.38%), MRSA-SK1 (43.65%), *C. neoformans* ATCC 90112 (34.25%), *C. albicans* ATCC 90028 (26.25%), *C. albicans* NCPF 3153 (23.20), *P. marneffei* clinical isolate (14.36%), *M. gypseum* SH-MU4 (12.71%), *A. baumannii* NPRC AB007 (7.73%%), *A. baumannii* NPRC AB005 (4.97%), *E. coli* ATCC 25922 (3.31%) and *P. aeruginosa* ATCC 27853 (0.55%), respectively (Table 12 and Figure 13).

Table 12 Number of active crude extracts and soil fungi tested at a concentration of 200 µg/ml

No. of crude extracts/No. soil fungi	Test microorganisms												Total
	Bacteria						Yeasts				Filamentous fungi		
	SA	MRSA	PA	EC	AB005	AB007	CA28	CA53	CN12	CN13	MG	PM	
Active crude extracts/total crude extracts (%)	211/543 (38.86)	133/543 (24.91)	2/541 (0.37)	6/541 (1.11)	9/531 (1.69)	14/531 (2.64)	76/539 (14.10)	68/539 (12.62)	104/535 (19.44)	180/534 (33.71)	38/534 (7.12)	40/531 (7.53)	311/543 (57.27)
Active soil fungi/total selected soil fungi (%)	110/181 (60.77)	79/181 (43.65)	1/181 (0.55)	6/181 (3.31)	9/181 (4.97)	14/181 (7.73)	48/181 (26.52)	42/181 (23.20)	62/181 (34.25)	93/181 (51.38)	26/181 (14.36)	23/181 (12.71)	145/181 (79.56)

SA = *Staphylococcus aureus* ATCC 25923

MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922

PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028

CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4

PM = *Penicillium marneffei* clinical isolate

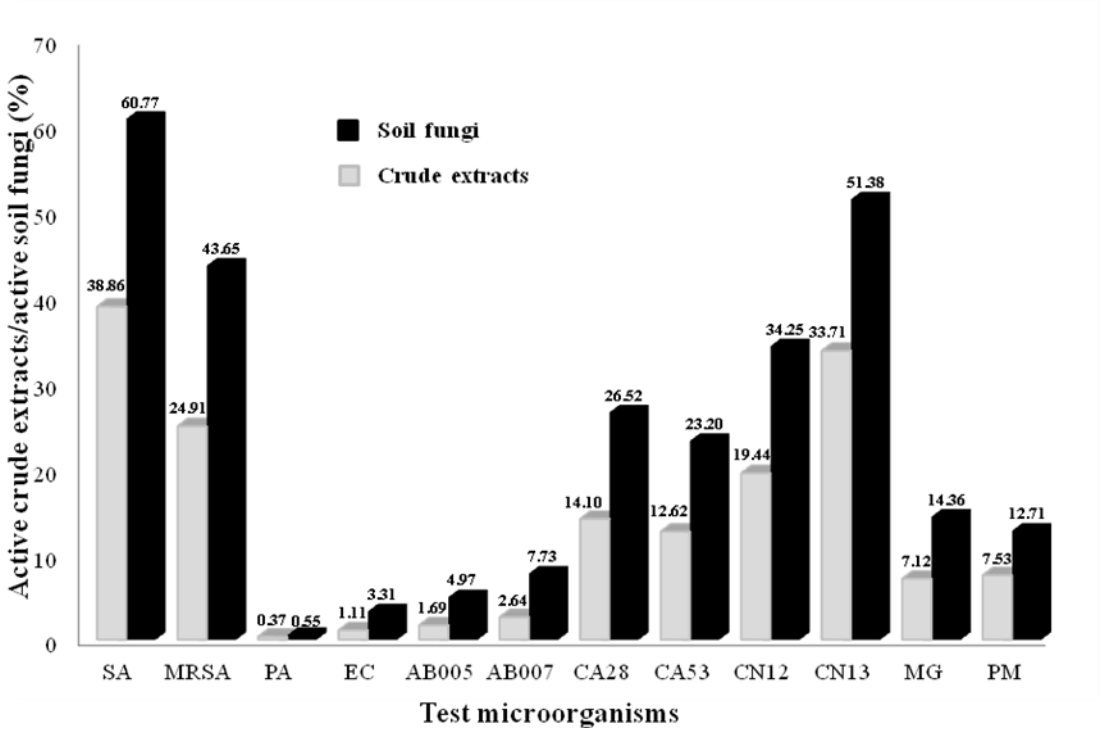

Figure 13 Antimicrobial activity of fungal crude extracts and soil fungi at a concentration of 200 µg/ml

SA = *Staphylococcus aureus* ATCC 25923 MRSA = Methicillin-resistant *S.aureus* SK1

EC = *Escherichia coli* ATCC 25922 PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028 CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4 PM = *Penicillium marneffei* clinical isolate

Most of fungal crude extracts (20.07%) at a concentration of 200 µg/ml were active against one test strain and 14.92, 6.08, 6.70, 2.76, 2.03, 1.66, 2.21, 0.55, 0.37% of crude extracts exhibited activity against 2, 3, 4, 5, 6, 7, 8, 9 and 10 test microorganisms, respectively. RSPG 198 CH was the only crude extract (0.18%) that showed activity toward all test microorganisms (Figure 14).

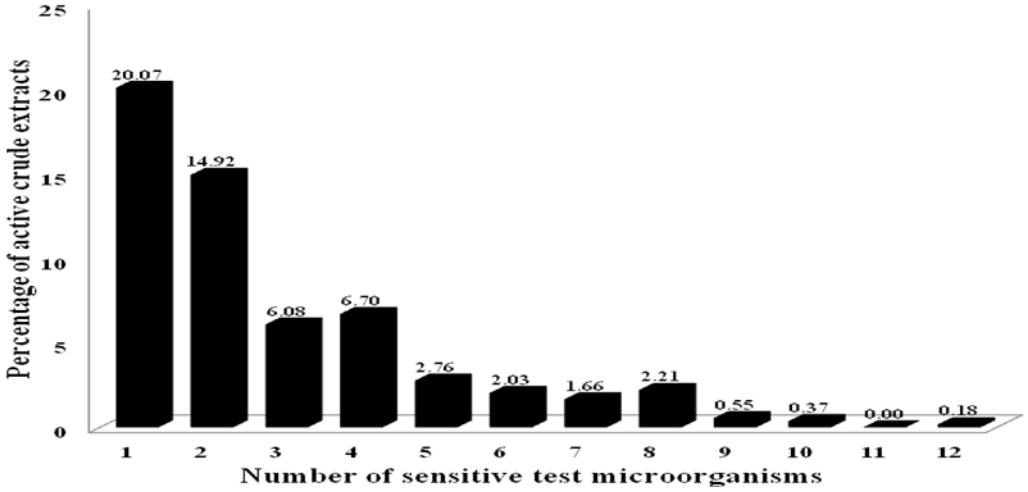

Figure 14 Number of susceptible test microorganisms inhibited by fungal crude extracts at a concentration of 200 µg/ml

Among the active crude extracts, cell hexane (CH) was the greatest active extracts (61.88%) followed by cell ethyl acetate (CE, 58.01%) and broth ethyl acetate (BE, 53.59%) (Figure 15).

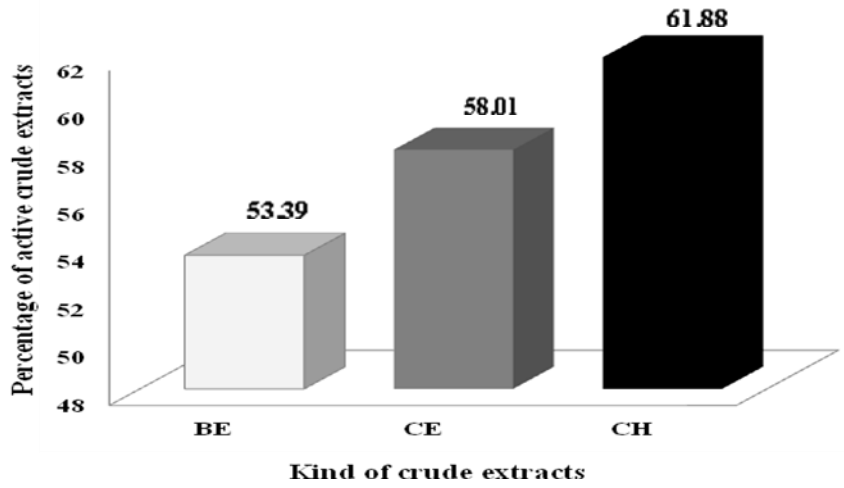

Figure 15 Types of active crude extracts against test microorganisms at a concentration of 200 µg/ml

BE = broth ethyl acetate extracts; CE = cell ethyl acetate extracts; CH = cell hexane extracts

Broth ethyl acetate (BE) extracts were highly active against *S. aureus* ATCC 25923 followed by *C. neoformans* ATCC 90113, MRSA-SK1, *C. neoformans* ATCC 90112, *C. albicans* ATCC 90028, *C. albicans* NCPF 3153, *P. marneffei* clinical isolate, *M. gypseum* SH-MU4, *A. baumannii* NPRC AB007, *A. baumannii* NPRC AB005 and *E. coli* ATCC 25922 with 34.81, 30.94, 25.41, 20.44, 13.81, 11.60, 9.94, 9.39, 4.42, 3.31 and 2.76%, respectively. Fungal BE extracts had no activity to *P. aeruginosa* ATCC 27853 (Figure 16).

Cell ethyl acetate (CE) extracts displayed the greatest activity toward *C. neoformans* ATCC 90113 (35.00%) followed by *S. aureus* (33.15%), MRSA SK-1 (20.44), *C. neoformans* ATCC 90112 (14.44%), both strains of *C. albicans* (11.05%), *M. gypseum* SH-MU4 (5.56%), *P. marneffei* clinical isolate (4.97%), *A. baumannii* NPRC AB007 (2.22%), *A. baumannii* NPRC AB005 (0.56%) and *P. aeruginosa* ATCC 27853 (0.55%) but CE was not active against *E. coli* ATCC 25922 (Figure 16).

Cell hexane (CH) extracts exhibited a broad range activity against all test microorganisms with *S. aureus* ATCC 25923 was the most susceptible strain (48.62%) followed by *C. neoformans* ATCC 90113 (35.26%), MRSA-SK1 (27.62%), *C. neoformans* ATCC 90112 (23.56%), *C. albicans* ATCC 90028 (17.51%), *C. albicans* NCPF 3153 (14.92%), *P. marneffei* clinical isolate (7.65%), *M. gypseum* SH-MU4 (6.36%), both strains of *A. baumannii* (1.18%), *P. aeruginosa* ATCC 27853 (0.56%) and *E. coli* ATCC 25922 (0.56%) (Figure 16).

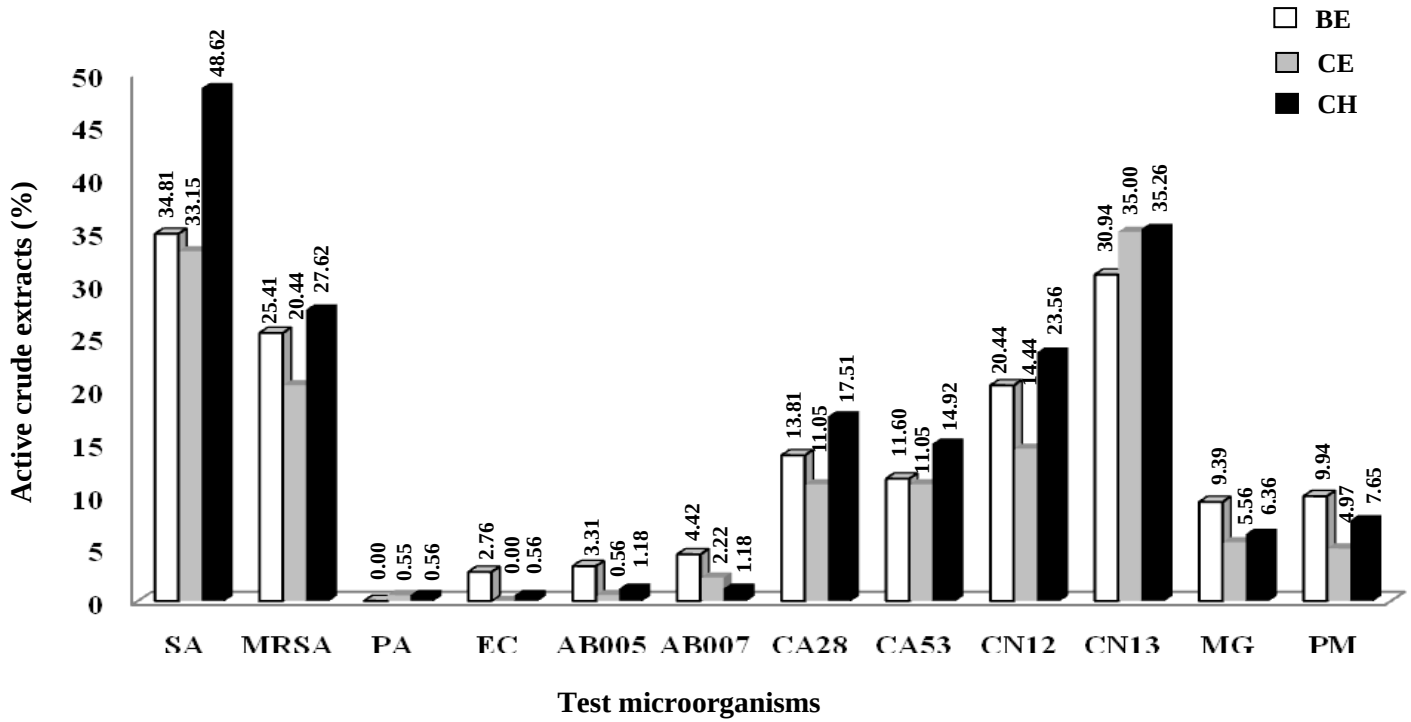

Figure 16 Percentage of each type of active crude extracts against each test microorganism at a concentration of 200 µg/ml

BE = Broth ethyl acetate extracts CE = Cell ethyl acetate extracts CH = Cell hexane extracts

SA = *Staphylococcus aureus* ATCC 25923 MRSA = Methicillin-resistant *S. aureus* SK1 EC = *Escherichia coli* ATCC 25922

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005 AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

PA = *Pseudomonas aeruginosa* ATCC 27853 CA28 = *Candida albicans* ATCC 90028 CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4 PM = *Penicillium marneffei* clinical isolate

3.3.2 Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) or minimum fungicidal concentrations (MFCs)

Active crude extracts from preliminary test were further examined for their MICs by a colorimetric broth microdilution test. The results showed that the MICs values varied among each test microorganism. The MIC values ranged from the lowest to the highest concentrations were as follows: *C. neoformans* ATCC 90113 (1-200 µg/ml); *S. aureus* ATCC 25923, MRSA SK-1 and both strains of *C. albicans* (2-200 µg/ml); *C. neoformans* ATCC 90112 (4-200 µg/ml); *P. marneffei* clinical isolate (16-200 µg/ml); *M. gypseum* SH-MU4 (32-200 µg/ml); *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (128-200 µg/ml) and both strains of *A. baumannii* (200 µg/ml) (Table 13).

MBC and MFC values of crude extracts were in ranges of 2->200 µg/ml and 4->200 µg/ml, respectively. The lowest MBC/MFC was found against MRSA (2 µg/ml) followed by *C. neoformans* ATCC 90113 (4 µg/ml); *S. aureus* ATCC 25923, *C. albicans* NCPF 3153 and *C. neoformans* ATCC 90112 (8 µg/ml); *C. albicans* ATCC 90028 and *P. marneffei* clinical isolate (16 µg/ml) and *M. gypseum* (128 µg/ml). However, the active crude extracts had no bactericidal activity against Gram-negative bacteria (MBC >200 µg/ml) (Table 13).

The MIC values could be divided into 3 levels as strong activity (MIC $\leq$ 8 µg/ml), moderate activity (MIC = 16-64 µg/ml) and weak activity (MIC = 128-200 µg/ml). Most fungal crude extracts exhibited weak activity followed by moderate and strong activity. The strong activity was the highest against *C. neoformans* ATCC 90113 (23 extracts) followed by *S. aureus* ATCC 25923 (14 extracts), MRSA (12 extracts), *C. neoformans* ATCC 90112 (7 extracts), *C. albicans* NCPF 3153 (3 extracts) and *C. albicans* ATCC 90028 (2 extracts). None of the extracts showed strong activity against filamentous fungi and Gram-negative bacteria (Figure 17).

Table 13 MIC and MBC or MFC ranges of active fungal crude extracts against each test microorganism

Test	Test microorganisms											
	Bacteria						Yeasts				Filamentous fungi	
	SA	MRSA	PA	EC	AB005	AB007	CA28	CA53	CN12	CN13	MG	PM
MIC (µg/ml)	2-200	2-200	128-200	128-200	200	200	2-200	2-200	4-200	1-200	32-200	16-200
MBC/MFC (µg/ml)	8->200	2->200	>200	>200	>200	>200	16->200	8->200	8->200	4->200	128->200	16->200
Vancomycin (MIC/MBC)	0.5/1	1/2										
Gentamicin (MIC/MBC)			0.25/2	0.5/1								
Colistin (MIC/MBC)					80/160	80/80						
Amphotericin B (MIC/MFC)							0.125/0.25	0.125/0.5	0.125/0.125	0.125/0.25		1/2
Miconazole (MIC/MFC)											1/32	

SA = *Staphylococcus aureus* ATCC 25923

MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922

PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028

CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flycytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4

PM = *Penicillium marneffei* clinical isolate

MIC = minimum inhibitory concentration (µg/ml)

MBC = minimum bactericidal concentration (µg/ml)

MFC = minimum fungicidal concentration (µg/ml)

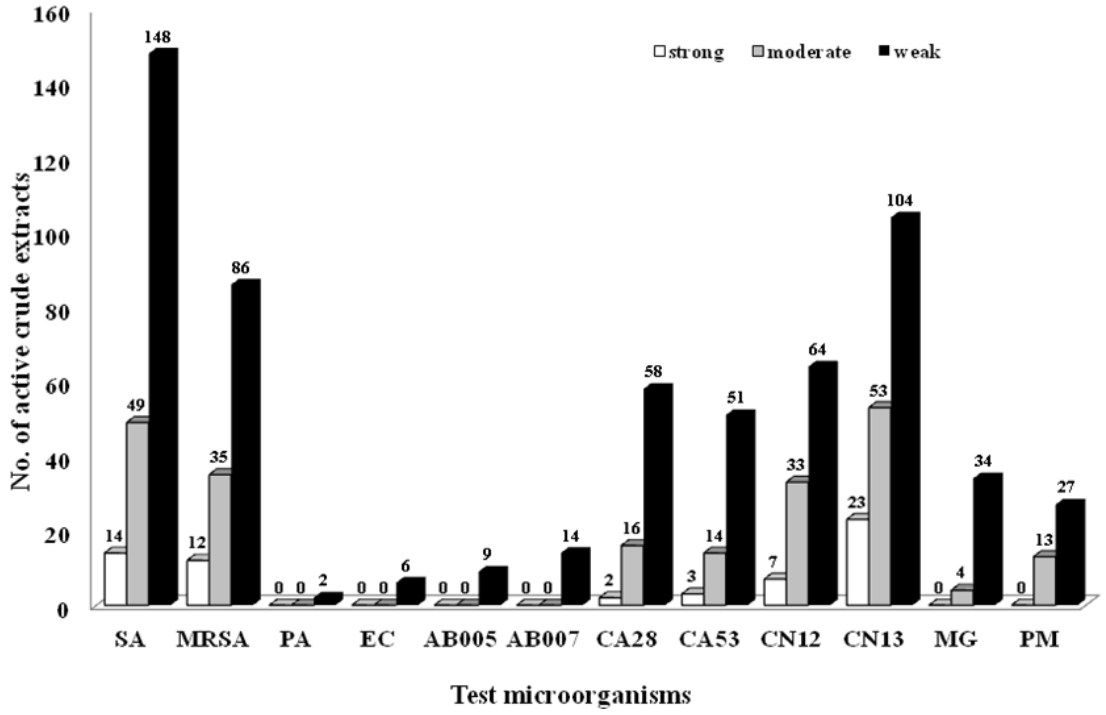

Figure 17 Number of active crude extracts having strong, moderate and weak activity against each test microorganism

SA = *Staphylococcus aureus* ATCC 25923 MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922 PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028 CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4 PM = *Penicillium marneffei* clinical isolate

strong activity = $\leq$ 8 µg/ml moderate activity = 16-64 µg/ml

weak activity = 128-200 µg/ml

Antibacterial activity

Two hundred and twenty-three crude extracts revealed antibacterial activity with MIC/MBC in ranges of 2-200/2->200 µg/ml. Strongly active extracts were found against Gram-positive bacteria. RSPG 206 CH was the best crude extract against *S.aureus* ATCC 25923 and MRSA SK-1 with MIC/MBC value of 2/8 and 2/2 µg/ml. For Gram-negative bacteria, fungal crude extracts showed only weak activity with MIC/MBC values ranging from 128-200/>200 µg/ml. (Table 14 and 15).

Antifungal activity

Two hundred and eleven crude extracts had antifungal activity with MIC/MFC values ranging from 1-200/4->200 µg/ml. The potential crude extracts represented strong activity toward *C. albicans* and *C. neoformans* whereas only moderate activity was found against *M. gypseum* and *P. marneffei*. RSPG 27 CH presented the best activity toward *C. albicans* ATCC 90028 and *C. albicans* NCPF 3153 with MIC/MFC value of 2/32 and 2/64 µg/ml. RSPG 206 CH was the best crude extract against *C. neoformans* ATCC 90112 with MIC/MFC equal to 4/8 µg/ml. RSPG 179 CH exhibited the best anti-*C. neoformans* ATCC 90113 and anti-*P. marneffei* activity with MIC/MFC value of 1/8 and 16/16 µg/ml. RSPG 199 CE showed the best anti-*M. gypseum* activity with MIC/MFC equal to 32/>200 µg/ml. (Table 14 and 15).

Table 14 Number of active fungal crude extracts against each test microorganism at each MIC and MBC or MFC value

Concentration (µg/ml)	No. of active crude extracts (n)																							
	SA (n=211)		MRSA (n=133)		PA (n=2)		EC (n=6)		AB 005 (n=9)		AB 007 (n=14)		CA 28 (n=76)		CA 53 (n=68)		CN 12 (n=104)		CN 13 (n=180)		MG (n=38)		PM (n=40)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
2	2	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	3	0	0	0	0	0
4	3	0	5	0	0	0	0	0	0	0	0	0	1	0	1	0	4	0	7	1	0	0	0	0
8	9	2	6	1	0	0	0	0	0	0	0	0	0	0	1	1	3	2	12	3	0	0	0	0
16	9	0	11	1	0	0	0	0	0	0	0	0	1	2	2	0	4	2	16	5	0	0	5	2
32	10	0	12	1	0	0	0	0	0	0	0	0	6	2	4	3	9	0	15	10	1	0	3	2
64	30	5	12	5	0	0	0	0	0	0	0	0	9	3	8	2	20	6	22	12	3	0	5	4
128	53	8	39	4	1	0	2	0	0	0	0	0	16	5	17	3	20	4	34	19	13	4	18	8
200	95	21	47	17	1	0	4	0	9	0	14	0	42	11	34	8	44	20	70	35	21	5	9	6
>200		175		103		2		6		9		14		53		51		70		95		29		18

SA = *Staphylococcus aureus* ATCC 25923

MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922

PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028

CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4

PM = *Penicillium marneffei* clinical isolate

MIC = minimum inhibitory concentration (µg/ml)

MBC = minimum bactericidal concentration (µg/ml)

MFC = minimum fungicidal concentration (µg/ml)

Table 15 Potential crude extracts presenting strong activity (MIC <10 µg/ml) or the best MIC against each test microorganism

Crude extracts/ Standard drug	MIC/MBC or MFC µg/ml											
	SA	MRSA	PA	EC	AB005	AB007	CA28	CA53	CN12	CN13	MG	PM
RSPG27 BE							4/16	4/32	4/64	8/128		64/128
RSPG27 CE	8/>200								4/64	2/32		32/64
RSPG27 CH							**2/32**	**2/64**		8/128		64/>200
RSPG28 CH	2/>200	4/>200										
RSPG50 BE	8/>200								8/16	8/32		
RSPG50 CE									4/8	4/4		
RSPG52 BE	8/>200	8>200								8/200		
RSPG58 BE	4/200	4/>200			200/>200	200/>200			8/200	8/200		
RSPG63 CE										4/>200		
RSPG63 CH										4/>200		
RSPG88 BE					200/>200	200/>200						
RSPG95 CH	8/200	4/>200										
RSPG105 CH									8/16	8/16		
RSPG157 CE	4/8	8/8						8/8				
RSPG162 CH	4/>200	8/>200										
RSPG178 BE				128/>200	200/>200	200/>200						
RSPG178 CE										4/16		16/32
RSPG178 CH										2/8		
RSPG179 BE				200/>200	200/>200	200/>200						32/128
RSPG179 CE										4/16		16/64
RSPG179 CH					200/>200	200/>200				**1/8**	64/200	**16/16**

Table 15 (cont.) Potential crude extracts presenting strong activity (MIC <10 µg/ml) or the best MIC against each test microorganism

Crude extracts/ Standard drug	MIC/MBC or MFC µg/ml											
	SA	MRSA	PA	EC	AB005	AB007	CA28	CA53	CN12	CN13	MG	PM
RSPG180 BE										8/64		
RSPG180 CE										8/16		
RSPG180 CH										8/32		
RSPG196 BE					200/>200							
RSPG198 CH			128/>200	128/>200	200/>200	200/>200						
RSPG199 BE						200/>200						
RSPG199 CE	8/128	8/64				200/>200					32/>200	
RSPG199 CH	8/64											32/32
RSPG202 BE						200/>200						
RSPG202 CE	8 /128	8/64									64/>200	64/64
RSPG202 CH												64/64
RSPG204 BE						200/>200						
RSPG204 CE						200/>200						
RSPG204 CH	8/64	8/16							**4/8**	8/8		16/>200
RSPG206 CH	**2/8**	**2/2**								8/16		
RSPG214 CH										4/32		
RSPG229 BE		4/>200										
RSPG230 CE										8/>200		

Table 15 (cont.) Potential crude extracts presenting strong activity (MIC <10 µg/ml) or the best MIC against each test microorganism

Crude extracts/ Standard drug	MIC/MBC or MFC µg/ml											
	SA	MRSA	PA	EC	AB005	AB007	CA28	CA53	CN12	CN13	MG	PM
RSPG230 CH		4/200										
RSPG231 CE										4/128		
RSPG231 CH										2/32		64/>200
Vancomycin	0.5/1	1/2										
Gentamicin			0.25/2	0.5/1								
Colistin					80/160	80/80						
Amphotericin B							0.125/0.25	0.125/0.5	0.125/0.125	0.125/0.25		1/2
Miconazole											1/32	

SA = *Staphylococcus aureus* ATCC 25923

MRSA = Methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC 25922

PA = *Pseudomonas aeruginosa* ATCC 27853

AB005 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005

AB007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB007

CA28 = *Candida albicans* ATCC 90028

CA53 = *Candida albicans* NCPF 3153

CN12 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain

CN13 = *Cryptococcus neoformans* ATCC 90113, flucytosine-resistant strain

MG = *Microsporum gypseum* SH-MU4

PM = *Penicillium marneffei* clinical isolate

MIC = minimum inhibitory concentration (µg/ml)

MBC = minimum bactericidal concentration (µg/ml)

MFC = minimum fungicidal concentration (µg/ml)

Bold = Study on possible mechanism of action by SEM

3.4 Study on possible mechanisms of action of the active crude extracts by scanning electron microscopy (SEM)

Strongly active crude extracts comprising RSPG 27 CH, 179 CH, 204 CH and 206 CH were selected to study the possible mechanism of action by SEM at four times their MIC concentrations. Scanning electron micrographs of *S. aureus* and MRSA SK-1 are shown in Figures 18-19. The untreated bacterial cells showed normal and smooth surface morphology (Figures 18a and 19a), while the cells treated with RSPG 206 CH (Figures 18c and 19c) showed pronounced morphological changes consistent with cell damage similar to the cells treated with vancomycin (Figures 18b and 19b). Broken cells with pores (arrows) and cytoplasmic protrusion were observed in both treated cells.

Figures 20 and 21 show scanning electron micrographs of *C. albicans* ATCC 90028 and NCPF 3153. The normal cells are shown in Figures 20a and 21a. The cells treated with RSPG 27 CH were markedly swollen as compared to the controls. They were completely deformed. Deep wrinkles and collasped cells were also observed (Figures 20c and 21c) as well as in cells treated with amphotericin B (Figures 20b and 21b).

Scanning electron micrographs of *C. neoformans* ATCC 90112 and 90113 are shown in Figures 22 and 23. Crude extracts treated cells exhibited dramatically cell damage with leakage of cellular contents (Figures 22c and 23c) similar to the amphotericin B treated cells (Figures 22b and 23b).

In addition, RSPG 179 CH was also tested with *P. marneffei*. Only slightly morphological changes such as wrinkle and flattened mycelia were observed in the sample treated with crude extract whereas broken hyphae were shown in the amphotericin B treated cell as compared with the control mycelia (Figure 24).

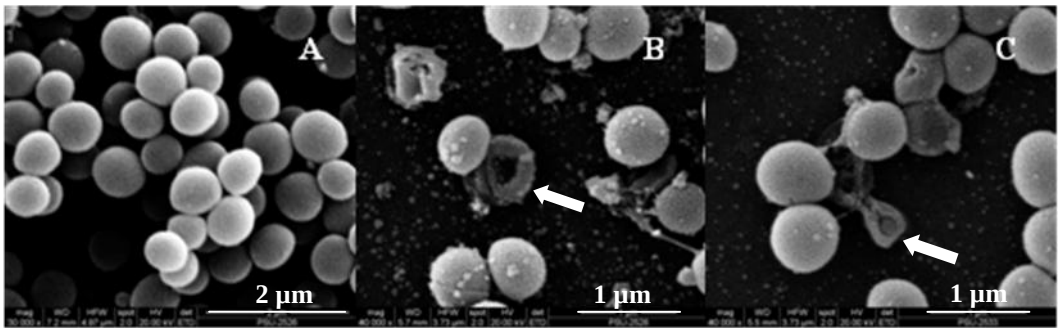

Figure 18 Scanning electron micrographs of *S. aureus* ATCC 25923 (SA) after 24 hours of incubation at 35°C

(A) SA + 1% DMSO (B) SA + 4 MIC Vancomycin (C) SA + 4 MIC RSPG 206 CH

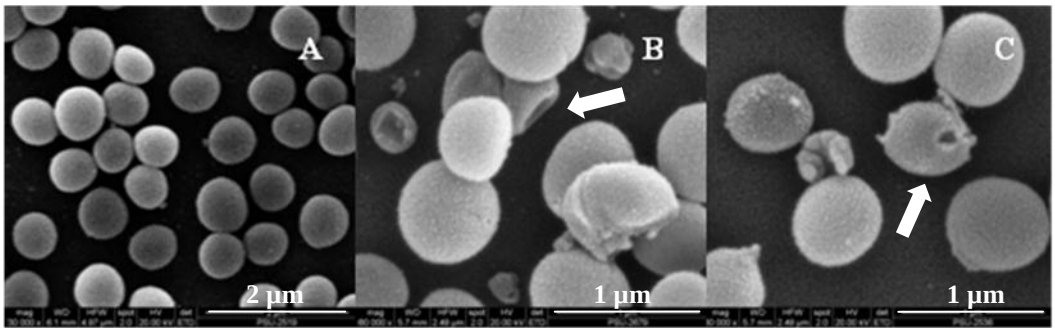

Figure 19 Scanning electron micrographs of methicillin-resistant *S. aureus* (MRSA) SK-1 after 24 hours of incubation at 35°C

(A) MRSA + 1% DMSO (B) MRSA + 4 MIC Vancomycin

(C) MRSA + 4 MIC RSPG 206 CH

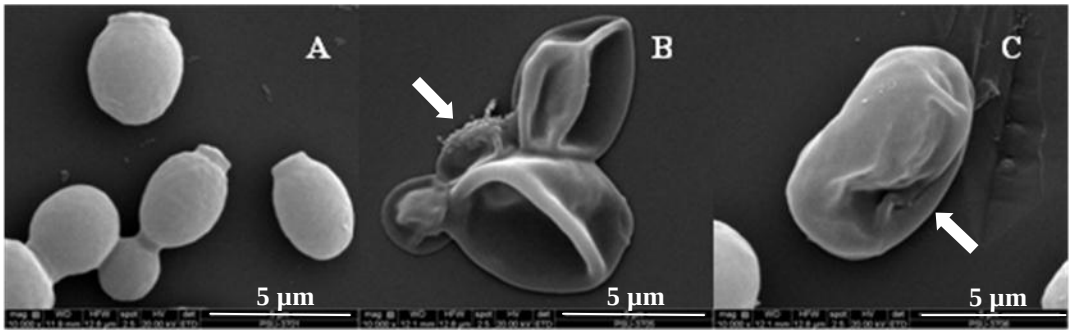

Figure 20 Scanning electron micrographs of *C. albicans* ATCC 90028 (CA28) after 24 hours of incubation at 35°C

(A) CA28 + 1% DMSO (B) CA28 + 4 MIC Amphotericin B

(C) CA28 + 4 MIC RSPG 27 CH

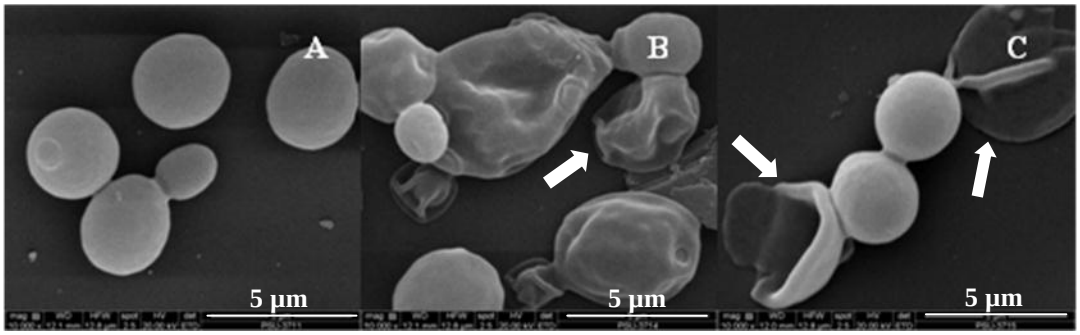

Figure 21 Scanning electron micrographs of *C. albicans* NCPF 3153 (CA53) after 24 hours of incubation at 35°C

(A) CA53 + 1% DMSO (B) CA53 + 4 MIC Amphotericin B

(C) CA53 + 4 MIC RSPG 27 CH

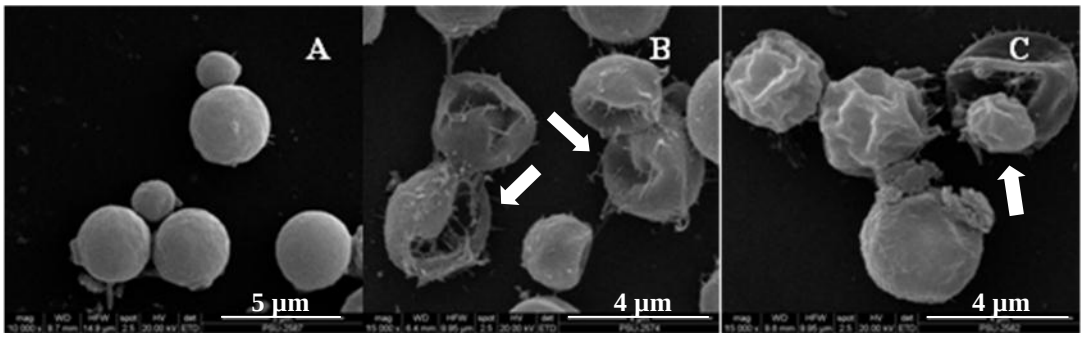

Figure 22 Scanning electron micrographs of *C. neoformans* ATCC 90112 (CN12) after 48 hours of incubation at 25°C

(A) CN12 + 1% DMSO (B) CN12 + 4 MIC Amphotericin B

(C) CN12 + 4 MIC RSPG 204 CH

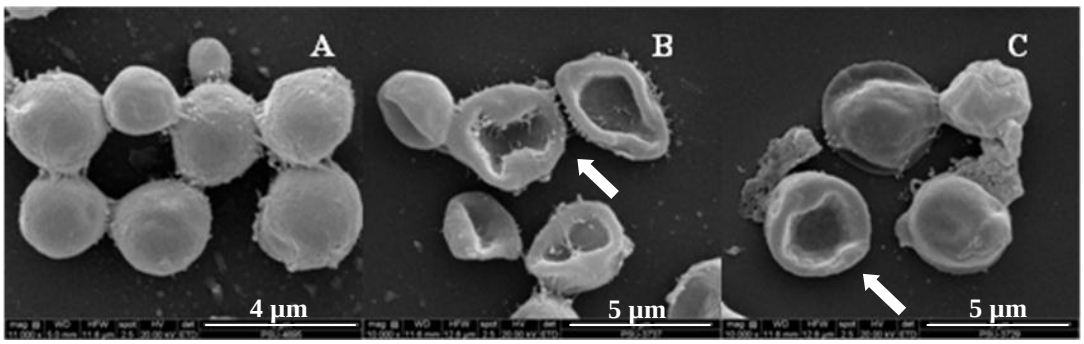

Figure 23 Scanning electron micrographs of *C. neoformans* ATCC 90113 (CN13) after 48 hours of incubation at 25°C

(A) CN13 + 1% DMSO (B) CN13 + 4 MIC Amphotericin B

(C) CN13 + 4 MIC RSPG 179 CH

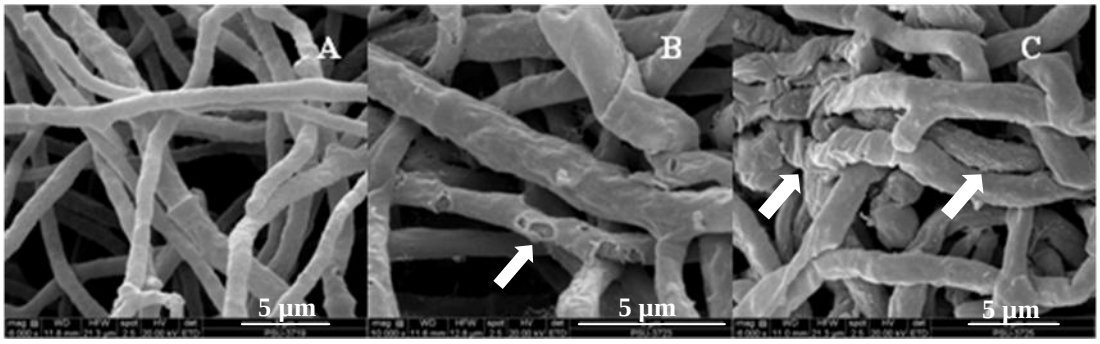

Figure 24 Scanning electron micrographs of *P. marneffei* clinical isolate (PM) after 3 days of incubation at 25°C

(A) PM + 1% DMSO (B) PM + 4 MIC Amphotericin B

(C) PM + 4 MIC RSPG 179 CH

3.5 Identification of interesting soil fungi

3.5.1 Morphological identification

Soil fungi that presented strong antimicrobial activity and/or interesting NMR profiles (Table 16) were identified based on morphology together with molecular data. From morphological characteristics, interesting soil fungi could be identified into 6 genera and 1 group of unidentified fungi as follows:

Table 16 Selected soil fungi presenting strong antimicrobial activity and/or interesting NMR profile

No.	code RSPG	Fungal group	Note
1	24	*Trichoderma*	Interesting NMR profile.
2	27	*Trichoderma*	Strong anti-yeast activity and interesting NMR profile.
3	28	*Trichoderma*	Strong anti-SA, anti-MRSA activity and interesting NMR profile.
4	37	*Fusarium*	Interesting NMR profile.
5	50	*Paecilomyces*	Strong anti-CN12 and anti-CN13 activity.
6	52	*Paecilomyces*	Interesting NMR profile.
7	58	*Paecilomyces*	Strong anti-SA, anti-MRSA activity and interesting NMR profile.
8	63	*Penicillium*	Interesting NMR profile.
9	82	Unidentified	Interesting NMR profile.
10	88	Unidentified	Interesting NMR profile.
11	93	*Penicillium*	Interesting NMR profile.
12	95	*Penicillium*	Strong anti-MRSA activity and interesting NMR profile.
13	99	*Penicillium*	Interesting NMR profile.
14	105	*Penicillium*	Strong anti-CN12 and anti-CN13 activity.
15	138	*Penicillium*	Interesting NMR profile.
16	157	*Penicillium*	Strong anti-SA and anti-CA53 activity.
17	161	*Penicillium*	Interesting NMR profile.

SA = *Staphylococcus aureus* ATCC 25923 MRSA = Methicillin-resistant *S.aureus* SK1 EC = *Escherichia coli* ATCC 25922

AB005/007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005/AB007 CA28/53 = *Candida albicans* ATCC 90028/NCPF 3153

CN12/CN13 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain/ATCC 90113, flucytosine-resistant strain

PM = *Penicillium marneffei* clinical isolate MIC = minimum inhibitory concentration Strong activity = MIC < 10 µg/ml

Table 16 (cont.) Selected soil fungi presenting strong antimicrobial activity and/or interesting NMR profile

No.	code RSPG	Fungal group	Note
18	162	*Penicillium*	Strong anti-MRSA activity.
19	178	*Aspergillus*	Anti-PM and anti-AB activity.
20	179	*Aspergillus*	Anti-PM, strong anti-CN13 activity and interesting NMR profile.
21	180	*Aspergillus*	Anti-EC and anti-AB activity.
22	185	Unidentified	Interesting NMR profile.
23	196	*Aspergillus*	Anti-AB activity.
24	197	*Aspergillus*	Interesting NMR profile.
25	198	*Aspergillus*	Active against all of test microorganisms and interesting NMR profile.
26	199	*Aspergillus*	Interesting NMR profile.
27	202	*Aspergillus*	Anti-AB activity.
28	204	*Aspergillus*	Strong anti-CN12 activity.
29	206	*Aspergillus*	Strong anti-SA and anti-MRSA activity.
30	214	*Absidia*	Strong anti-CN13 activity.
31	227	*Fusarium*	Interesting NMR profile.
32	229	*Fusarium*	Strong anti-MRSA activity and interesting NMR profile.
33	230	*Fusarium*	Strong anti-MRSA activity.
34	231	*Fusarium*	Strong anti-MRSA activity.

SA = *Staphylococcus aureus* ATCC 25923 MRSA = Methicillin-resistant *S.aureus* SK1 EC = *Escherichia coli* ATCC 25922

AB005/007 = Multidrug-resistant *Acinetobacter baumannii* NPRC AB005/AB007 CA28/53 = *Candida albicans* ATCC 90028/NCPF 3153

CN12/CN13 = *Cryptococcus neoformans* ATCC 90112, flucytosine-susceptible strain/ATCC 90113, flucytosine-resistant strain

PM = *Penicillium marneffei* clinical isolate MIC = minimum inhibitory concentration Strong activity = MIC < 10 µg/ml

***Absidia* sp.**

Group member: RSPG214

Descriptions:

RSPG214 grew rapidly on PDA. The colony was cottony and gray in color. Spores were produced in sporangium. Mycelium was non-septate, however a septum was observed (arrow) below a sporangium in a sporangiophore. (Figure 25).

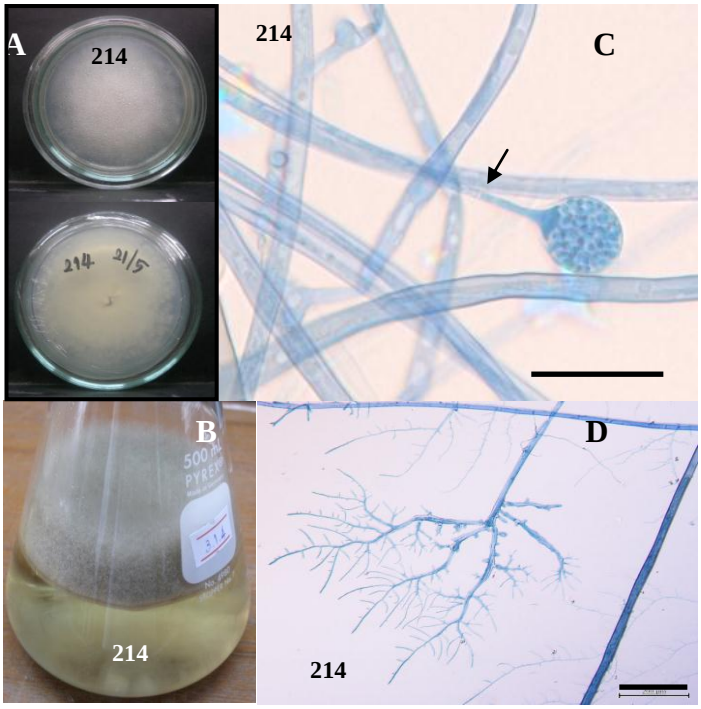

Figure 25 Morphological characteristics of *Absidia* sp. RSPG214

A: colony on PDA (7 days)

B: fermentation broth (21 days)

C-D: microscopic morphology

Arrow: septum in sporangiophore

Scale bar: D = 50 µm, E = 200 µm

***Aspergillus* spp.**

Group members: RSPG178, 179, 180, 196, 197, 198, 199, 202, 204, 206

Descriptions:

The *Aspergillus* group could be divided into 2 groups relied on their color presented on agar medium as yellow (RSPG178, 179, 180) and green (RSPG196, 197, 198, 199, 202, 204, 204). Most of their colonies were floccose and grew rapidly except RSPG199, 202 and 204 which had powdery colonies with floccose hyphae at the center and grew slower. The microscopic features showed septate and hyaline hyphae. They produced the aspergillum-like spore-bearing structure. Conidiophores varied from short to long with spiny or smooth surface. Conidial shapes were varied from globose to subglobose (Figure 26).

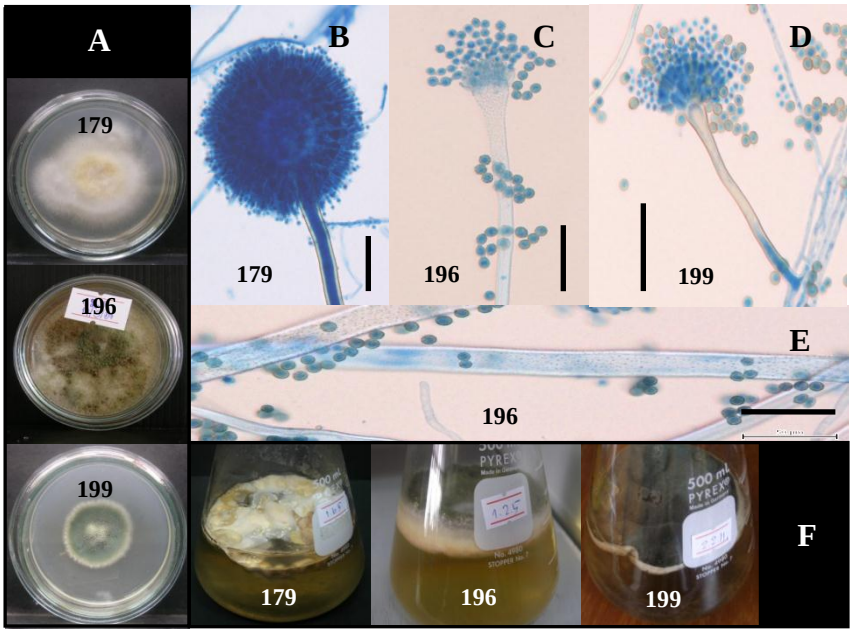

Figure 26 Morphological characteristics of *Aspergillus* spp.

A: colony on PDA

B-D: the aspergillum-like spore-bearing structure

E: spiny conidiophores

F: fermentation broth (21 days)

Scale bar: 50 µm

***Fusarium* spp.**

Group members: RSPG37, 63, 227, 229, 230, 231

Descriptions:

The fungal colonies were fast growing on PDA. The colors of colonies were white to pale orange or pale yellow. They produced both macro- and microconidia. Macroconidia presented 3-5 septate, fusiform, slightly curved and stout. Microconidia were 1-2 celled, fusiform to ovoid, straight or curved (Figure 27).

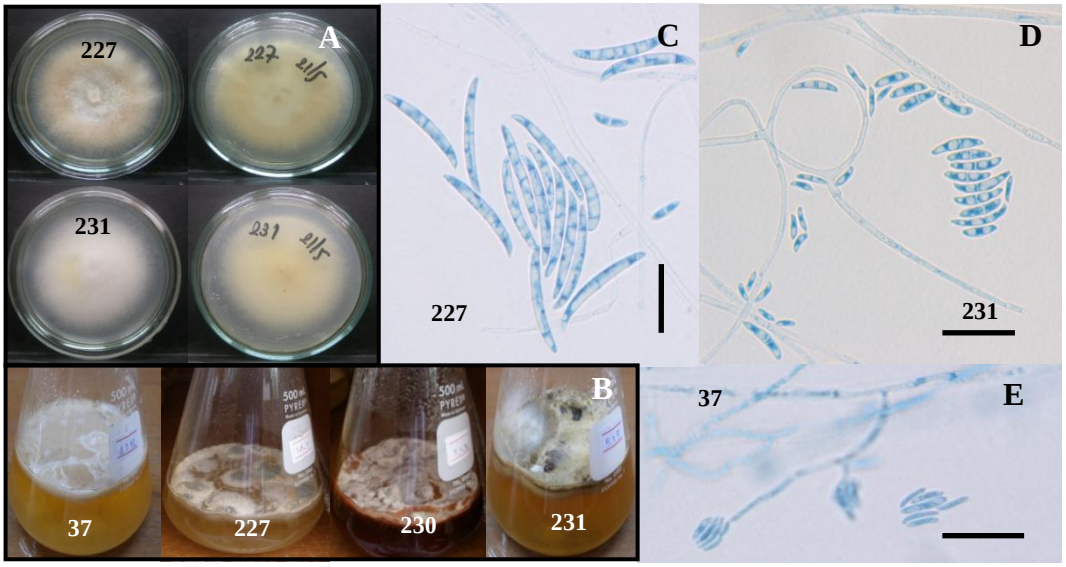

Figure 27 Morphological characteristics of *Fusarium* spp.

A: colony on PDA (7days)

B: fermentation broth (21 days)

C-E: macro- and microconidia

Scale bar: 50 µm

***Paecilomyces* spp.**

Group members: RSPG50, 52, 58

Descriptions:

The colonies of RSPG50, 52 and 58 were velvety, initially white and become violet. The reverse was white and brown. The microscopic features were hyaline septate hyphae, taper phialides with a long chain of oval conidia (Figure 28).

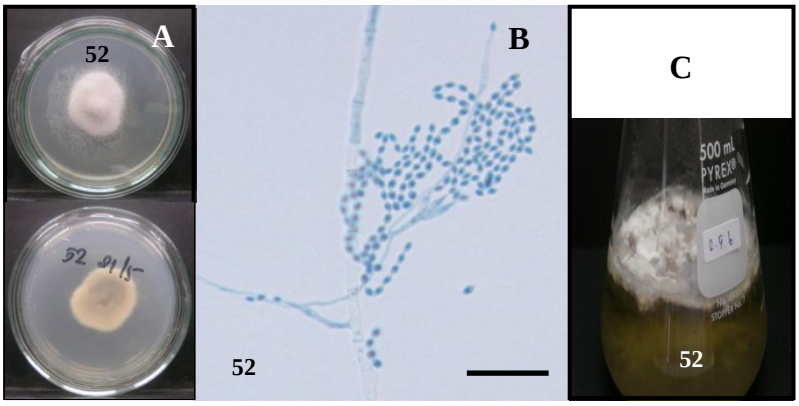

Figure 28 Morphological characteristics of *Paecilomyces* sp. RSPG52

A: colony on PDA (7 days)

B: microscopic morphology

C: fermentation broth (21 days)

Scale bar: 50 µm

***Penicillium* spp.**

Group members: RSPG93, 95, 99, 105, 138, 157, 161, 162

Descriptions:

Colonies of *Penicillium* group were white to green, deep green or gray-green, woolly or velvety, corrugated, moderate to rapid growth. RSPG93 produced yellow-brown soluble pigment in PDA while RSPG161 presented exudates production. The brush-like spore-bearing structure was observed from this group (Figure 29).

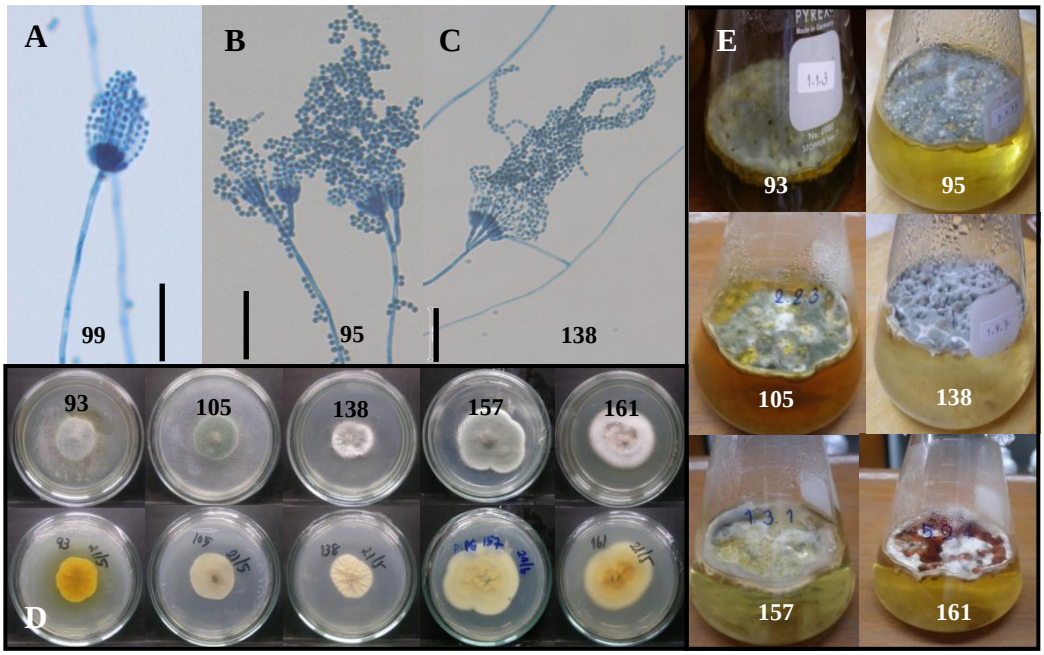

Figure 29 Morphological characteristics of *Penicillium* spp.

A-C: the brush-like spore-bearing structure

D: colony on PDA (7 days)

E: fermentation broth (21 days)

Scale bar: 50 µm

***Trichoderma* spp.**

Group members: RSPG24, 27, 28

Descriptions:

Colonies of RSPG24, 27 and 28 were fast growing on PDA. The woolly colonies were initially white and became patches green when the conidia were produced. The microscopic morphology was hyaline septate hyphae, branched conidiophores, flask-shaped phialides and globose to ovoid conidia (Figure 30).

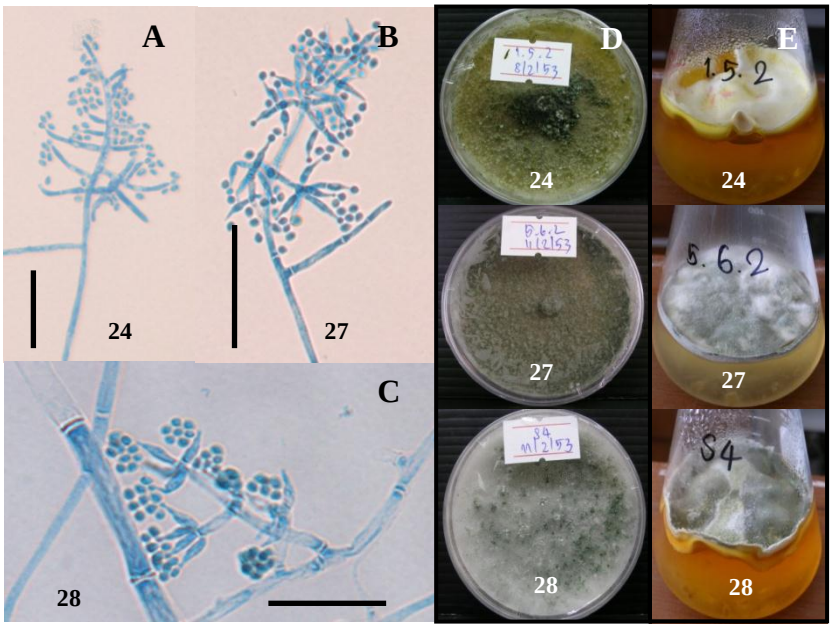

Figure 30 Morphological characteristics of *Trichoderma* spp.

A-C: microscopic morphology

D: colony on PDA

E: fermentation broth (21 days)

Scale bar: 50 µm

Unidentified fungi

Group members: RSPG82, 88, 185

Descriptions:

The colony on PDA of RSPG82 was corrugated, white with orange in reverse whereas RSPG88 was flossy and white to pale yellow. RSPG185 was slow growing, corrugated and yellow. Their microscopic features represented hyaline septate hyphae without reproductive structure (Figure 31).

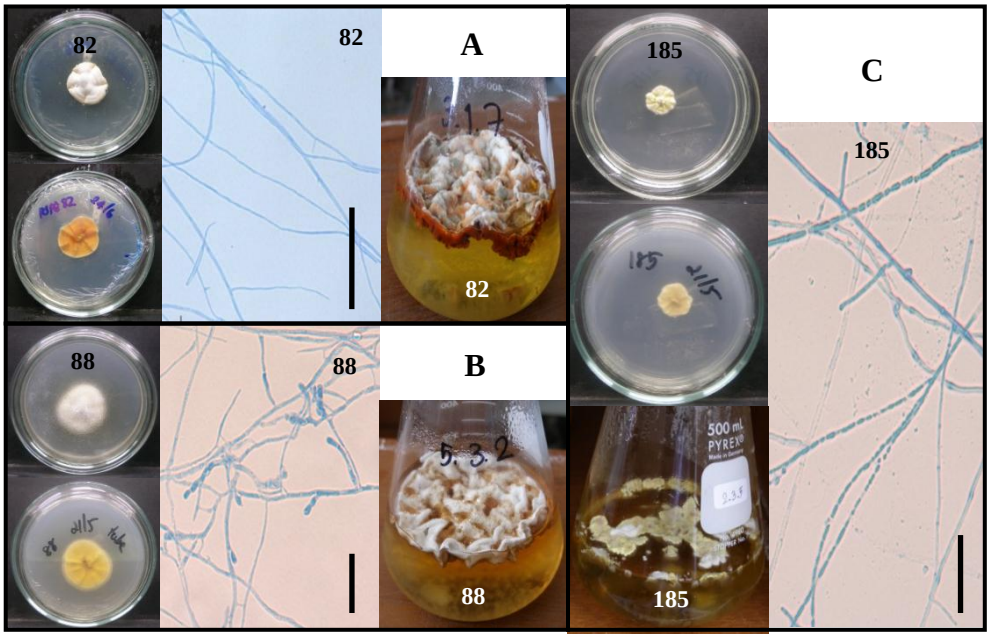

Figure 31 Morphological characteristics of unidentified fungi

A: RSPG82

B: RSPG88

C: RSPG185

Scale bar: 50 µm

3.5.2 Molecular identification

The identification of interesting soil fungi was confirmed by molecular technique. ITS sequences data were used to reconstruct a maximum parsimonious tree (MPTs). With molecular data, interesting soil fungi could be classified into 3 classes, 3 orders and 7 genera as follows:

Class Eurotiomycetes, Order Eurotiales

Nineteen isolates of soil fungi were clustered in the order Eurotiales in 2 genera, *Aspergillus* and *Penicillium*. Eleven out of 19 isolates (RSPG178, 179, 180, 185, 196, 197, 198, 199, 202, 204 and 206) were placed in various *Aspergillus* species. *P. griseofulvum* JX231006 and *P. citrinum* JQ647899 were used as the outgroups. Maximum parsimony analysis from 98 taxa of 431 total characters yielded 100 MPTs with tree length 168 steps, CI of 0.8810, HI of 0.1190, RI of 0.9903 and RC of 0.8724. One hundred and four characters were parsimony-informative and 8 variable characters were parsimony-uninformative.

The best tree of *Aspergillus* estimated by the Kishino-Hasegawa (KH) test is shown in Figure 32 RSPG178, 179 and 180 were placed in a subclade A comprising several strains of *A. sclerotiorum*, *A. fresenii* AY338961, *A. elegans* EU165705, *A. bridgeri* EF661404, *A. bridgeri* EF661398, *A. persii* EF661399, *A. bridgeri* EF200084 and *A. persii* FR733836 supported by bootstrap values of 64%, with *A. sclerotiorum* AY373866 as the most similar taxon (99% nucleotide identity). Therefore, RSPG178, 179 and 180 could be identified as *A. sclerotiorum* based on nucleotide sequence homology.

RSPG196 and 198 were located in a subclade B. RSPG196 grouped with several *A. nomius* strains with 99.4-100% nucleotide identity and strong bootstrap values (96%). RSPG196 can be named as *A. nomius*. RSPG198 had affiliation with *A. bombycis* supported by high bootstrap values (92%) and identical nucleotide identity (100%). Thus, RSPG198 should be referred to *A. bombycis*.

RSPG185 grouped well within a subclade C and formed a sister group to unknown species of *Aspergillus* (*Aspergillus* sp. HQ288052, *Aspergillus* sp. DQ092528, *Aspergillus* sp. HQ832962 and *Aspergillus* sp. EF669595). The

nucleotide identities within the group were 95.7-96.5%. However, RSPG185 had no closely related species with moderate bootstrap support (70%), therefore, it can only be reffered to *Aspergillus* sp.

RSPG197 and 206 clustered within a subclade D consisting of four species from *Aspergillus* section *Usti* including *A. ustus*, *A. keveii*, *A. insuetus* and *A. minutus* (100% bootstrap support). The nucleotide identities within the group were 99.4-100%. The molecular data of ITS rDNA sequence revealed that RSPG197 and 206 belonged to *Aspergillus* section *Usti*.

Three soil fungal isolates, RSPG 199, 202 and 204 were closely related and well placed in a subclade E containing several *A. unguis* strains (teleomorph: *Emericella unguis*) and *Aspergillus* sp. HM589221 supported by high bootstrap values and nucleotide identity of 100%. Therefore, RSPG199, 202 and 204 should be named as *A. unguis*.

Eight isolates of soil fungi (RSPG82, 93, 95, 99, 105, 138, 161 and 162) were located in the genus *Penicillium*. Maximum parsimony analysis from 117 taxa of 473 total characters yielded 100 MPTs with tree length of 393 steps, CI of 0.6947, HI of 0.3053, RI of 0.9620 and RC of 0.6683. Fifty-five variable characters were parsimony-uninformative and 139 characters were parsimony-informative. *A.versicolor* JX845289 and *Trichocoma paradox* JF417485 were used as outgroups.

Figure 33 shows the best topology of *Penicillium* tree analysed by the KH test. RSPG95, 138 and 162 were clustered in a subclade A. RSPG95 and 162 were closely related and formed a subgroup with various strains of *P. citrinum* with 100% nucleotide identity and 100% bootstrap values. Therefore, these two isolates could be identified as *P. citrinum*. While RSPG138 formed a sister group to *P. roseopurpureum* AF455437, *P. roseopurpureum* AF455492, *P. rosepurpureum* GU723456 and *P. copicola* JN617685. This group was established by 60% bootstrap support. The most closely related species of RSPG138 was *P. copticola* JN617685 with 99.5% nucleotide sequence identity. Thus, it could be identified as *P. copticola*.

RSPG99 grouped with several strains of *Penicillium* sp. in a subclade B supported by moderate bootstrap support but without any closely associated taxa. Its nucleotide identity with this grouping was relative low (78.0-80.6%). As the result of ITS rDNA analysis, this isolate can only be referred to *Penicillium* sp.

RSPG82 was placed in a subclade C containing various *P. scelerotiorum* strains supported by robust bootstrap values (100%) and nucleotide identity of 99.5-100%. RSPG82 could be identified as *P. sclerotiorum*.

RSPG93 belonged to a subclade D including several strains of *P. herquei*. Although the grouping was not supported by bootstrap analysis, the nucleotide identities within the group were 99.7-100%. Therefore, RSPG93 should be named as *P. herquei*.

RSPG161 was placed in a subclade E and formed a subgroup with *Penicillium* sp. GU565135, *Penicillium* sp. JN545832 and *P. pimiteouiense* GQ924903 supported by moderate bootstrap values (68%). RSPG161 had affinity with *Penicillium* sp. GU565135 and *P. pimiteouiense* GQ924903 with 100% nucleotide identity. RSPG161 could be referred to *P. pimiteouiense*.

Finally, RSPG105 grouped in a subclade F supported by bootstrap value (100%). RSPG105 was closely related to *P. aculeatum* strains with 99.0-99.5% nucleotide identity. ITS rDNA analysis suggested that it could be identified as *P. aculeatum*.

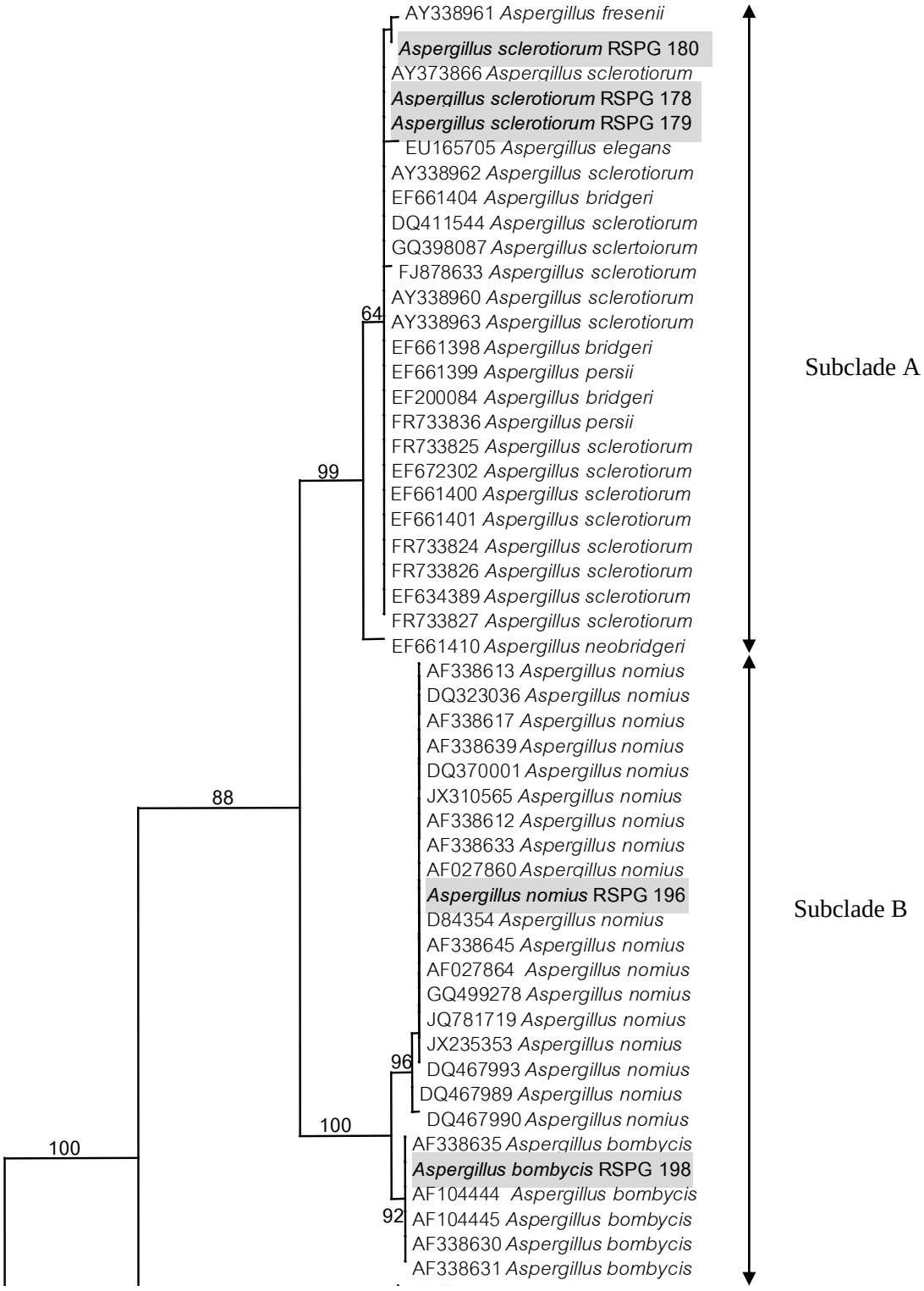

Figure 32 Phylogenetic tree of *Aspergillus* spp. based on ITS1-5.8S-ITS2 sequences. The number of each branch point represents percentage bootstrap support (≥50 %) from Maximum Parsimony with 100 replications are shown on the branch. *P. griseofulvum* JX231006 and *P. citrinum* JQ647899 were used as outgroups. Length; 168 steps; consistency index (CI); 0.8810; retention index (RI); 0.9903; homoplasy index (HI); 0.1190; rescaled consistency index (RC); 0.8724.

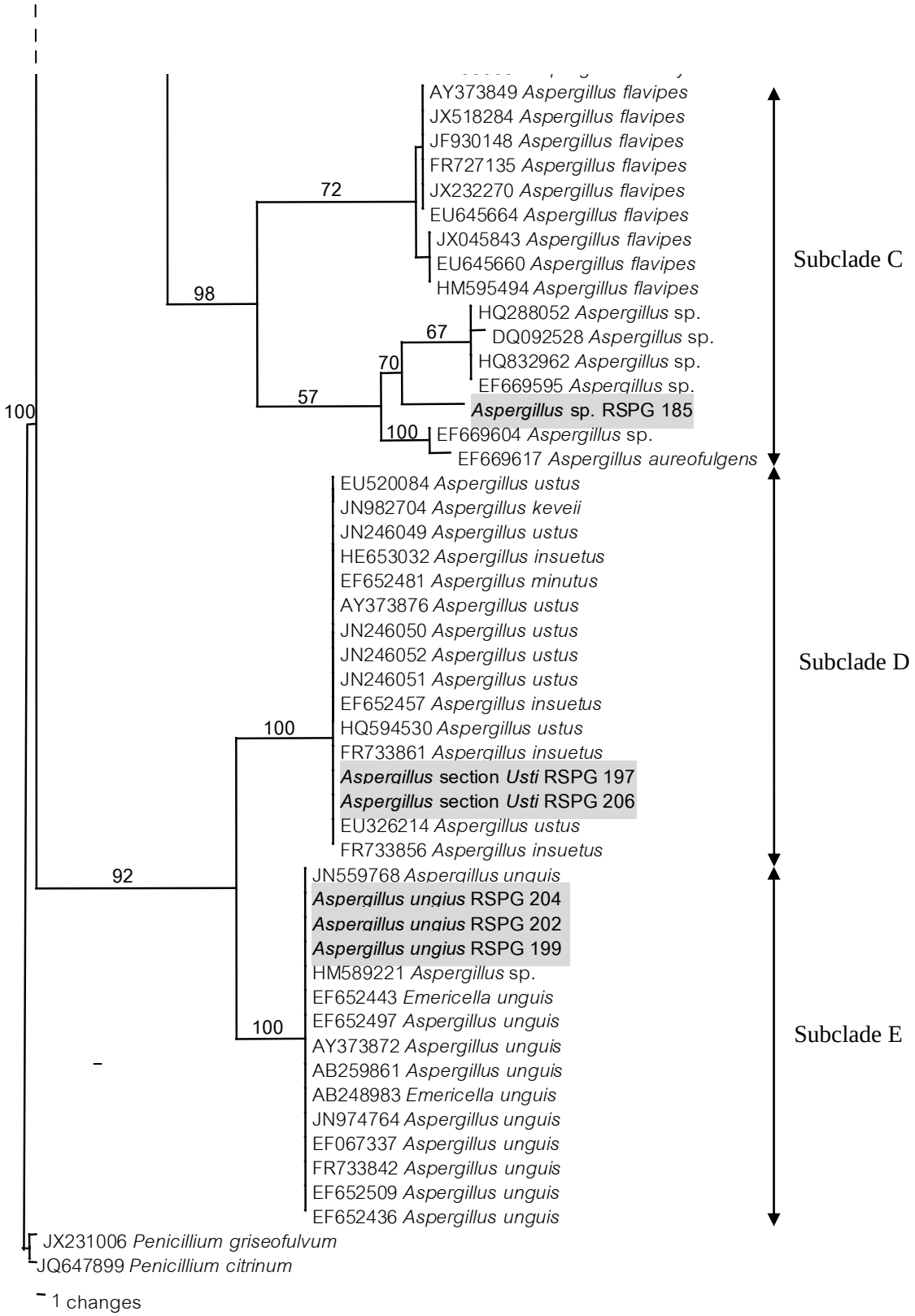

Figure 32 (cont.)

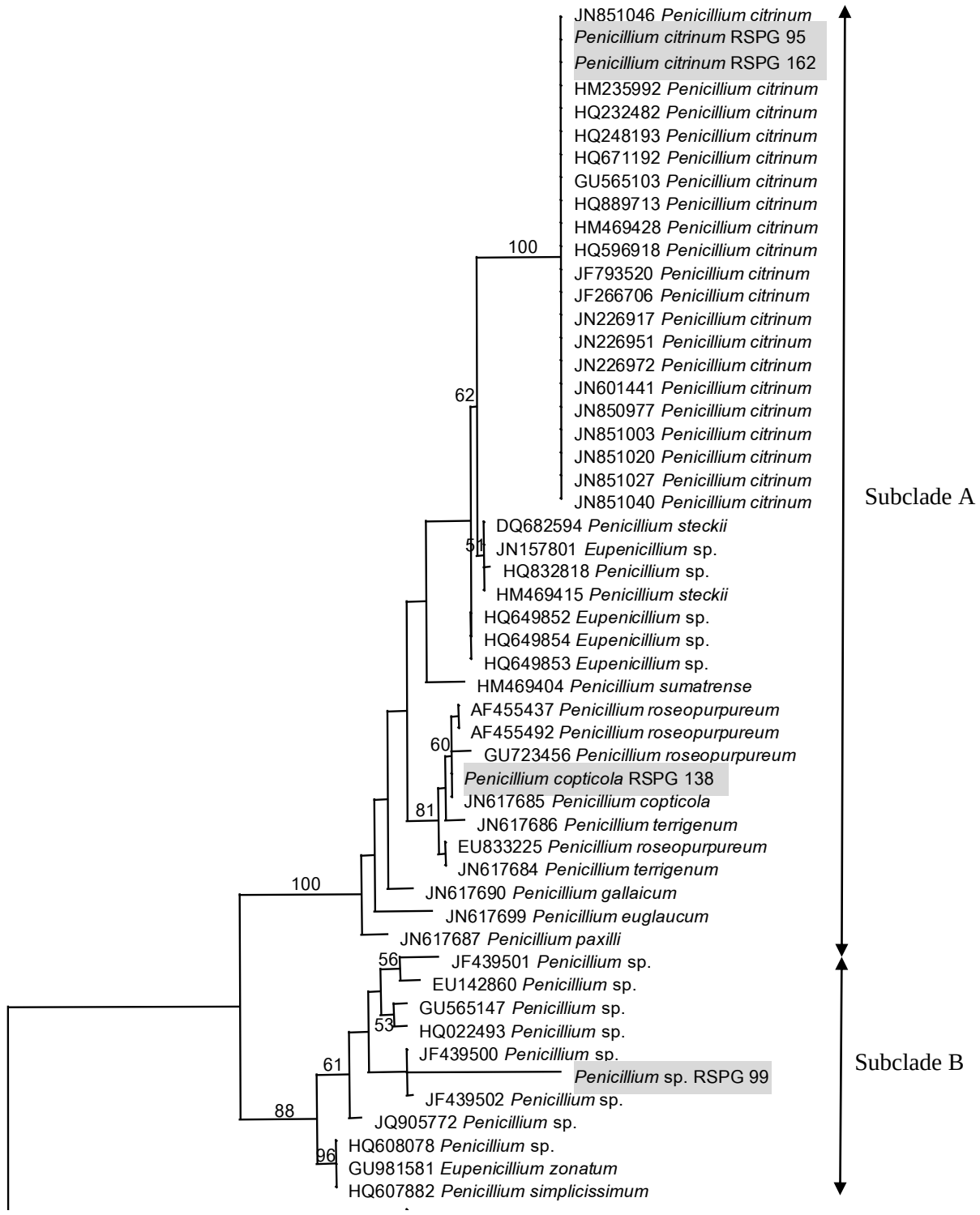

Figure 33 Maximum parsimonious tree of *Penicillium* spp. based on ITS1-5.8S-ITS2 sequences. The number on each branch presents percentage of bootstrap values support (≥50 %) with 100 replications. *A. versicolor* JX845289 and *Trichocoma paradox* JF417485 were used as outgroups. Length; 393 steps; consistency index (CI); 0.6947; retention index (RI); 0.9620; homoplasy index (HI); 0.3053; rescaled consistency index (RC); 0.6683.

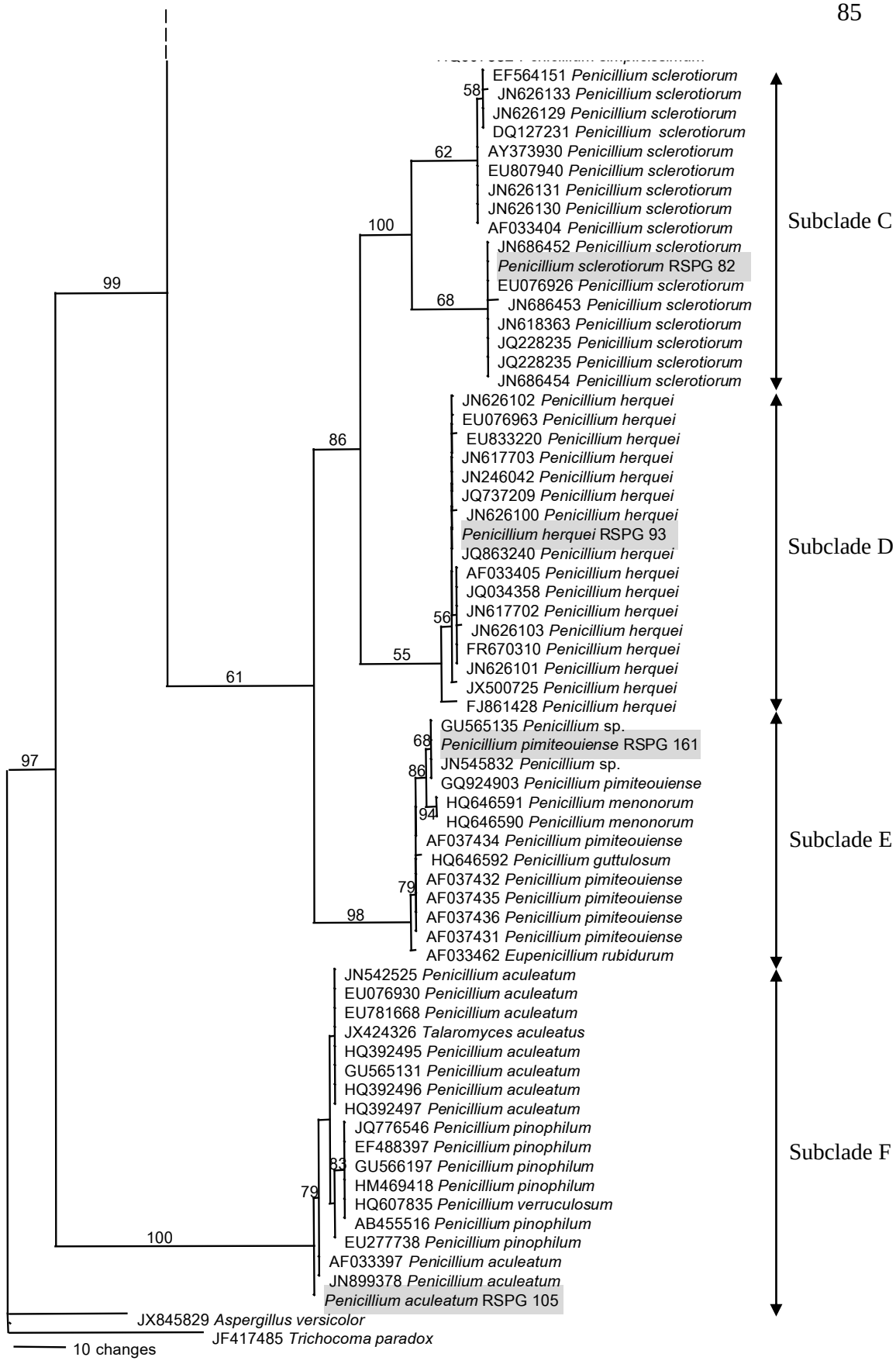

Figure 33 (cont.)

Class Sordariomycetes, Order Hypocreales

Thirteen fungal isolates were placed in the Order Hypocreales within four genera *Trichoderma*, *Paecilomyces*, *Myrothecium* and *Fusarium*. *Bionectria lucifer* AF210683 and *B. epichloe* JN198444 were used as outgroups. Maximum parsimony analysis from 66 taxa of 563 total characters yielded 1000 MPTs with tree length 389 steps, CI of 0.8123, HI of 0.1877, RI of 0.9767 and RC of 0.7934. Thirty-nine variable characters were parsimony-uninformative and 182 characters were parsimony-informative. The best tree estimated by the KH test is shown in Figure 34

The soil fungi RSPG24, 27 and 28 were located in subclade A. RSPG24 had affiliation with *T. reesei* (teleomorph: *Hypocrea jecorina*) supported by high bootstrap values and nucleotide identity of 100%. Therefore, RSPG24 should be referred to *T. reesei*. RSPG27 was clustered with *T. brevicompactum* with 100% bootstrap values and 100% nucleotide identity. Thus, this isolate was identified as *T. brevicompactum*. RSPG28 was closely related to the sequences of *Hypocrea lixii* (anamorph : *T. harzianum*) with bootstrap values and nucleotide identity of 100% and 99.8-100%, respectively. This fungus did not produce any ascospores, therefore, RSPG28 should be name as *T. harzianum*.

Three soil fungal isolates, RSPG50, 52 and 58 grouped within subclade B containing several *Paecilomyces lilacinus* strains including clinical isolate *Purpureocillium lilacinum* with 99.5-100% nucleotide identity and 100% bootstrap values. The ITS phylogeny supported the identification of RSPG50, 52 and 58 as *P. lilacinus*.

RSPG88 was placed in subclade C with several *M. roridum* strains. The nucleotide identities within the group were 98.3-99.7%. RSPG88 was referred to *M. roridum* with 100% bootstrap support.

Six isolates of soil fungi (RSPG37, 63, 227, 229, 230 and 231) were clustered in subclade D. RSPG37, 63, 227, 229 and 231 grouped with *F. oxysporum* EU625403, *Fusarium* sp. EU750689, *F. solani* FJ719812, *F. solani* AM412643 and *F. solani* AM 412603 supported by bootstrap values of 100%. RSPG37 and 229 were grouping together and most related to *F. solani* AM412643 with 96.1-97.3% ITS sequence identity thus, RSPG37 and 229 can be referred to *F. solani*. RSPG63, 227

and 231 were identical to *F. solani* FJ719812, *Fusarium* sp. EU750689 and *F. oxysporum* EU625403 with 99.3-99.7% sequence identity. Considering the morphology of RSPG63, 227 and 231, the colonies were white to pale orange or pale yellow. Blue color was occurred on colony when cultured in PDB. Macroconidia were slightly curved, stout and had 3-5 septate. Microconidia had 1-2 septa and large. Thus, RSPG63, 227 and 231 should be named as *F. solani* (Nelson *et al*., 1983). While RSPG230 were grouped with several strains of *F. solani* and *Fusarium* sp. EF687945 with 93% bootstrap support. Its ITS rDNA sequence was closely related to *F. solani* AM412623 with 99.7% nucleotide identity. Therefore, RSPG230 was identified as *F. solani*.

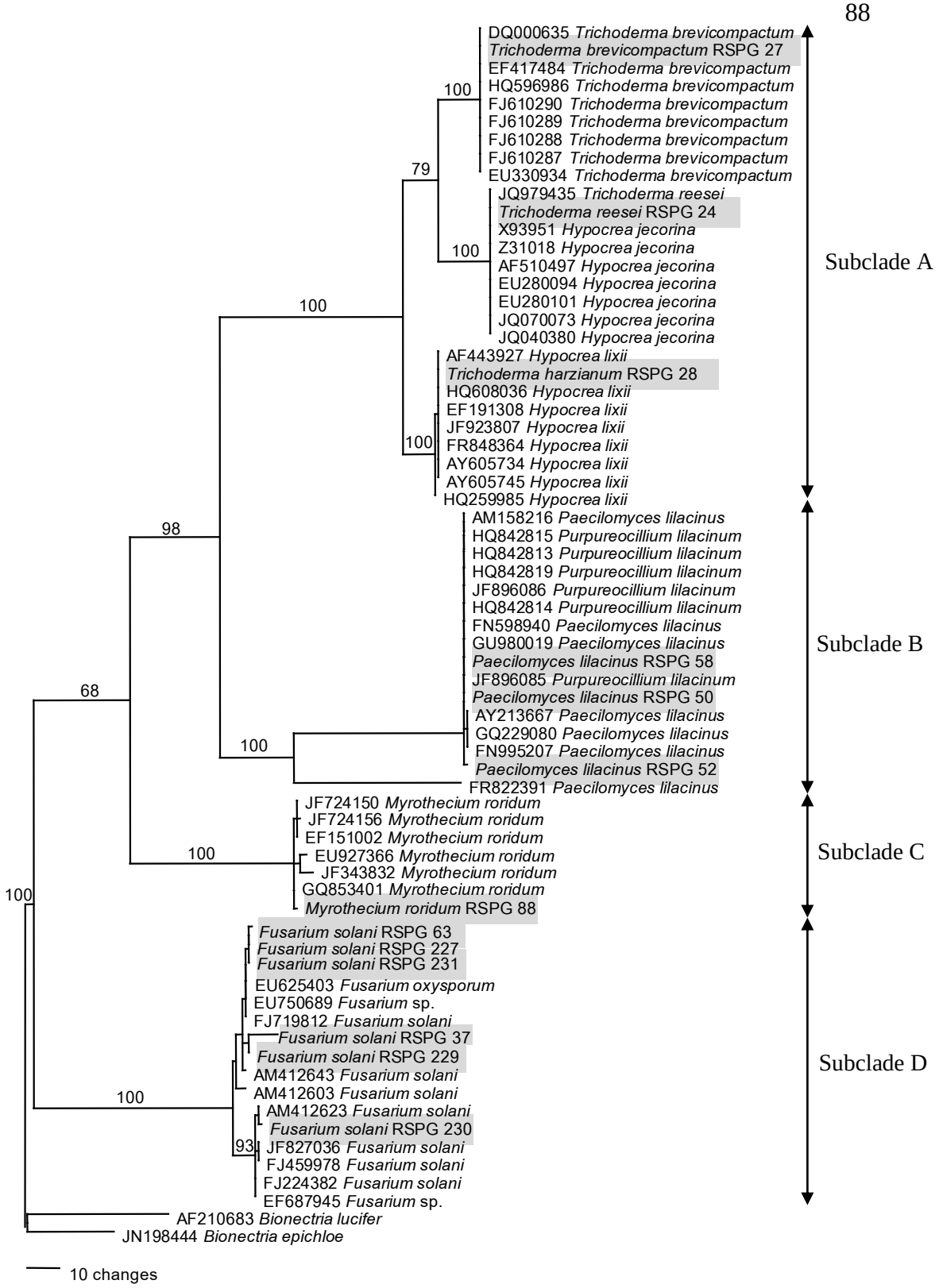

Figure 34 Phylogenetic tree based on ITS1-5.8S-ITS2 sequences of soil fungi in order Hypocreales. The number on each branch represents percentage of bootstrap values support (≥50 %) with 100 replications. *Bionectria lucifer* AF210683 and *B. epichloe* JN198444 were used as outgroups. Length; 389 steps; consistency index (CI); 0.8123; retention index (RI); 0.9767; homoplasy index (HI); 0.1877; rescaled consistency index (RC); 0.7934.

Class Zygomycetes, Order Mucorales

RSPG 214 was located in the Mucorales comprising several *Absidia* species. Maximum parsimony analysis from 24 taxa of 767 total characters, with *Cunninghamella elegans* FJ792589 and *C. bertholletiae* FJ345351 were used as outgroups. The numbers of constant characters, parsimony-uninformative and parsimony-informative characters were 242, 33 and 492, respectively. Four most parsimonious trees were obtained from heuristic searches. The best tree calculated by the KH maximum parsimony test is shown in Figure 35 with tree length of 1961 steps, CI of 0.5502, RI of 0.7267 RC of 0.3998 and HI of 0.4498.

RSPG 214 had affiliation with *A. repens*, *A. psychrophilia*, *A. heterospora*, *A. anomala* and *A. spinosa*, but no closely associated with other known species from GenBank database. Its nucleotide identity in this grouping was relative low (50.2-72.1%) with weak bootstrap support. Thus, RSPG 214 was identified to be *Absidia* sp.

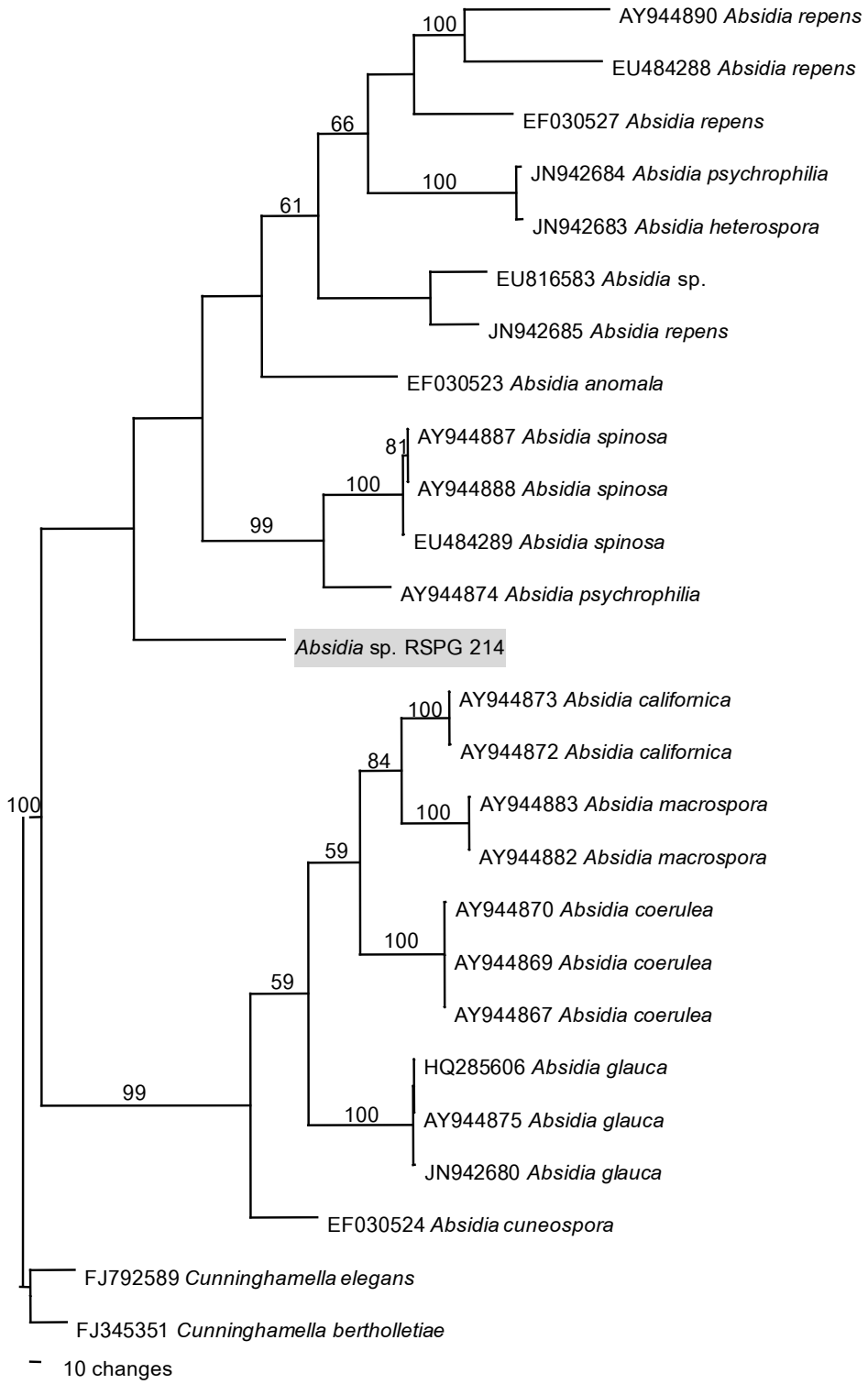

Figure 35 Maximum parsimonious tree of RSPG214 based on ITS1-5.8S-ITS2 sequences. The number on each branch represents percentage of bootstrap values support (≥50 %) with 1000 replications. *Cunninghamella elegans* FJ792589 and *C. bertholletiae* FJ345351 were used as outgroups. Length; 1961 steps; consistency index (CI); 0.5502; retention index (RI); 0.7267; homoplasy index (HI); 0.4498; rescaled consistency index (RC); 0.3998.

CHAPTER 4

DISCUSSIONS

4.1 Number of isolated soil fungi

Soil fungi were isolated from soil samples that collected in January, April, June and August 2010 from Plant Genetic Conservation Project area, Rajjaprabha dam, Suratthani province. The fungal density of soil samples were in the ranges of 4.2×10^4-1.1×10^6 CFU/g. The average fungal density from this study was in the same range as in other studies. Chutia and Ahmed (2012) isolated soil fungi from different virgin forest floor in India and reported mean of fungal population of 9.9×10^4 CFU/g. Makut and Owolewa (2011) isolated antibiotic-producing soil fungi from Nigeria and recorded the total fungal count as 1.6-4.7×10^4 TFU/g. In Thailand, Boonleang (1999) reported the fungal density of soil samples from Forest-Reviving as the Royal Suggestions and Plant Germplasm Forest Project in Nakhon Ratchasima province of 1.80×10^4-1.09×10^5 CFU/g. Soil samples from RSPG area, Rajjaprabha dam were very strongly acid to slightly acid soil with the pH value of 4.90-6.43 (Yongchalermchai *et al*., 2011) that encourage the population of fungi. Generally, the numbers of fungi in soil were varied in range of 10^5-10^6/g of soil (Metting, 1993).

The lowest density of soil fungi was found in April, whereas the highest density was found in August. Soil properties e.g. pH and moisture content affect to a survival of fungi inhabiting in soil (Rohilla and Salar, 2012). Most of fungi require high water availability for their growth (Levetin *et al*., 2001; Burge, 2006). From the collection times in 2010, the highest average temperature and the lowest average rainfall were presented in April (http://www.tmd.go.th/programs/uploads /yearlySummary/T_weather2553.pdf). High temperatures and low rainfall decrease

water availability in soil and limit the fungal growth. Moreover, fungal hyphae and conidia in soil are exposed to ultraviolet radiation which has effects on fungal viability and spore germination (Levetin *et al*., 2001). Therefore, high moisture and low temperature from rain in August are a suitable condition to promote the growth of soil fungi.

4.2 Fungal species identified by morphology

Soil fungi were isolated by dilution plate method on RBC agar. This medium was supplemented with chloramphenicol and rose bengal to prevent bacterial growth. Rose bengal also slows down the growth of fungal colonies decreasing the tendency of fast-growing fungi to spread quickly over the plate before slow-growing fungi form colonies (Garrett, 1981). The isolated fungal species, *Penicillium*, *Aspergillus*, *Trichoderma*, *Gongronella*, *Fusarium*, *Mucor* and *Cunninghamella* were common genera found in soil samples. Dilution plate method usually employed to isolate fungi which produce conidia (Rao, 1970) but this technique has some disadvantages. Firstly, no single culture medium is suitable for isolation of all fungal species. Thus, some species are not occurred because their population numbers are too low or grow rather slowly when compared with the common species. The secondary disadvantage is the original suspension of soil. Some of heavy fungal fragment and spore fuse with heavier part of soil and are sunk to the bottom of the shaking vessel before a sample has been pipetted off to make a dilution. So, this fungal group was not appeared on the isolation medium (Garrett, 1981). Other techniques used for isolation of soil fungi are Warcup soil plate, immersion plate and tube and baiting technique. The Warcup soil plate allows the isolation of fungi that are not representing with the dilution plate method and potential to isolate a wider range of fungal species (Garrett, 1981). Immersion plate and tube employ to study the microbes in a natural way in their habitats (Subbarao, 1999). Baiting technique is a semiselective method used for isolation of various fungi such as *Pythium*, *Chaetomium*, *Rhizoctonia*, dermatophytes and water fungi (Rao, 1970; Windham and

Lucas, 1987; Simpanya and Baxter, 1996; Deechouy, 2013). A sexual stage of fungi has been obtained under controlled laboratory conditions for example Rodriguez and Owen (1992) successfully induced the sexual stage of *Colletotrichum musae* by controlling light and growth temperature.

From this study, *Penicillium* is the most frequent isolated genus followed by *Aspergillus*. Deechouy (2013) also isolated soil fungi from RSPG area and found that the most isolated genera from dilution plate method using GANA and TSM were *Penicillium* followed by *Aspergillus* and *Trichoderma.* Chutia and Ahmed (2012) also reported that *Penicillium* and *Aspergillus* were abundant species isolated from virgin forest floor in India. These two genera can be found in all natures due to their ability to adapt to different environments (Wahegaonkar *et al*., 2011).

4.3 Antimicrobial activity of fungal crude extracts

Soils are traditionally the major source of antibiotic and/or new compound producing fungi. Antibiotics provided by fungi are currently used in pharmaceuticals especially cephalosporin, fusidic acid and penicillin (Takahashi *et al*., 2008; Gharaei-Fathabad *et al*., 2009; Petit *et al*., 2009; Makut and Owolewa, 2011). Several researches indicated that soil fungi served as a good source of antimicrobial agents (Qureshi, 2003; Liermann *et al*., 2009; Kumar *et al*., 2010; Ali *et al*., 2011; Senthilkumar *et al*., 2011).

In this study, antimicrobial activity of 181 soil fungi was evaluated from their crude extracts toward 12 human pathogens comprising bacteria (6 strains), yeasts (4 strains) and filamentous fungi (2 strains). Three hundred and eleven crude extracts from 145 selected soil fungi exhibited antimicrobial activity against at least one test strain. This result is similar to Mya (2011), Helen *et al*. (2012) and Jaiswal *et al*. (2012) that isolated soil fungi from different places exhibited antimicrobial activity against one or more test microorganisms.

Among the tested microorgnisms, Gram-positive bacteria (*S. aureus* ATCC 25923 and MRSA SK-1) were more susceptible than Gram-negative bacteria (*A. baumannii* AB007, *A. baumannii* AB005, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and yeasts (*C. neoformans* and *C. albicans*) were more susceptible than the filamentous fungi (*P. marneffei* and *M. gypseum*). Several studies also found the less inhibition of Gram-negative bacteria by soil fungi (Corte *et al*., 2000; Makut and Owolewa, 2011; Chutia and Ahmed, 2012) and by other natural products (Kelmanson *et al*., 2000; Mahesh and Satish, 2008; Wagate *et al*., 2010). In Gram-negative bacteria, their outer membrane displays an important role as a permeability barrier that prevents exposure of susceptible sites to antimicrobial agents (Kaye *et al*., 2004). On the other hand, Gharaei-Fathabad *et al.* (2009) reported that *C. albicans* was the lowest susceptible strain when examined with fungal crude extracts dissolved in chloroform and tested by disc diffusion method. The polarity of the compounds can affect the diffusion of compounds onto the culture medium (Jiang, 2011). Different solvents used to dissolve crude extracts and different techniques used to evaluate activity may give different results of antimicrobial assay.

Even most of fungal crude extracts were active against only one test strain (20.07%) but overall of antimicrobial activity testing showed that fungal crude extracts were able to inhibit more than one test strain. RSPG 198 CH was the only crude extract presented activity to all of the test microorganisms. The results indicated that crude extracts from soil fungi had a wide range of antimicrobial activity. Many investigators including Corte *et al*. (2000); Takahashi *et al*. (2008); Gharaei-Fathabad *et al*. (2009); Petit *et al*. (2009); Makut and Owolewa (2011); Chutia and Amhed (2012) also reported that soil fungi showed a broad antimicrobial spectrum against various strains of test microorganisms.

Considering the type of crude extracts, cell hexane was the highest active crude extracts (CH, 61.88%) followed by cell ethyl acetate (CE, 58.01%) and broth ethyl acetate (BE, 53.59%). This could explain that active substances are retained in fungal mycelium and contained cell-bound components. Active extracts may have low polarity and are better dissolved in hexane than ethyl acetate (Buatong, 2011; Prapagdee *et al*., 2012). This work is correlated with Buatong *et al*. (2011) and

Jeenkeawpieam *et al*. (2012) who evaluated antimicrobial activity of crude extracts of endophytic fungi from mangrove plants and *Rhodomyrtus tomentosa*. In contrast, Preedanon (2008) found that BE extracts from marine-derived fungi were the greatest active extracts. Kuephadungphan *et al*. (2013) studied on antimicrobial activity of entomopathogenic fungi and found that CE extracts were more active than the BE and CH extracts. Different groups of fungi produce different attributes of bioactive metabolites and different solvent extractions provide different compounds. Ethyl acetate is a medium polarity solvent that is used to extract compounds of intermediate polarity whereas non-polar solvent, hexane, is used to solubilize greatly lipophilic compounds (Seidel, 2006). Unionized, low polarity and higher lipid solubility agents are well known to easily permeate cell membrane than inverse agents (Peck *et al*., 2008).

Active crude extracts from preliminary test were further evaluated for their MICs and MBCs/MFCs. The results showed that the MICs/MBCs or MFCs were varied in range of 1-200/2->200 µg/ml. The lowest MIC was active against *C. neoformans* ATCC 90113 (RSPG 179 CH) while the lowest MBC was found in MRSA SK-1 (RSPG 206 CH). It is interesting to notice that *C. neoformans* ATCC 90113 and MRSA are drug resistant strains. *C. neoformans* ATCC 90113, encapsulated opportunistic yeast is resistant to flucytosine antifungal drug by a single mutational event within the pyrimidine salvage pathway (Perfect and Cox, 1999). MRSA, a methicillin-resistant *S. aureus* presents the expression of PBP2a that has a lower penicillin-binding (PBP) affinity and higher rates of release of the bound drug and can take over the transpeptidation (cross-linking) reactions of the host PBPs (Stapleton and Taylor, 2002). This indicates that active fungal crude extracts may act on different or new targets (Buatong *et al*., 2011). Strongly active crude extracts including RSPG 27 BE, 50 BE, 52 BE, 105 CH, 157 CE, 178 CE, 178 CH, 179 BE, 179 CE, 180 BE, 180 CH, 196 BE, 202 CH, 204 CH and 206 CH possessed bactericidal and/or fungicidal activity with MBCs/MFCs of 1 to 4 folds of MICs**.** They will be further investigated for active constituents. Results from this study have revealed that soil fungi from Rajjaprabha dam can be a possibly source of bioactive natural products.

4.4 Study on possible mechanisms of action of the active crude extracts by scanning electron microscopy (SEM)

The best active crude extracts comprising RSPG 27 CH, 179 CH, 204 CH and 206 CH were chosen for study on possible modes of action by SEM and compared with standard drugs. RSPG 27 CH was highly active toward both strains of *C. albicans*. RSPG 179 CH presented activity to *C. neoformans* ATCC 90113 and *P. marneffei*. RSPG 204 CH was tested with *C. neoformans* ATCC 90112 whilst RSPG 206 CH was examined with both strains of *S. aureus*.

From the results, the cells treated with RSPG 27 CH exhibited cell collapse or lysis (Figure 20c and 21c). The NMR profile of RSPG 27 CH demonstrated one major compound as trichodermin (Rukachaisirikul *et al*., personal communication). Trichodermin is a member of trichothecenes mycotoxin group that acts as an inhibitor of the elongation and termination steps in the protein synthesis (Westerberg *et al*., 1976; Reino *et al*., 2008). Kim *et al*. (2012) suggested that cells treated with antifungal agent were damaged initially form the interior, as indicated by cell collapse. Furthermore, the cells treated with RSPG 179 CH and 204 CH displayed cell leaked or lacerated (Figure 23c and 24c) similar to amphotericin B treated cells (Figure 23b and 24b). Amphotericin B is a polyene macrolide antifungal agent which is produced by the soil actinomycetes *Streptomyces nodosus*. Amphotericin binds to ergosterol in the fungal cell membrane resulting in the formation of pores, allowing leakage of potassium and other cellular components that lead to cell death (Chapman *et al*., 2003). This may be concluded that these fungal crude extracts may have an effect on fungal cell wall or cell membrane.

Vancomycin is a tricyclic glycopeptide that effectively inhibits Gram-positive bacteria. Vancomycin binds to D-Ala-D-Ala C terminus of pentapeptide resulting in blocking the production of peptidoglycan and inhibiting bacterial cell wall synthesis (Courvalin, 2006). The SEM micrographs of cells treated with vancomycin and RSPG 206 CH (Figure 18-19) revealed morphological changes consistent with

cell damage. The results illustrated that RSPG 206 CH may have the active materials affecting on bacterial cell wall.

However, the results from this experiment were only a preliminary study on possible mechanisms of active crude extracts. The true mechanism should be further studied and confirmed by other techniques such as transcription-translation assays, whole-cell protein synthesis, translation elongation assay, translation termination assay, cytoplasmic-membrane depolarization assay, bacteriolysis, loss of 260-nm-absorbing material, measurement of plasma membrane fluorescence anisotropy and determining released glucose and trehalose (Shinabarger *et al*., 1997; Friedrich *et al*., 2000; Carson *et al*., 2002; Kim *et al*., 2009).

4.5 Identification of interesting soil fungi by morphological characteristics and molecular technique

4.5.1 Morphological identification

Interesting soil fungi could be identified by morphological characteristics into 6 genera including *Absidia*, *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium* and *Trichoderma*. In general, the identification of fungi at the species level by cultural method could be done using several media such as malt extract agar (MEA) for microscopic analysis and colony characters (Rivera and Seifert, 2011), Czapek yeast agar (CYA) for colony characters (Pitt, 1973), Czapek agar (CZ) for ability to grow and sporulate in the absence of ammonia (Raper and Thom, 1949), yeast extract sucrose agar (YES) to stimulate colony pigmentation by enhancing secondary metabolite production (Filenborg *et al*., 1990), oatmeal agar (OA) to stimulate sclerotial or ascomatal development (Rivera and Seifert, 2011) and creatine sucrose agar (CREA) to test for acid production (Frisvad, 1993). However, this technique is time-consuming and laborious. In this study, only PDA was used. Therefore, the sporulating fungi were identified by morphology only at genera level

and were then confirmed by molecular method. Fungi that did not produce any reproductive structures were identified only by molecular method.

4.5.2 Molecular identification

Nowadays, molecular technique is an important tool for classification of fungi. It provides specificity, sensitivity and time-saving for identifying the fungi at diverse taxonomic levels. In this study, selected soil fungi were identified by molecular technique based on ITS1-5.8S-ITS2 rDNA. The results showed that active soil fungi were identified into 3 classes (Eurotiomycetes, Sordariomycetes, Zygomycetes) and 3 orders (Eurotiales, Hypocreales, Mucorales).

Class Eurotiomycetes, Order Eurotiales

Nineteen soil fungal isolates were well placed in the Class Eurotiomycetes, order Eurotiales. Eleven isolates belonged to a genus *Aspergillus* and 8 isolates were classified in a genus *Penicillium*. Species of *Aspergillus* and *Penicillium* are well-known as the important bioactive compound producers with a wide variety of bioactivities (Bugni and Ireland, 2004).

RSPG178, 179 and 180 were identified as *A. sclerotiorum*. The colonies on PDA were white to yellow and floccose with small sclerotia. They produced globose vesicles and smooth globose conidia. Their crude extracts displayed the best activity against *C.neoformans* ATCC 90113 with MICs value less than 10 µg/ml, whereas the other test microorganisms were inhibited at higher concentrations. Various compounds including ochratoxins A and B, penicillic acid, xanthomegnins, 2'-oxoasterriquinol D Me ether, scleramide and cytotoxic indole-3-ethenamide have been reported from *A. sclerotiorum* (Varga *et al*., 1996; Frisvad *et al*., 2004; Whyte *et al*., 2000; Wang *et al*., 2011). Ochartoxins are mycotoxin produced by several species of *Aspergillus* and *Penicillium* (Bayman and Baker, 2006). Ochratoxin A has been shown to inhibit protein synthesis, mitochondrial respiration and increase oxidative stress in the cells (Schilter *et al*., 2005). Penicillic acid was found to down-regulate quorum-sensing regulating gene in *P. aeruginosa* with the concentration of 60%

(Liaqat *et al*., 2008). Xanthomegnin showed genotoxic activity and interrupted cell respiration (Mori *et al*., 1984 and Kawai *et al*., 1976).

RSPG196 can be named as *A. nomius*. Most of related *A. nomius* strains from the database were isolated from soil, silkworm excrement, arthropods and subterranean termites (www.ncbi.nlm.nih.gov). The colony on PDA was white to green, floccose and rapid growth. It presented globose to subglobose conidia and long stipe. The broth ethyl acetate extract (BE) had mild activity to *A. baumanii* AB005. The secondary metabolites of RSPG196 are being examined by Rukachaisirikul and co-workers. Secondary metabolites produced by *A. nomius* including aflatoxin B and G, n-butylhexadeconoate, 3-hydrobutylhexahydroxylpyrrolo- pyrazinedione and cytotoxic antiinsectan aspernomine (Olsen *et al*., 2008; Staub *et al*., 1992; Meenupriya and Thangaraj, 2012.). *A. nomius* exhibited a high variation of morphology and hardly to discriminate from *A. flavus* (Zotti *et al*., 2011). Mycotic keratitis, onychomycosis and pneumonia caused by *A. nomius* have been reported (Manikandan *et al*., 2009; Zotti *et al*., 2011; Caira *et al*., 2012).

RSPG198 was identified as *A. bombycis*. The colony texture on PDA was floccose, pale greenish-yellow in color and rapid growth. Its conidia were globose with spinule. RSPG198 CH showed a broad range antimicrobial activity against all test microorganisms (MICs value of 64-200 µg/ml) with interesting signal of NMR profile (Rukachaisirikul *et al*., personal communication). *A. bombysis* produced aflatoxin B and G in addition to kojic acid. Furthermore, this fungus can be found in silkworm cultivation and may infect the wild silkmoth (*Bombyx mandarina*) (Goto *et al*., 2003).

RSPG185 was referred to unidentified *Aspergillus* species based on molecular identification. Most of related *Aspergillus* taxa from GenBank were obtained from the marine habitat (www.ncbi.nlm.nih.gov). The colony of RSPG185 on PDA was yellow, white edge, rough and slow growth. The sequence alignment of RSPG185 was distincted from other taxa in a subclade (Figure 36). The sequence alignment of RSPG185 in position 3, 69 and 109 presented A, T and C, while other taxa displayed G, C and A. Futhermore, in position 46, other taxa had the nucleotide

base as C or T but it was missing in RSPG185. Consequently, RSPG185 had no close reference species, and then it can only be identified as *Aspergillus* sp. RSPG185 showed interesting NMR profile although its crude extracts revealed mild activity against *S. aureus*. Nine known compounds comprising fumitremorgin C, aspochalasin J, asperpentyn, 2-(1'-methylethenyl)benzofuran-5-carboxylic acid, 4-hydroxy-3-prenylbenzoic acid, eutypinic acid, 2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid, *N*-prenyl fumitremorgin C and aszonalenin together with 14 new compounds were found from this isolate (Rungsaiwattana, 2011). This result indicates that soil fungi in particular *Aspergillus* spp. are a good source of novel compounds.

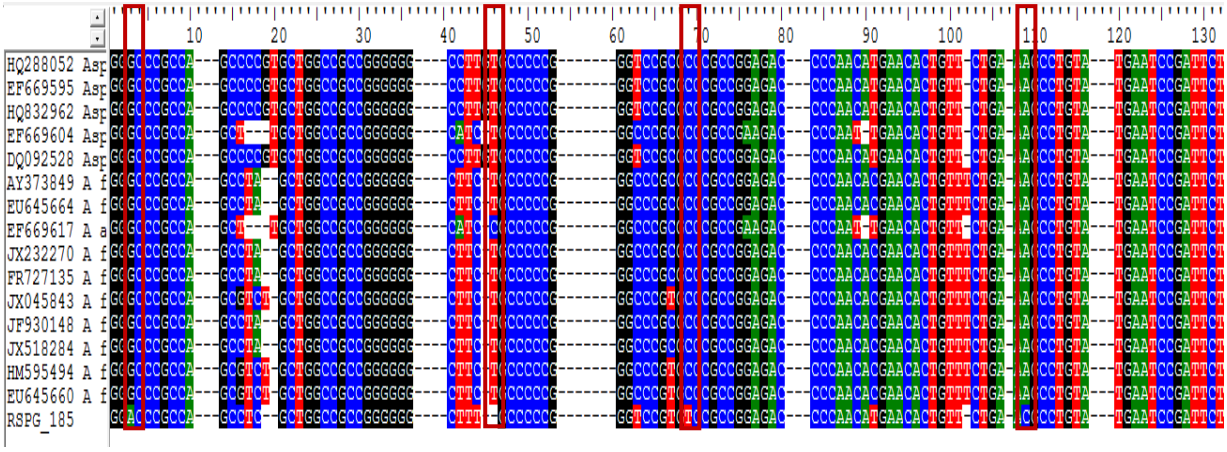

Figure 36 Partial ITS rDNA sequences alignment of RSPG185 and closely related taxa. Distinct nucleotides of RSPG185 compared with other sequences are marked.

RSPG197 and 206 were classified in *Aspergillus* section *Usti*. They grouped with many *Aspergillus* species isolated from soil and patients (www.ncbi.nlm.nih.gov). The colonies were floccose, white to green and rapid growth on PDA. RSPG197 had interesting NMR profile, whereas the cell hexane extract (CH) of RSPG206 demonstrated strong activity (MICs = 2-8 µg/ml) against both strains of *S. aureus* and *C. neoformans* ATCC 90113. Examples of secondary metabolites from *Aspergillus* section *Usti* include ustic acid, mycotoxin austocystins, austalides, niduol, asperugins, drimans, pergillin, ophiobolins G and H (Samson *et al*., 2011). Pergillin was a nontoxic fungal metabolite that moderately inhibited plant

growth (Cutler *et al*., 1980). Ophiobolins G and H inhibited the growth of *B. subtilis* and ophiobolin H also induced hyperacusia in day-old chicks (Cutler *et al*., 1984).

RSPG 199, 202 and 204 can be named as *A. unguis*. Most of related *A. unguis* strains from GenBank were reported from marine habitat, soil, patients and PlaoYai (*Croton oblongifolius*) (www.ncbi.nlm.nih.gov). The colonies on PDA were deep green, white margin, powdery, floccose hyphae at the center and moderate growth. The microscopic features were short stipe and green conidia. RSPG199CE, 199CH, 202CE and 204CH displayed strong activity against both strains of *S. aureus* and *C. neoformans* with MICs value of 4-8 µg/ml. Moreover, their crude extracts inhibited *A. baumannii* AB007, *C. albicans*, *M. gypseum* and *P. marneffei* with MICs value of 16-200 µg/ml. RSPG199 showed interesting NMR profile (Rukachaisirikul *et al*., personal communication). Several secondary metabolites consist of phospholipase A_2 inhibitor folipastatin, antibacterial depside guisnol, 2-chlorounguinol, emeguisins A→C, 3-ethyl-5,7-dihydroxy-3,6-dimethylphthalide, nornidin, antibacterial depsidone nidulin, unguinol, haiderin, rubinin, shirin and narsin were previously described from *A. unguis* (Kamal *et al*., 1970; Turner and Aldridge, 1983; Kawahara *et al*., 1988a; Kawahara *et al*., 1988b; Hamano *et al*., 1992; Nielsen *et al*., 1999).

RSPG95 and 162 had affiliation with *P. citrinum*. They grouped with various *P. citrinum* strains found from gorgonian sea fans, germinated *Aquilaria crassna* seed, agricultural soil, insect, oil palm, wood, orchid *(Cymbidium insigne*) and liquor brewing environment (www.ncbi.nlm.nih.gov). The fungal colonies were gray-green, velvety, moderate growth and cream to pale yellow in reverse on PDA. The microscopic morphology showed 1 or 2-stage branched and green globose conidia. The fungal crude extracts displayed antibacterial (Gram-positive bacteria) and antifungal (*C. neoformans*) activity with MICs value of 4-200 µg/ml. Hydroxyisovaleramide was the major compound reported from RSPG162 (Rukachaisirikul *et al*., personal communication) while the compounds from RSPG95 are being examined. There are many reports on secondary metabolites from *P. citrinum* such as 5 new polyketides including coniochaetones C and D, (3R,4S)-6,8-dihydroxy-1,1-dimethyl-3,4,5-trimethylisochroman, penicillanthranins A and B along with 13 known compounds from sea fan-derived fungus *P. citrinum* (Khamthong *et*

al., 2012). Mazumder, *et al*. (2002) extracted mycotoxin citrinin from *P. citrinum*, which was active against both Gram-positive and Gram-negative bacteria.

RSPG138 could be referred to *P. copticola*. The colony on PDA was white to gray-green in the front, corrugated, moderate growth and white in reverse. The crude extracts of RSPG138 were active against *S. aureus*, *C. albicans* ATCC 90028, both strains of *C. neoformans* and *P. marneffeii* with weak activity (128-200 µg/ml). Sporogen AO-1, petasol, 6-dehydropetasol, 7-hydroxypetasol, JBIR-27, (+) phomenone and new compounds were reported from RSPG138 (Deangrot, personal communication). Sporogen AO-1 represented antifunagal, anti-HIV and cytotoxic activity (Kang *et al*., 2003; Jayasuriya *et al*., 2005; Yurchenko *et al*., 2013). (+) Phomenone showed antimalarial activity with EC_{50} equal to 0.32 µg/ml (Kaur *et al*., 2009).

RSPG99 was identified as *Penicillium* sp. Most of related *Penicillium* species from GenBank were found mainly from Zijin mountain, China (www.ncbi.nlm.nih.gov). The colony on PDA was white, velvety, moderate growth and produced diffusible yellow pigment into the medium. The sequence alignment of RSPG99 presented 37 nucleotide deletions and 20 bases that were different from other taxa in a subclade (Figure 37). Thus, it had a long branch length and did not group with any known species. RSPG99 crude extracts showed mild activity against Gram-positive bacteria and *C. neoformans* ATCC 90113. Questinol, dihydrogeodin, 2-(3-chloro-4-methyl-γ-resorcyloyl)-5-hydroxy-*m*-anisic acid methyl ester, sulochrin, methyl asterrate, asterric acid, asperpentyn, emodin, coniochaetone B, GKK1032B, methyl dichloroasterrate, methyl 8-hydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate and 3 novel compounds were isolated from RSPG99 (Rukachaisirikul *et al*., personal communication).

RSPG82 was referred to *P. sclerotiorum*. It grouped with several *P. sclerotiorum* isolates found from soil and Japanese bamboo (*Polygonum cuspidatum*) (www.ncbi.nlm.nih.gov). The colony on PDA was white, moderate growth and orange in reverse. The broth ethyl acetate extract of RSPG82 displayed interesting NMR profile (Rukachaisirikul *et al*., personal communication) with weak antifungal activity against *C. neoformans* ATCC 90113 and *M. gypseum*. Sclerotiorin was a

highly active compound from *P. sclerotiorum* that acted as an inhibitor of aldose reductase and lipase (Negishi *et al*., 1998; Chidananda *et al*, 2006). The antimicrobial activities of sclerotiorin, isochromophilone VI and pencolide extracted from *P. sclerotiorum* have been reported by Lucas *et al*. (2007). Two new azaphilone derivatives, penicilazaphilones A and B and 1 new isocoumarin, penicilisorin were found from *P. sclerotiorum* (Arunpanichlert *et al*., 2010). The low cellulolytic activity xylanase was produced by *P. sclerotiorum*. This was useful for some process in pulp and paper industry (Knob and Carmona, 2008).

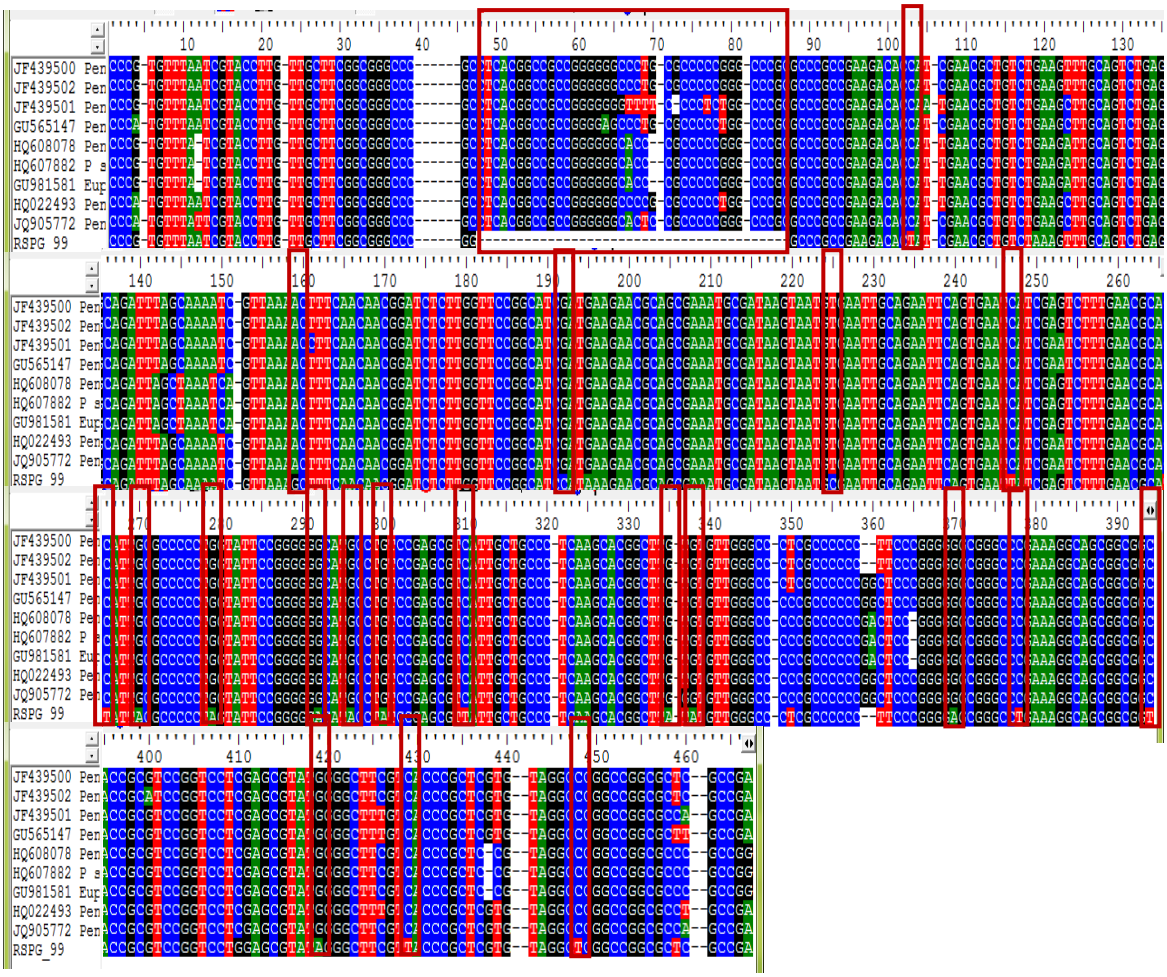

Figure 37 Partial ITS rDNA sequences alignment of RSPG99 and closely related taxa. Distinct nucleotides of RSPG99 compared with other sequences are marked.

RSPG93 had affiliation with *P. herquei*. The closest relatives of RSPG93 were reported from pearl oyster (*Pinctada martensii*) (www.ncbi.nlm.nih.gov). The colony was deep green, velvety and moderate growth. It produced yellow-brown soluble pigment in PDA. The microscopic morphology showed 2-stage branched conidiophores, smooth conidia and spiny stipe. The compounds produced by RSPG93 were investigated by Rukachaisirikul *et al*. (data not shown). RSPG93 crude extracts presented activity against Gram-positive bacteria, *C. neoformans* and filamentous fungi with MICs value of 32-200 µg/ml. According to Omura and co-workers (1979), an alkaloid named herquline has been found from *P. herquei*. Herquline weakly inhibited blood platelet aggregation induced by adenosine diphosphate, however the antimicrobial activity has not been reported. The RSPG93 broth ethyl acetate extract (BE) displayed very good antioxidant activity to superoxide anion with IC_{50} equal to 0.45 mg/ml (Hutadilok-Towattana *et al*., personal communication). Morimoto *et al*. (1987) had pointed out that an antioxidative compound, herqueinone derivative PHR was isolated from *P. herquei* and had synergistic effect on tocopherol.

RSPG161 can be named as *P. pimiteouiense*. The closest relatives of this isolate were reported from Thai medicinal plants and vineyard ecosystem (www.ncbi.nlm.nih.gov). The colony on PDA was velvety, white to green with exudate production. The reverse of colony was white. The microscopic features were short stipe and rough conidia. RSPG161 crude extracts displayed anti-*S. aureus* (2 strains) and anti-yeast activity with MICs value of 32-200 µg/ml. Its NMR profile showed interesting signal (Rukachaisirikul *et al*., personal communication). Peterson *et al*. (1999) isolated *P. pimiteouiense* from polycystic kidney cell cultures but ocharatoxin A, a cause of nephritis was not detected. The antimicrobial activity from *P. pimiteouiense* has not been reported before.

RSPG105 was classified as *P. aculeatum*. The colonies grown on PDA were deep green, white edge, powdery and white with brown in reverse. The microscopic morphology presented 3-stage branched and smooth conidia. The compounds produced by RSPG105 are under investigation. Its crude extracts presented activity against Gram-positive bacteria, *C. albicans* ATCC 90028 and *C.*

neoformans with MICs value of 8-128 µg/ml. Okuda and co-workers (1984) found the antibiotic penitricin from *P. aculeatum*. High amount of dextranase were produced by this fungus (Prabhu, 1984). In addition, dextranase from *P. aculaetum* could be used in co-immobilizates for the direct conversion of sucrose into isomalto-oligosaccharides (Erhardt *et al*., 2008).

Class Sordariomycetes, Order Hypocreales

RSPG24, 27 and 28 were well placed in the genera *Hypocrea/Trichoderma* reported from the GenBank database. Colonies of RSPG24, 27 and 28 were wooly and rapid growth on PDA. The colors of colonies were white from the front and reverse except RSPG24, the reverse was yellow. The colonies became patches green when they produced conidia. The microscopic morphology was septate hyaline hyphae, branched conidiophores, flask-shaped phialides and green conidia. *Trichoderma* species are widely distributed in all types of soil (Reino *et al*., 2008). They were used as enzyme producer and biocontrol agent (Nielsen *et al*., 2005). Several secondary metabolites from *Trichoderma* spp. have been reported comprising anthraquinones, daucanes, simple pyrones, koninginins, trichodermamides, viridians, viridiofungins, nitrogen heterocyclic compounds, azaphilones, trichothecenes, bisorbicillinoids, statins, trichodenones and cyclopentenone derivatives etc. (Reino *et al*., 2008).

RSPG24 was identified as *T. reesei*. Most of related *Hypocrea*/*Trichoderma* species from the database were industrial and standard (ATCC) strains (www.ncbi.nlm.nih.gov). The crude extracts of RSPG24 were active against *S. aureus*, MRSA, *C. albicans* NCPF 3153, *C. neoformans* ATCC 90113 and *M. gypseum* with moderate to weak activity (MIC16-200 µg/ml). The BE extract of RSPG24 had good antioxidant activity against hydroxyl anion with IC_{50} value of 1.22 mg/ml (Hutadilok-Towattana *et al*., personal communication). RSPG24 produced sorbicillin derivatives (3′-hydroxysorbicillin, 2’,3’-dihydrosorbicillin and sorbicillin), phenol derivative (tyrosol) and trichoacorenol (Rungsaiwattana, 2011). Anti-*C. albicans* and anti-*A. fumigatus* activity of 2’,3’-dihydrosorbicillin and sorbicillin have

been reported by Zhao *et al*. (2012). Tyrosol is an antioxidant that can protect cells from oxidative damage (Giovannini *et al*., 1999).

RSPG27 was identified as *T. brevicompactum*. Most of related species from GenBank were found mainly from banana roots (www.ncbi.nlm.nih.gov). Its crude extracts were active against *S. aureus*, both strains of *C. albicans* and both strains of *C. neoformans* with MICs value of 2-8 µg/ml. The growths of MRSA, *M. gymseum* and *P. marneffei* were suppressed by RSPG27 crude extracts with MICs value of 16-200 µg/ml. The main compound from RSPG27 was trichodermin (Rukachaisirikul *et al*., personal communication), which had been reported to inhibit elongation and termination step in protein synthesis (Westerberg *et al*., 1976). Moreover, trichodermin had highly cytotoxic activity against human solid tumor cell lines (Choi *et al*., 1996).

RSPG28 can be referred to *T. harzianum*. The crude extracts from RSPG28 could inhibit both strains of *S. aureus* and *C. neoformans* with MICs value of 2-128 µg/ml. Chromone derivative (5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone), isocoumarin derivative (diaportinol) and epoxy-δ-lactone derivative (nafuredin) were isolated from RSPG28 (Satpradit, 2011). Liu *et al*. (2012) extracted 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone from marine-derived fungus *Hypocrea virens* but its antimicrobial activity was not reported. Nafuredin is a helminth-specific inhibitor of electron-transport enzyme, complex I, which exhibits anthelmintic activity against nematode in sheep and pig (Shiomi *et al*., 2001; Omura and Shiomi, 2007).

RSPG50, 52 and 58 were identified as *P. lilacinus*. Most of related species from GenBank were reported from tropical Pacific Ocean, show cave, metallic surface in a hydro-electric power station and patients (www.ncbi.nlm.nih.gov). The colonies of these three isolates were velvety, initially white and become violet. The reverse was white and brown. The microscopic features were hyaline septate hyphae, taper phialides and form long chain of oval conidia. Their crude extracts showed activities against 10 strains of tested microorganisms, except for *E. coli* and *P. aeruginosa*, with MICs value in range of 4-200 µg/ml. *P.*

lilacinus was frequently isolated from terrestrial and marine environments as well as from nematode eggs (Elbandy *et al*., 2009). It can cause severe mycoses in immunocompromised patients and immunocompetent hosts (Carey *et al*., 2003; Pastor and Guarro, 2006). The clinical strains have been named *Purpureocillium lilacinum* (Luangsa-ard *et al*., 2011) while the environmental strains are identified as *P. lilacinus*. Leucinostatin A, P168, kojic acid, phomaligol A and methylphomaligol A have been reported from *P. lilacinus*. Leucinostatin A showed antitumor and antimicrobial activities against Gram-positive bacteria and fungi (Mori *et al*., 1982). P168 exhibited activity against filamentous fungi, yeasts and bacteria (Isogai *et al*., 1981). Kojic acid, phomalogol A and methylphomaligol A had cytotoxic activity against several human solid tumor cell lines (Elbandy *et al*., 2009).

RSPG88 was identified as *M. roridum* based on ITS rDNA sequence analysis. It grouped with various species reported from GenBank database found from cotton, melon, soybean, water hyacinth, *Salvia* spp. and *Hemionitis arifolia* (www.ncbi.nlm.nih.gov). The colony of RSPG88 was flossy and white to pale yellow. RSPG88 crude extracts showed activity against *S. aureus*, *A. baumannii* and yeasts with the MIC values of 64-200 µg/ml. Isoepiepoformin was the major compound isolated from RSPG88 (Rukachaisirikul *et al*., personal communication). *M. roridum* is a soil fungus found worldwide (Fish *et al*., 2012). It has been reported as an endophyte and plant pathogen (Worapong *et al*., 2009). *M. roridum* is able to produce cellulase and xylanase enzymes in submerged cultures (Okunowo *et al*., 2010).

RSPG37, 63, 227, 229, 230 and 231 were classified as *F. solani*. The closest relatives of our soil fungi were reported from mulberry, lettuce and cilantro roots (www.ncbi.nlm.nih.gov). Colonies of the isolated fungal strains were white, pale orange or pale yellow. Macroconidia had 3-5 septa, slightly curved and stout. Microconidia were large and had 1-2 septa. Their crude extracts displayed active activity against Gram-positive bacteria, yeasts and filamentous fungi with MIC values of 2-200 µg/ml. Fusarpyrones A and B, two new pyrone derivatives, were isolated from the soil fungus *F. solani* PSU-RSPG37 together with eight known compounds, anhydrofusarubin, fusarubin, 5-hydroxy-8-methoxy-2,4-dimethylnaphtha[1,2,b]furan -6,9-dione, 2,3-dihydro-5-hydroxy-8-methoxy-2,4-dimethyl naphtho[1,2,b]furan-6,9-

dione, javanicin, fusalanipyrone, p-hydroxyacetophenone, and tyrosol. Anhydro-fusarubin, fusarubin, 5-hydroxy-8-methoxy-2,4-dimethyl naphtho[1,2,b]furan-6,9-dione and javanicin displayed cytotoxic acivity to KB, MCF-7, NCH-137 and vero cell line (Trisuwan *et al*., 2013).

Naphthoquinone derivatives (javanicin, 2,3-dihydro-5-hydroxy-8-methoxy-2,4-dimethylnaphtho[1,2-b]furan-6,9-dione, and solaniol) and tyrosol were isolated from RSPG229 (Rukachaisirikul *et al*., personal communication). Moreover, RSPG227 also produced naphthoquinone derivative (javanicin) and phenol derivatives (tyrosol, 4-hydroxyphenylacetic acid, monaspilosin and aspergillol B) (Rukachaisirikul *et al*., personal communication). Kornsakulkarn *et al*. (2011) isolated javanicin from an endophytic fungus *Fusarium* sp. that presented anti-*C. albicans* activity with IC_{50} of 6.16 mg/ml. Salaniol was the member of trichothecenes mycotoxin group which was produced by *F. solani* (Ishii *et al*., 1971).

Class Zygomycetes, Order Mucorales

RSPG214 was classified in the order Mucorales, Class Zygomycetes. This fungus was shown to grow rapidly on PDA. The colony was cottony, gray in color and had no pigment production. The microscopic morphology was shown to be non-septate hyphae and sporangiospores produced in sporangium. Its ITS rDNA sequence analysis of RSPG214 revealed that it grouped with several species of *Absidia* but had no affinity with any known species. The alignment of this group showed highly nucleotide variation between taxa (Figure 38) therefore RSPG214 can only be referred to *Absidia* sp. The CH extract of RSPG214 had strong activity against *C. neoformans* ATCC 90113 with MIC value of 4 µg/ml. A genus *Absidia* is usually found as saprobe in soil and decaying organic matters (Ho *et al*., 2004; Antoniadou, 2009). Moreover, it can be found to be human pathogen such as *Absidia corymbifera* that caused zygomycosis in immunocompromised patients (Ribes *et al*., 2000). *Absidia* spp. were applied in microbial biotransformation and enzyme production. Villemain *et al*. (2006) used *Absidia fusca* to degrade two polycyclic aromatic hydrocarbons (PAHs): anthracene and fluoranthene. Chen *et al*. (2012) used *A. corymbifera* to transform 20(*S*)-protopanaxatriol and obtained novel compounds

which showed cytotoxic activity to human prostate tumor cell lines. *A. corymbifera* DY-9 was isolated from soil in Korea and used as extracellular chitin deacetylase producer which may be applied into the industrial production of chitosan and chitosan oligomers (Zhao *et al*., 2010). Furthermore, *Absidia coerulea* was used to produce hydrocortisone (anti-inflammatory drug) in moderate pressure bioconversion system (Jia and Cui, 2009). Although there were many researches of *Absidia* sp. on biotechnological aspects, however their antimicrobial metabolite applications have not been reported.

Morphological identification was identified selected soil fungi into only genus level whereas molecular technique could be identified fungi into genus and species level. RSPG88 was classified as unidentified fungi by morphology because it did not produced any reproductive structures but it can be named as *Myrothecium roridum* via molecular data. In contrast, RSPG157 was not successfully amplified in PCR reaction thus it was identified as *Penicillium* sp. by morphology. Pallen *et al*. (1992) suggested that PCR failure may be result from defective PCR reagents, mistakes in setting up the PCR, or presenting of inhibitory substances in the sample.

In conclusion, the selected strains of soil fungi from this study were identified based on morphology and molecular data into 7 genera, 16 species and 1 section (Table 17). Most of them were usually found from all kinds of environments. Occasionally, they cause diseases to human, plant and animal. Their crude extracts showed antimicrobial activities against several strains of test microorganisms, especially Gram-positive bacteria and yeast *C. neoformans*. Additionally, our interesting soil fungi produced a wide range of secondary metabolites both new and known compounds that may be used in future application.

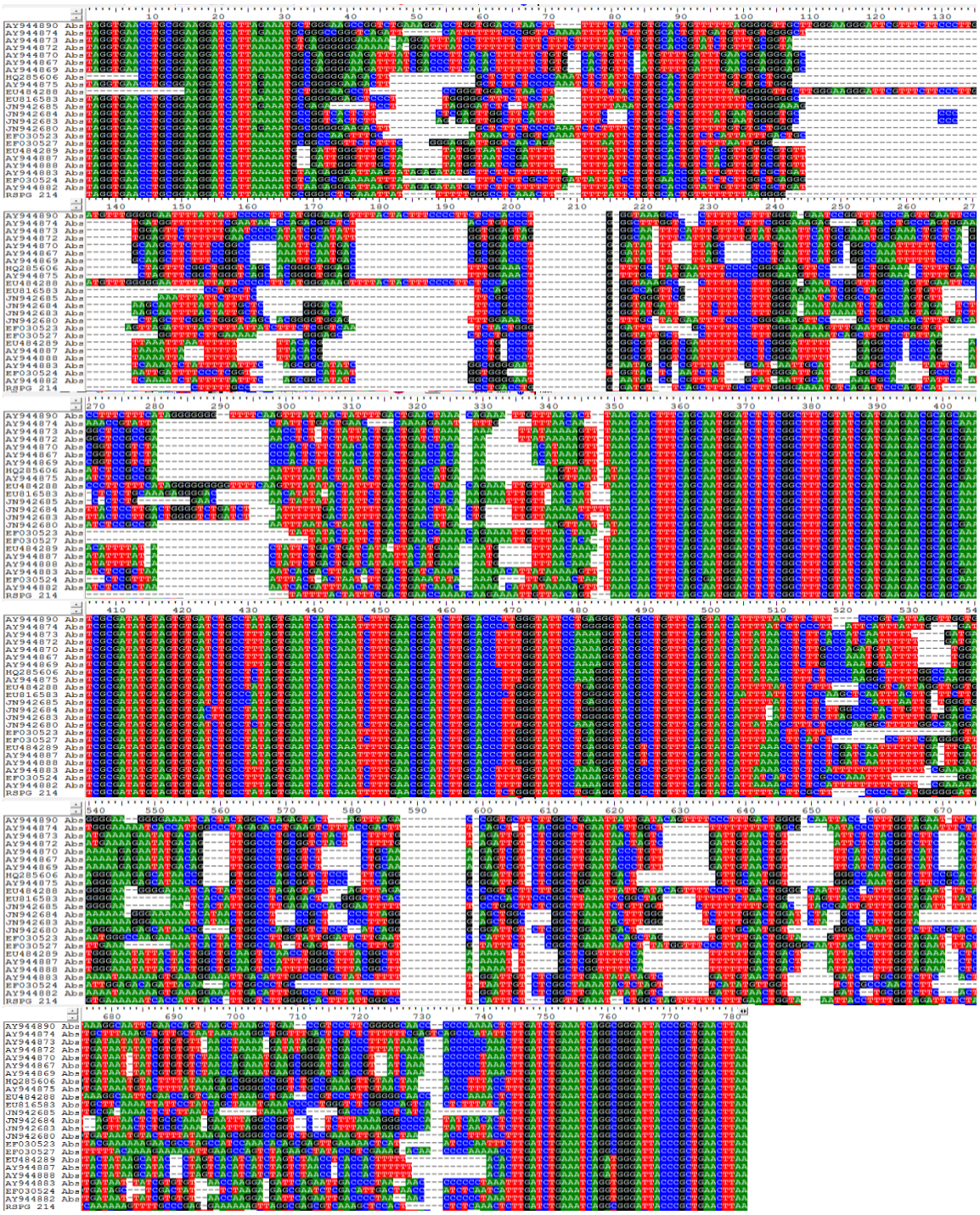

Figure 38 Partial ITS rDNA sequences alignment of RSPG214 and closely related taxa

Table 17 Identification of selected soil fungi by morphological and molecular characteristics

Class/Order	RSPG code	BCC code	Genbank accession number	Identification technique	
				Morphology	Molecular (ITS rDNA sequences)
Eurotiomycetes/ Eurotiales (20 isolates)	82	56875	KC478540	Unidentified	*Penicillium sclerotiorum*
	93	56877	KC478541	*Penicillium* sp.	*Penicillium herquei*
	95	56878	KC478536	*Penicillium* sp.	*Penicillium citrinum*
	99	56879	KC478542	*Penicillium* sp.	*Penicillium* sp.
	105	56846	KC478547	*Penicillium* sp.	*Penicillium aculeatum*
	138	56847	KC478549	*Penicillium* sp.	*Penicillium copticola*
	157	56848	ND	*Penicillium* sp.	ND
	161	56849	KC478543	*Penicillium* sp.	*Penicillium pimiteouiense*
	162	56850	KC478535	*Penicillium* sp.	*Penicillium citrinum*
	178	56851	KC478521	*Aspergillus* sp.	*Aspergillus sclerotiorum*
	179	56852	KC478520	*Aspergillus* sp.	*Aspergillus sclerotiorum*
	180	56853	KC478519	*Aspergillus* sp.	*Aspergillus sclerotiorum*
	185	56854	KC478518	Unidentified	*Aspergillus* sp.
	196	56855	KC478550	*Aspergillus* sp.	*Aspergillus nomius*
	197	56856	KC478526	*Aspergillus* sp.	*Aspergillus* section *Usti*
	198	56857	KC478548	*Aspergillus* sp.	*Aspergillus bombycis*
	199	56858	KC478522	*Aspergillus* sp.	*Aspergillus unguis*
	202	56859	KC478523	*Aspergillus* sp.	*Aspergillus unguis*
	204	56860	KC478524	*Aspergillus* sp.	*Aspergillus unguis*
	206	56861	KC478525	*Aspergillus* sp.	*Aspergillus* section *Usti*
Sordariomycetes/ Hypocreales (13 isolates)	24	56867	KC478546	*Trichoderma* sp.	*Trichoderma reesei*
	27	56868	KC478544	*Trichoderma* sp.	*Trichoderma brevicompactum*
	28	56869	KC478545	*Trichoderma* sp.	*Trichoderma harzianum*
	37	56870	KC478529	*Fusarium* sp.	*Fusarium solani*
	50	56871	KC478537	*Paecilomyces* sp.	*Paecilomyces lilacinus*
	52	56872	KC478539	*Paecilomyces* sp.	*Paecilomyces lilacinus*
	58	56873	KC478538	*Paecilomyces* sp.	*Paecilomyces lilacinus*
	63	56874	KC478530	*Fusarium* sp.	*Fusarium solani*
	88	56876	KC478534	Unidentified	*Myrothecium roridum*
	227	56863	KC478531	*Fusarium* sp.	*Fusarium solani*
	229	56864	KC478532	*Fusarium* sp.	*Fusarium solani*
	230	56865	KC478528	*Fusarium* sp.	*Fusarium solani*
	231	56866	KC478533	*Fusarium* sp.	*Fusarium solani*
Zygomycetes/ Mucorales (1 isolate)	214	56862	KC478527	*Absidia* sp.	*Absidia* sp.

ND = Not done

CHAPTER 5

CONCLUSIONS

A total of 629 isolates of soil fungi were isolated from 100 soil samples collected from Plant Genetic Conservation Project area (RSPG) at Rajjaprabha dam, Suratthani province in January, April, June and August, 2010. The fungal density of soil samples were in the range of $4.2x10^4$-$1.1x10^6$ CFU/g. The highest fungal density was found in August whereas the lowest was in April.

Three hundred and ninety-eight out of 629 isolates (63.26%) were classified into 27 genera by their morphology with *Penicillium* was the most abundant group (21.94%) followed by *Aspergillus* (18.60%), *Trichoderma* (4.77%), *Gongronella* (3.66%), *Fusarium* and *Mucor* (2.07%), *Cunninghamella* (1.91%) and other genera (0.16-1.59%).

Selected soil fungi (181 isolates) were cultivated in PDB for 3 weeks at 25°C and extracted via ethyl acetate or hexane to obtain broth ethyl acetate extracts (BE), cell ethyl acetate extracts (CE) and cell hexane extracts (CH) from each isolate. Fungal crude extracts were evaluated for their antimicrobial activity against 12 human pathogens. The results showed that 311 crude extracts (57.27%) from 145 selected isolates (80.11%) exhibited activity toward at least one test strain. Fungal crude extracts showed the most activity against *S. aureus* ATCC 25923 followed by *C. neoformans* ATCC 90113, MRSA SK-1, *C. neoformans* ATCC 90112, *C. albicans* ATCC 90028, *C. albicans* NCPF 3153, *P. marneffei* clinical isolate, *M. gypseum* SH-MU4, *A. baumannii* AB007, *A. baumannii* AB005, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. The MIC/MBC or MFC values were varied in range of 1-200/2->200 µg/ml.

Strongly active crude extracts including RSPG27CH, 179CH, 204CH and 206CH were further studied on the possible mode of action by SEM. The treated cells showed pronounced morphological changes consistent with cell damage when compared to untreated cells. The results illustrated that these fungal crude extracts cause deformation of the test microorganisms. In RSPG27CH, trichodermin was a major component and was known to act on fungal protein synthesis.

The identification of 34 isolates of interesting soil fungi was confirmed by molecular technique based on ITS1-5.8S-ITS2 rDNA. They could be classified as *Absidia* sp. (RSPG214), *Aspergillus* sp. (RSPG185), *A. bombycis* (RSPG198), *A. sclerotiorum* (RSPG178, 179, 180), *Aspergillus* section *Usti* (RSPG197, 206), *A. unguis* (RSPG199, 202, 204), *F. solani* (RSPG37, 63, 227, 229, 230, 231), *M. roridum* (RSPG88), *Paecilomyces lilacinus* (RSPG50, 52, 58), *Penicillium* sp. (RSPG99), *P. aculeatum* (RSPG105), *P.citrinum* (RSPG95, 162), *P. copticola* (RSPG138), *P. herquei* (RSPG93), *P. pimiteouiense* (RSPG161), *P. sclerotiorum* (RSPG82), *T. brevicompactum* (RSPG27), *T. harzianum* (RSPG28) and *T. reesei* (RSPG24). The results form this study provided that soil fungi isolated from Rajjaprabha dam are a good source for antimicrobial agents.

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Appendix

1. Chemical

1.1 Normal saline solution (0.85% NaCl) 100 ml

NaCl 0.85 g

Distilled water 100 ml

Autoclave at 121°C/15 min, 15 pound/inch^2

1.2 McFarland Standard

No. of McFarland	0.5	1	2	3	4	5	6	7	8	9	10
1% $BaCl_2$	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
1% H_2SO_4	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9.0
Approximate cell density ($\times 10^8$/ml)	1.5	3	6	9	12	15	18	21	24	27	30

1% $BaCl_2$: 1.175% w/v $BaCl_2.2H_2O$ = 0.048M $BaCl_2$; 1% H_2SO_4: 1% w/v H_2SO_4 = 0.18 M H_2SO_4

1.3 Phosphate buffer solution (PBS) pH 7 200 ml

0.2 M KH_2PO_4 50 ml

0.2 M NaOH 29.1 ml

Adjust volume into 200 ml with distilled water and mix thoroughly.

1.4 1.8% Resazurin (stock solution) 20 ml

Resazurin 0.36 g

Distilled water 20 ml

Resazurin solution was sterilized via 0.45 µm millipore filter and kept in the dark at 4°C until used.

1.5 0.5 M NaOH 100 ml

NaOH	2 g
Distilled water	100 ml

1.6 1 M Tris-HCl pH 8 100 ml

Tris-HCl (15.76 g) was dissolved in distilled water, adjusted pH to 8 with NaOH solution and adjusted to final volume with distilled water.

1.7 50X TAE buffer 1000 ml

Tris base	242 g
0.5 M EDTA	100 ml
Glacial acetic acid	57.1 ml

Adjust to final volume with distilled water.

1.8 6X Loading dye 250 ml

80% glycerol	93.6 ml
0.5 M EDTA	3 ml
Bromophenol blue	0.3 g
Xylene cyanol FF	0.3 g

Adjust with distilled water for final volume and mix thoroughly before storing at room temperature.

1.9 CTAB lysis buffer 100 ml

5 M NaCl	28 ml
10% CTAB	20 ml
5 M Tris-HCl	2 ml

0.5 M EDTA	5 ml

Add distilled water to the final volume of 100 ml and autoclave.

1.10 5 M NaCl 100 ml

NaCl	29.22 g
Distilled water	100 ml

Autoclave at 121°C/15 min, 15 pound/inch^2

2. Media

2.1 Potato infusion 1000 ml

Peeled potato	1 kg
Distilled water	1000 ml

Peeled potatoes were cut into small pieces (1 x 1 cm^2) and boiled in distilled water until boiling 10-15 minutes after that boiled potatoes were discarded. Potato infusion was adjusted to 1000 ml with distilled water and stored at -20ºC.

2.2 Potato dextrose agar (PDA) 1000 ml

Potato infusion	200 ml
D-glucose	20 g
Agar	15 g
Distilled water	800 ml

2.3 Potato dextrose broth (PDB) 1000 ml

Potato infusion	200 ml
D-glucose	20 g
Distilled water	800 ml

3. List of ITS primer pairs for successfully PCR amplification of each soil fungal isolate

No.	Code RSPG	Fungal name	Primers	Additive reagent (4M Betaine)
1	24	*Trichoderma reesei*	ITS1F/ITS4	-
2	27	*Trichoderma brevicompactum*	ITS5/ITS4	-
3	28	*Trichoderma harzianum*	ITS5/ITS4	-
4	37	*Fusarium solani*	ITS1F/ITS4	-
5	50	*Paecilomyces lilacinus*	ITS1F/ITS4	-
6	52	*Paecilomyces lilacinus*	ITS1F/ITS4	-
7	58	*Paecilomyces lilacinus*	ITS1/ITS4	-
8	63	*Fusarium solani*	ITS1F/ITS4	-
9	82	*Penicillium sclerotiorum*	ITS5/ITS4	-
10	88	*Myrothecium roridum*	ITS5/ITS4	-
11	93	*Penicillium herquei*	ITS5/ITS4	-
12	95	*Penicillium citrinum*	ITS1/ITS4	-
13	99	*Penicillium* sp.	ITS5/ITS4	-
14	105	*Penicillium aculeatum*	ITS5/ITS4	-
15	138	*Penicillium copticola*	ITS5/ITS4	+
16	161	*Penicillium pimiteouiense*	ITS5/ITS4	-
17	162	*Penicillium citrinum*	ITS5/ITS4	-
18	178	*Aspergillus sclerotiorum*	ITS1F/ITS4	-
19	179	*Aspergillus sclerotiorum*	ITS5/ITS4	-
20	180	*Aspergillus sclerotiorum*	ITS1F/ITS4	-
21	185	*Aspergillus* sp.	ITS5/ITS4	-
22	196	*Aspergillus nomius*	ITS5/ITS4	-
23	198	*Aspergillus bombycis*	ITS5/ITS4	-
24	197	*Aspergillus* section *Usti*	ITS5/ITS4	-
25	199	*Aspergillus unguis*	ITS5/ITS4	-
26	202	*Aspergillus unguis*	ITS5/ITS4	-

No.	Code RSPG	Fungal name	Primers	Additive reagent (4M Betaine)
27	204	*Aspergillus unguis*	ITS5/ITS4	-
28	206	*Aspergillus* section *Usti*	ITS5/ITS4	-
29	214	*Absidia* sp.	ITS1F/ITS4	-
30	227	*Fusarium solani*	ITS5/ITS4	-
31	229	*Fusarium solani*	ITS1/ITS4	-
32	230	*Fusarium solani*	ITS5/ITS4	-
33	231	*Fusarium solani*	ITS5/ITS4	-

*RSPG157 was not successfully amplified in PCR reaction.

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Bachelor of Science (Microbiology)	Prince of Songkla University	2006
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Scholarship Awards during Enrolment

- Centre of Excellence for Innovation in Chemistry (PERCH-CIC)
- Plant Genetic Conservation Project Under The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG)
- Natural Products Research Center of Excellence (NPRC)
- Teacher Assistant (TA)

List of Publication and Proceeding

Borwornwiriyapan, K., Phongpaichit, S., Sakayaroj, J. and Rukachaisirikul, V. 2013. *Penicillium* spp. isolated from soil from Surat Thani province and their antimicrobial activity. The 1st Academic Science and Technology Conference 2013, 18th March 2013, The Ambassador Hotel, Bangkok, Thailand.

Poster presentation

Borwornwiriyapan, K., Phongpaichit, S., Sakayaroj, J. and Rukachaisirikul, V. 2011. Antimicrobial activity of *Trichoderma* spp. isolated from soils from Surat Thani, Thailand. Asian Mycological Congress 2011 & the 12th International

Marine and Freshwater Mycology Symposium, 7-11th August 2011, Convention Center, University of Incheon, Incheon, The Republic of Korea.

Borwornwiriyapan, K., Phongpaichit, S., Sakayaroj, J. and Rukachaisirikul, V. 2011. Antimicrobial activity of *Aspergillus* spp. isolated from soils from Surat Thani, Thailand. 1st International Congress on Natural Products, 17-18th October 2011, Khao Lak Emerald Beach Resort & Spa, Phang Nga, Thailand.

Borwornwiriyapan, K., Phongpaichit, S., Sakayaroj, J. and Rukachaisirikul, V. 2013. Screening of soil fungi from Rajjaprabha dam, Surat Thani province which produced antimicrobial substances. The International Congress for Innovation in Chemistry (PERCH-CIC Congress VIII) "Chemistry for Creative Economy", 5-8th May 2013, Jomtien Plam Beach Hotel & Resort Pattaya, Chonburi, Thailand.

List of Awards

- The best poster presentation in major of Basic Science from the 1st Academic Science and Technology Conference 2013
- Outstanding poster presentation in major of Innovation in Bioactive Natural Products from the International Congress for Innovation in Chemistry (PERCH-CIC Congress VIII)