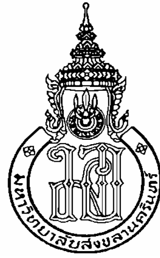


Endophytic Fungi Producing Antimicrobial Substances from Mangrove Plants in the South of Thailand

Jirayu Buatong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

Prince of Songkla University

2010

Copyright of Prince of Songkla University

Thesis Title	Endophytic Fungi Producing Antimicrobial Substances from Mangrove Plants in the South of Thailand
Author	Miss Jirayu Buatong
Major Program	Master of Science in Microbiology

Major Advisor:

……………………………………………
(Assoc. Prof. Dr.Souwalak Phongpaichit)

Co-advisor:

……………………………………………
(Prof. Dr.Vatcharin Rukachaisirikul)

Examining Committee:

………………………………Chairperson
(Dr.Metta Ongsakul)

……………………………………………
(Dr.Jariya Sakayaroj)

……………………………………………
(Assoc. Prof. Dr.Souwalak Phongpaichit)

……………………………………………
(Prof. Dr.Vatcharin Rukachaisirikul)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Microbiology

……………………………………………
(Assoc. Prof. Dr.Krerkchai Tongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์ เชื้อราเอนโดไฟท์ที่ผลิตสารต้านจุลินทรีย์จากพืชป่าชายเลนในภาคใต้ของประเทศไทย

ผู้เขียน นางสาวจิรายุ บัวทอง

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2552

บทคัดย่อ

วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อคัดเลือกเชื้อราเอนโดไฟท์ที่สร้างสารต้านจุลินทรีย์จากพืชป่าชายเลน โดยทำการแยกเชื้อราเอนโดไฟท์จากพืชป่าชายเลน 18 ชนิด ใน 4 จังหวัดทางภาคใต้ของประเทศไทยได้ทั้งหมด 619 isolates อัตราการแยกราเอนโดไฟท์เฉลี่ย คิดเป็น 9.8 isolates/ต้น หรือ 0.49 isolates/ชิ้นตัวอย่าง พบอัตราการแยกสูงสุดในต้นถั่วขาว (*Bruguiera cylindrica*) 2.1 isolates/ต้น หรือ 1.05 isolates/ชิ้นตัวอย่าง สามารถแยกเชื้อราเอนโดไฟท์ได้จากส่วนกิ่งมากที่สุด (39%) สุ่มเลือกเชื้อราเอนโดไฟท์ที่มีลักษณะโคโลนีแตกต่างกัน 150 isolates เพาะเลี้ยงในอาหาร potato dextrose broth (PDB) แล้วนำไปสกัดด้วยวิธีทางเคมี นำสารสกัดทั้งหมด 385 สารสกัดมาทดสอบฤทธิ์ต้านจุลินทรีย์ก่อโรค 7 ชนิด ได้แก่ *Staphylococcus aureus* ATCC25923, Methicillin-resistant *Staphylococcus aureus* SK1 (MRSA-SK1), *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028, *Cryptococcus neoformans* ATCC90112 และ *Microsporum gypseum* MU-SH4 ด้วยวิธี colorimetric microdilution และ หาค่า minimal

inhibitory concentration (MIC) และ minimal bactericidal concentration (MBC) หรือ minimal fungicidal concentration (MFC) พบว่ามีสารสกัดที่แสดงฤทธิ์ต้านจุลินทรีย์ทั้งหมด 181 สารสกัด (47%) จากเชื้อราเอนโดไฟท์ 92 isolates (61.33%) โดยสารสกัดส่วนใหญ่แสดงฤทธิ์ต้านเชื้อ MRSA-SK1 (31.95%) และ *S. aureus* ATCC25923 (28.31%) ให้ค่า MIC/MBC อยู่ในช่วง 4-200/64-200 µg/ml และ 8-200/64-200 µg/ml ตามลำดับ และมีสารสกัดเพียง 0.52% ที่แสดงฤทธิ์ต้านเชื้อ *P. aeruginosa* ATCC27853 (MIC 200 µg/ml และ MBC > 200 µg/ml) สำหรับฤทธิ์ต้านเชื้อราพบว่าสารสกัด 25.45% และ 11.69% สามารถต้านเชื้อ *M. gypseum* MU-SH4 (MIC 4-200 µg/ml และ MFC 8-200 µg/ml) และ *C. neoformans* ATCC90112 (MIC 8-200 µg/ml และ MFC 8-200 µg/ml) และ 7.53% แสดงฤทธิ์ต้านเชื้อ *C. albicans* ATCC90028 (MIC 32-200 µg/ml และ MFC 32-200 µg/ml) ในการศึกษาครั้งนี้ไม่พบสารสกัดที่สามารถต้านเชื้อ *E. coli* ATCC25922 โดยราเอนโดไฟท์ MA34 สร้างสารต้านเชื้อ *S. aureus* ATCC25923 และ MRSA-SK1 ได้ดีที่สุด สารสกัดจากราเอนโดไฟท์ MA96 ต้านเชื้อ *M. gypseum* MU-SH4 ได้ดีที่สุด ราเอนโดไฟท์ MA12 และ MA194 สามารถต้านเชื้อ *C. neoformans* ATCC90112 ได้ดีที่สุด ราเอนโดไฟท์ MA99 และ MA194 สามารถต้านเชื้อ *C. albicans* ATCC90028 ได้ดีที่สุด มีราเอนโดไฟท์เพียง 2 isolates คือ MA82 และ MA125 ที่สามารถต้านเชื้อ *P. aeruginosa* ATCC27853 เมื่อนำราเอนโดไฟท์ 21 isolates ที่สร้างสารต้านจุลินทรีย์ และ/หรือมี NMR profile ที่น่าสนใจ ไปจัดจำแนกโดยอาศัยลักษณะทางสัณฐานวิทยาและวิธีทางชีวโมเลกุลโดยวิเคราะห์ลำดับเบส ribosomal DNA-Internal Transcribed Spacers (ITS1-5.8S-ITS2, ITS) และ large subunit (LSU) สามารถจัดจำแนกเชื้อราเอนโดไฟท์อยู่ใน order ต่างๆ 5 orders ได้แก่ Capnodiales, Diaporthales, Hypocreales, Pleosporales และ Xylariales และ พบว่า MA12 และ MA99 จัดเป็น Mycosphaerellaceae species และ *Pestalotiopsis* sp. ตามลำดับ ส่วน MA96 MA125 และ MA194 จัดเป็น

Phomopsis spp. สำหรับ MA34 จัดจำแนกได้ในระดับ order คือ Xylariales สำหรับ MA82 ยังไม่สามารถจำแนกชนิดได้ ผลการศึกษาครั้งนี้แสดงให้เห็นว่าราเอนโดไฟท์จากพืชป่าชายเลนมีความหลากหลายและเป็นแหล่งของผลิตภัณฑ์ธรรมชาติที่มีฤทธิ์ต้านจุลินทรีย์

Thesis Title Endophytic Fungi Producing Antimicrobial Substances from Mangrove Plants in the South of Thailand
Author Miss Jirayu Buatong
Major Program Microbiology
Academic Year 2009

ABSTRACT

The main aim of this work was to investigate endophytic fungi from mangrove plants that can produce antimicrobial substances. A total of 619 endophytic fungi were isolated from 18 mangrove plants obtained from 4 provinces, in southern Thailand. The average rate of endophytic fungal isolation was 9.8 isolates per plant or 0.49 isolates per sample disc. The highest isolation rate was obtained from *Bruguiera cylindrica* (21 isolates/plant or 1.05 isolates/disc) and the majority of isolates investigated were from branches (39%). One hundred and fifty isolates of endophytic fungi were selected based on their different colonial morphologies and were grown in potato dextrose broth (PDB), harvested and extracted with chemical solvents. Three hundred and eighty-five crude extracts were obtained and tested against seven pathogenic microorganisms that included *Staphylococcus aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* SK1 (MRSA-SK1), *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028, *Cryptococcus neoformans* ATCC90112 and *Microsporum gypseum* MU-SH4. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC) were determined by using a colorimetric microdilution method. In total, 181 extracts (47%) from 92 endophytic fungal isolates (61.33%) were inhibitory. Most of these extracts were active against MRSA-SK1 (31.95%) and *S. aureus* ATCC25923 (28.31%) with MICs/MBCs that ranged from 4-200/64-200 μg/ml and 8-200/64-200 μg/ml, respectively. Only 0.52 % of the extracts inhibited *P. aeruginosa* ATCC27853 (MIC 200 μg/ml, MBC>200 μg/ml). For the antifungal activity 25.45% and 11.69% inhibited *M. gypseum* MU-SH4 and *C. neoformans* ATCC90112 with MICs/MFCs

that ranged from 4-200/8-200 μg/ml and 8-200/8-200 μg/ml, respectively, while 7.53% were active against *C. albicans* ATCC90028 with MICs/MBCs values of 32-200/32-200 μg/ml. None of the extracts inhibited *E. coli* ATCC25922. The endophytic fungus MA34 displayed the strongest antibacterial activity against *S. aureus* ATCC25923 and MRSA-SK1. The endophytic fungus MA96 displayed the strongest antifungal activity against *M. gypseum* MU-SH4. The endophytic fungi MA12 and MA194 displayed the strongest antifungal activity against *C. neoformans* ATCC90112. The endophytic fungi MA99 and MA194 displayed the strongest antifungal activity against *C. albicans* ATCC90028. Only the endophytic fungi MA82 and MA125 displayed antibacterial activity against *P. aeruginosa* ATCC27853. Fungal identification was based initially on morphological characters and then by using the ribosomal DNA-Internal Transcribed Spacers (ITS1-5.8S-ITS2, ITS) and large subunit (LSU) analyses. This revealed that 21 isolates with antimicrobial activity and/or having extracts with interesting NMR profiles belonged to five orders; Capnodiales, Diaporthales, Hypocreales, Pleosporales and Xylarialas. Isolates MA12 and MA99 were identified as Mycosphaerellaceae species and *Pestalotiopsis* sp., respectively while MA96, MA125 and MA194 belonged to the *Phomopsis* spp. MA34 was identified as belonging to the order of Xylariales and MA82 was an unidentified endophytic fungus. Results from this study indicated that endophytic fungi from mangrove plants are diverse and could be a good source of antimicrobial natural products.

CONTENTS

		Page
บทคัดย่อ		iii
ABSTRACT		vi
ACKNOWLEDGEMENTS		viii
CONTENTS		ix
LIST OF TABLES		xi
LIST OF FIGURES		xii
LIST OF ABBREVIATIONS AND SYMBOLS		xvi
CHAPTER1	INTRODUCTION	
	1.1 Background and rationale	1
	1.2 Review of the literature	3
	1.3 Molecular characterization and phylogeny of fungi	15
	1.4 Objectives	22
CHAPTER2	MATERIALS AND METHODS	
	2.1 Materials	23
	2.2 Methods	28
CHAPTER 3	RESULTS	
	3.1 Isolation of endophytic fungi from mangrove plants	40
	3.2 Screening of fungal crude extracts for antimicrobial activity	42
	3.3 Determination of MIC and MBC or MFC	46
	3.4 Identification of endophytic fungi	54
CHAPTER 4	DISCUSSION	
	4.1 Endophytic fungal isolation from mangrove plants	88
	4.2 Antimicrobial assay	89
	4.3 Identification of active endophytic fungi	92

CONTENTS (CONT.)

	Page
CHAPTER 5 CONCLUSIONS	
5.1 Conclusions	102
5.2 Suggestion for future work	103
REFERENCES	104
APPENDIX 1	120
APPENDIX 2	123
VITAE	144

LIST OF TABLES

Table		Page
1	Distribution of mangrove forest area in Thailand	6
2	Chemical constituents and activities of some mangrove plants	8
3	Endophytic fungal secondary metabolites with anticancer, antibacterial and antifungal activities	11
4	Mangrove plant species collected for endophytic fungal isolation and origins	23
5	Primers used for the polymerase chain reaction (PCR) and DNA sequencing	37
6	Endophytic fungi isolated from various mangrove host plants	41
7	Selected endophytic fungi from various mangrove species and their antimicrobial activity against pathogenic microorganisms	44
8	Type of crude extracts of endophytic fungi having antimicrobial activity	45
9	Antimicrobial activity of crude extracts from endophytic fungi	49
10	MICs, MBCs or MFCs of selected active crude extracts from endophytic fungi	50
11	MIC, MBC or MFC values of active crude extracts against pathogenic microorganisms	53
12	Antimicrobial (AM), interesting NMR profile and morphological identification of 21 active isolates	56
13	Summary of the molecular identification of active endophytic fungi using ribosomal DNA sequence analyses	65
14	Mixture of McFarland Standard	124
15	MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics	125

LIST OF FIGURES

Figure		Page
1	Different species of true mangrove plants in mangrove forest	4
2	Novel compounds from endophytic fungi	13
3	The ribosomal RNA gene region of eukaryotic cell	18
4	Plant segments	28
5	Endophytic fungal isolation on CMA with antibiotics	29
6	Extraction of chemicals produced by endophytic fungi	31
7	ITS, LSU region and universal primers	36
8	Percentage of endophytic fungi isolated from each mangrove plant part	40
9	Primary assay against *C. neoformans* by the colorimetric microdilution method at 200 µg/ml	42
10	Percentage of types of active crude extracts from endophytic fungi tested at 200 µg/ml	45
11	Determination of MIC of crude extracts by colorimetric microdilution method	46
12	Antimicrobial activity of crude extracts against various microbes tested at 200 µg/ml	47
13	Number of test strains inhibited by crude extracts	49
14	Endophytic fungi 21 isolates produced strong antimicrobial activity and/or interesting NMR profile	59
15	Colonies and conidia of nine active endophytic fungi and their identification	61
16	Stromata production on PDA media of MA34 and MA132	64

LIST OF FIGURES (CONT.)

Figure		Page
17	Phylogram obtained from LSU rDNA sequence analysis of endophytic fungus MA12 and fungi from the Capnodiales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	69
18	Phylogram obtained from ITS rDNA sequence analysis of endophytic fungus MA12 and fungi from the Capnodiales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	70
19	The phylogram obtained from LSU rDNA sequence analysis of the endophytic fungi MA71, MA164 and fungi from the Pleosporales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes	73
20	The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungi MA71, MA164 and fungi from the Pleosporales, generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes	74

LIST OF FIGURES (CONT.)

Figure		Page
21	Phylogram obtained from LSU rDNA sequence analysis of endophytic fungi MA60, MA81, MA96, MA125, MA194 and fungi from the Diaportales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	77
22	Phylogram obtained from ITS rDNA sequence analysis of endophytic fungi MA2, MA60, MA81, MA96, MA194 and fungi from the Diaportales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	78
23	Phylogram obtained from LSU rDNA sequence analysis of endophytic fungus MA70 and fungi from the Hypocreales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	80
24	Phylogram obtained from ITS rDNA sequence analysis of endophytic fungus MA70 and fungi from the Hypocreales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes	81

LIST OF FIGURES (CONT.)

Figure		Page
25	The phylogram obtained from LSU rDNA sequence analysis of the endophytic fungi MA92, MA156, MA165, MA177 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes	85
26	The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungi MA21, MA92, MA165, MA177 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes	86
27	The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungus MA156 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes	87
28	Mangrove plants were collected for endophytic fungi isolation	120

LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree Celsius
g	=	Gram
mg	=	Milligram
µ	=	Micro
µg	=	Microgram
µl	=	Microliter
ml	=	Milliliter
NMR	=	Nuclear Magnetic Resonance
DMSO	=	Dimethyl sulfoxide
EtOAc	=	Ethyl acetate
Na_2SO_4	=	Sodium sulfate
MeOH	=	Methanol
ha	=	Hectare

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Endophytic fungi colonize healthy plant tissues without any symptoms of disease and have been found in all plant species investigated. Endophyte-infected plants can also gain protection from herbivores and pathogens due to the bioactive secondary metabolites that endophytes generate in their host plant tissues. Isolated endophytic fungi are known to produce a wide range of new or novel bioactive compounds and biologically active secondary metabolites. Novel antibiotics, immunosuppressants and anticancer compounds are only a few examples of the compounds detected after isolation, cultivation, purification and characterization of some endophytes (Sette *et al*., 2006).

Mangroves provide a unique and dominant ecosystem comprised of intertidal marine plants, mostly trees, predominantly bordering margins of tropical coastlines around the world. These halophytic (salt tolerant) plants thrive in intertidal areas that receive daily inundation between mean sea levels and the highest astronomical tides. They provide vital habitats and food for similarly adapted resident and transient fauna (Tomlinson, 1986; Duke *et al*., 1998). Some mangrove species or parts of mangroves have been used for medicinal purpose; such as the bark and seed of *Xylocarpus granatum* used for the treatment of diarrhea, amoebiasis, cholera and infected wounds; leaves of *Bruguiera* spp. have been used for pressure; the bark of *Rhizophora apiculata* was used to cure diarrhea, nausea, vomiting and amoebiasis (Bamroongrugsa, 1999). When a mangrove plant is stressed from environmental changes or being attacked by animals, insects and microbes, some endophytic fungi may be induced to produce bioactive metabolites that protect the host plant. Natural products from mangrove fungal endophytes having biological activity have been reported. Paecilin A and B produced by *Paecilomyces* sp. isolated from *Bruguiera*

gymnorrhiza had high anticancer activity (Chen *et al*., 2007). Cytosporone B produced by *Dothiorella* sp. from *Avicennia marina* had antifungal activity (Xu *et al*., 2005). Therefore, some mangrove fungal endophytes can be a good source of bioactive and novel substances.

1.2 Review of the literature

1.2.1 Mangrove forest

Mangrove forests form unique ecosystems in the intertidal regions along sheltered coasts where they grow abundantly in saline soil and brackish water subject to periodic fresh- and salt-water inundation. Tropical/subtropical mangrove plants are evergreen plants belonging to several families that have developed various specialized morphological and physiological traits to withstand variations in temperature and salinity (Pang, 2008).

1.2.2 The origin of mangroves

Scientists theorize that the earliest mangrove species originated in the Indo-Malayan region. This may account for the fact that there are far more mangrove species present in this region than anywhere else. Because of their unique floating propagules and seeds, certain of these early mangrove species spread westward, borne by ocean currents, to India and East Africa, and eastward to the Americas, arriving in Central and South America during the upper Cretaceous period and lower Miocene epoch, between 66 and 23 million years ago. During that time, mangroves spread throughout the Caribbean Sea across an open seaway which once existed where Panama lies today. Later, sea currents may have carried mangrove seeds to the western coast of Africa and as far south as New Zealand. This might explain why the mangroves of West Africa and the Americas contain fewer, but similar colonizing species, whereas those of Asia, India, and East Africa contain a much fuller range of mangrove species (http://www.wrm.org.uy/deforestation/mangroves/forest.html). True mangroves are mainly restricted to intertidal areas between the high water levels of neap and spring tides (Figure 1). True mangrove species belong to at least 20 different families. About 80 species of true mangrove tree/shrubs are recognized, of which 50-60 species make a significant contribution to the structure of mangrove

forests (Bandaranayake, 2002). The most common tree species are *Rhizophora*, *Avicennia*, *Bruguiera*, *Sonneratia* and *Xylocarpus*.

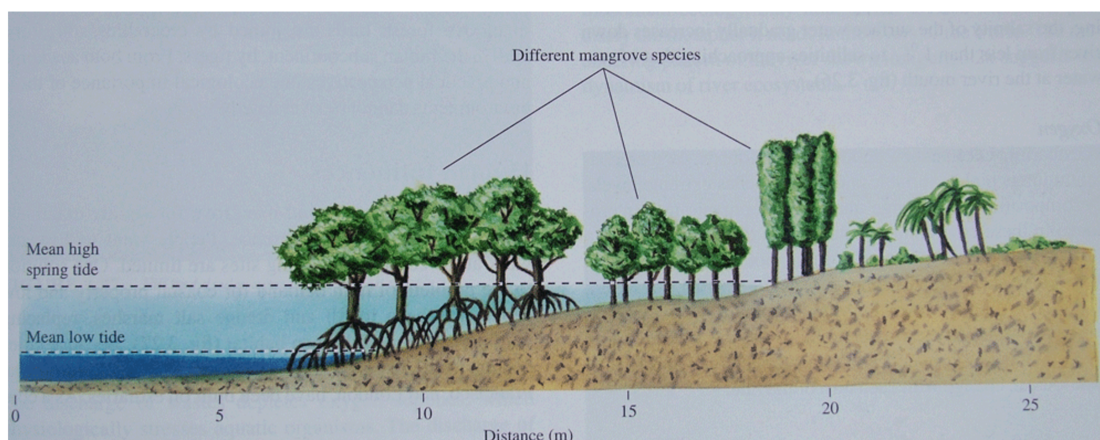


Figure 1 Different species of true mangrove plants in mangrove forest
Source: http://cyberlab.lh1.ku.ac.th/elearn/faculty/fisher/fi16/page_3.htm

1.2.3 Mangroves in Thailand

Mangrove forests are located in 23 coastal provinces in Thailand. In 1961 these forests covered 368,000 ha in Thailand, but the area has dropped to 240,000 ha by 2002. The major causes of the loss of mangrove forests are the timber and charcoal industries, while some areas were converted for urbanization, agriculture, and aquaculture especially shrimp farms and coastal resorts. There are 74 species of mangrove trees in Thailand and major areas are in the south of the country in Phang Nga province (Table 1). The major mangrove plants are in the family Rhizophoraceae; *Rhizophora* sp., *Ceriops* sp., *Bruguiera* sp., the family Sonneratiaceae; *Sonneratia* sp., the family Verbenaceae; *Avicennia* sp. and Meliaceae; *Xylocarpus* sp. Less than 1,500 square kilometres of mangrove forests are left in Thailand, most of which lie along the west peninsular coast from Ranong to Satun. Only 99 square kilometres of this vital habitat are currently protected, accounting for approximately 3% of the original area. The Andaman coast is the area in Thailand with the highest number of mangroves, however the mangrove trees here show little

of their former grandeur. Mangrove forests may seem dense and impenetrable now, but an undisturbed forest would be further blessed with dozens of epiphytes in the upper branches. The coastal people of Thailand have long cherished the mangroves as a source of foods, medicine and fuel. Medicines include treatments for lumbago, skin diseases, venereal diseases (VD) and impotency (http://www.geocities.com/~nesst/mangrove.htm).

Table 1 Distribution of mangrove forest area in Thailand

Province/Region	Province Area	Mangrove area in 2004 (sq. km.)	%
Bangkok	1565.22	3.09	0.20
Prachuap Khiri Khan	6367.62	2.54	0.04
Phetchaburi	6225.14	38.45	0.62
Samut Prakan	1004.09	6.35	0.63
Samut Songkhram	416.71	12.76	3.06
Samut Sakhon	872.35	33.32	3.82
Chanthaburi	6338.00	89.77	1.42
Cha Choeng Sao	5351.00	12.85	0.24
Chon Buri	4363.00	4.45	0.10
Trat	2819.00	103.70	3.68
Rayong	3552.00	16.72	0.47
Krabi	4708.51	350.14	7.44
Chumphon	6009.01	71.18	1.18
Trang	4917.52	330.36	6.72
Nakhon Si Thammarat	9942.50	280.12	2.82
Narathiwat	4475.43	99.95	2.23
Pattani	1940.36	37.07	1.91
Phang Nga	**4170.90**	**417.11**	**10.00**
Phatthalung	3424.47	97.21	2.84
Phuket	543.03	22.80	4.20
Ranong	3298.05	250.92	7.61
Song Khla	7393.89	46.47	0.63
Satun	2478.98	347.21	14.01
Surat Thani	12891.47	83.51	0.65
Whole Kingdom	**513,155.02**	**2,758.05**	**0.54**

Source: http://www.forest.go.th/stat/stat50/TAB6.htm

1.2.4 Bioactive compounds from mangrove plant

The mangrove forest is considered to be a dynamic ecotone (or transition zone) between terrestrial and marine habitats (Gopal and Chauhan, 2006). Mangroves have been a source of many biologically active compounds. Some mangrove plants have been used in herbal medicine (Bamroongrugsa, 1999), and extracts from mangroves have been reported to have antimicrobial activities against human, animal and plant pathogens (Table 2). Some of the activities of mangrove plants may be due to mutualistic fungal endophytes associated with the host plants (Selosse *et al*., 2004; Suryanarayanan *et al*., 1998).

Table 2 Chemical constituents and activities of some mangrove plants

Mangrove plant	**Tested for**	**Chemical constituents**
Avicennia offcinalis	Antiviral activity, biotoxicity on fingerlings of fish	Alkaloids, arsenic, carbohydrates, carotenoids, chlorophyll a, b, a+b, flavonoids, glycosides, lipids, proteins, polyphenols, saponins, tannins, triterpenoids
Bruguiera cylindrica	Antiviral and larvaicidal activity, biotoxicity on tobacco mosaic virus and fingerlings of fish	Minerals (Ca, Na, Mg, K), sulphur containing alkaloids, tannins
Rhizophora apiculata	Antiviral, larvicidal, antifungal, antifeedant, antimicrobial activity, antiviral properties against human immunodeficiency virus	Aliphatic alcohols aldehydes, and carboxylic acids, carotenoids, condensed and hydrolysable tannins, benzoquinones, lipids, n-alkanes, minerals, phenolic compounds, polysaccharides, steroids, triterpenes
Rhizophora mucronata	Antiviral, anti-HIV activity, growth hormone tests on plants, biotoxicity on figerlings of fish	Alkaloids, anthocyanidins, carbohydrates, carotenoids, chlorophyll a, b, a+b, condensed and hydrolysable tannins, gibberellins, flavonoids, insitols, lipids, minerals, polyphenols, procyanidins, priteins, saponins, steroid, triterpenes
Xylocarpus granatum	Antifungal activity, insect antifeedant activity	Alkaloids, amino acids; carbohydrates; carotenoids, chlorophyll a, b, a+b, fatty acids, flavonoids, hydrocarbons; limonoids, minerals, organic acids; polyphenols, protein, tannins, triterpenes, saponins,

Source: Adapted from Bandaranayake (2002)

1.2.5 Endophytic fungi

Endophytic fungi live in plant tissues, for all or part of their life cycles without causing any immediate negative effects (Hirsch and Braun, 1992). The plant is thought to provide nutrients to the microbe, while the microbe may produce factors that protect the host plant from attack by animals, insects or microbes (Yang *et al*., 1994). Endophytic fungi are found in almost all plants (Huang *et al*., 2001) and in a wide variety of plant tissue types, such as twigs, bark, leaf, and branches. (Tejesvi *et al*., 2005; Phongpaichit *et al*., 2006; Verma *et al*., 2007). Many endophytic fungi produce biologically active secondary metabolites in culture (Phongpaichit *et al*., 2006; 2007; Liu *et al*., 2008; Gong and Guo, 2009). They are a rich source of novel organic compounds such as penicillone, pyrenocine A and pyrenocine B from the endophytic fungus *Penicillium paxilli* PSU-A71 (Rukachaisirikul *et al*., 2007); 7-amino-4-metylcoumarin from the endophytic fungus *Xylaria* sp. YX-28 (Liu *et al*., 2008). They represent a relatively unexplored ecological source, and their secondary metabolism is particularly active because of their metabolic interactions with their hosts.

1.2.6 Endophytic fungi and host plant interaction

Endophytic fungi colonize the living, internal tissue of plants without causing any immediate, overt negative effect (Hirsch and Braun, 1992). The relationship of the endophyte to its host plant may have begun to evolve from the time that higher plants first appeared on the earth, hundreds of millions of years ago (Strobel, 2003). Some of them help to promote plant growth such as *Piriformospora indica* that lives in the root of barley, reprograms its host plant to salt-stress tolerance, disease resistance, and higher yield (Waller *et al*., 2005). Some endophytes produce toxic substances that can protect host plants from plant pathogens, insects, nematodes, and herbivores (Schardl and Phillips, 1997; Schwarz *et al*., 2004). In 2004, Germaine and coworkers found that endophytes have adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into

their own genomes. Fungal endophytes can modify plants at the genetic, physiological, and ecological levels (Weishampel and Bedford, 2006).

1.2.7 Natural products from endophytic fungi

Natural products still remain the most important source for discovering new and potential drug molecules. Large numbers of plants, microbial and marine sources have been tested for production of bioactive compounds. Since the discovery of taxol-producing endophytic fungi (Stierle *et al*., 1993; Strobel *et al*., 1996), the search for novel secondary metabolites has focused on endophytic fungi. Some of the compounds produced by endophytic fungi could be candidates for the discovery of new drugs.

1.2.7.1 Bioactive compounds

The number of people in the world with health problems caused by drug-resistant microorganisms and cancer are increasing at an alarming rate. Scientists continue to search for new sources of drugs and endophytic fungi are an interesting source. Natural products previously isolated from fungal endophytes have a broad spectrum of biological activity (Zhang *et al*., 2006). In addition, a significant number of interesting molecules are produced by endophytes, including alkaloids, steroids, terpenoids, isocoumarins, quinones, flavonoids, phenylpropanoids, lignans, peptides, phenolics, aliphatics, and volatile organic compounds, and many of them are biologically active (Tan and Zou, 2001; Gunatilaka, 2006; Zhang *et al*., 2006). Some reported bioactive secondary metabolites from endophytic fungi are shown in Table 3.

Table 3 Endophytic fungal secondary metabolites with anticancer, antibacterial and antifungal activities

Endophytic fungus	Host plant	Secondary metabolite	Biological activity (MIC or IC_{50})	Reference
Aspergillus sp.	*Cynodon dactylon*	Helvolic acid, monomethylsolochrin, ergosterol, 3β-hydroxy-5α,8α-epidioxy-ergosta-6,22-diene	*Helicobacter pylori* (MIC 8, 10, 20, 30µg/ml, respectively)	Li *et al*. (2005)
Colletotrichum gloeosporioides	*Justica gendarussa*	Taxol	Cytotoxic (IC_{50} 0.005-0.05 µM)	Gangadevi and Multhumary (2008)
Diaporthe sp. BCC6140	unidentified wood	Diaporthein B	Antimycobacterial (MIC 3.1 µg/ml)	Dettrakul *et al*. (2003)
Hypocreales sp.	*Knema laurina* (Blume) warb	Brefeldin A, 8-deoxy-trichothecin, trichothecolone, 7-hydroxytrichodermol, 7-hydroxyscirpene	Human epidermoid carcinoma of the mouth (KB), human breast cancer cells (BC-1) and human small cell lung cancer cells (NCI-H187). (IC_{50} 0.04-22.76 µM)	Chinworrungsee *et al*. (2008)
Penicillium paxilli PSU-A71	*Garcinia atroviridis*	Pyrenocin B	*Microsporum gypseum* MU-SH4-4 (MIC 32 µg/ml)	Rukachaisirikul *et al*. (2007)
Xylaria sp.	*Garcinia dulcis*	Sordaricin	*Candida albicans* ATCC90028 (MIC 32µg/ml)	Pongcharoen *et al*. (2008)
Phomopsis sp. usia5	*Urobotrya siamensis* (Opiliaceae)	3-Nitropropionic acid	Antimycobacterial (MIC 3.3 µM)	Chomcheon *et al*. (2005)
Unidentified endophytic fungus	*Castaniopsis fissa*	Ergosta-8(9),22-diene-3,5,6,7tetraol-(3β,5α,6β,7α,22*E*)	Bel-7402, NCI4460 and L-02 cell lines (IC_{50} 8.445, 5.03, 13.621 µg/mL, respectively)	Li *et al*. (2004)

1.2.7.2 Enzymes

Fungi are important source of enzymes that have many industrial uses, such as in paper manufacturing, animal feeds, bread making, juice and wine industries. Endophytic fungi usually produce the enzymes necessary for the colonization of plant tissues. Choi *et al*. (2005) reported that 21 endophytic fungal isolates from *Brucea javanica* produced amylase, cellulase and ligninase. The enzymes found indicated that most endophytes are degraders of the simple sugars and structural polysaccharides available in recently dead leaves and possibly wood. Jordaan and team (2006) isolated endophytic fungi from *Colophospermum mopane* and tested for their ability to produce amylase. They found that 6 isolates of *Alternaria* sp., 2 isolates of *Phoma* sp. and 3 isolates of *Phomopsis* sp. produced amylase. Furthermore, wood-inhabiting fungal endophytes of the Chilean tree *Drimys winteri* and *Prumnopitys andina* were isolated and produced lignocellulolytic enzymes. Endophytic fungi of the *Bjerkandera* sp. and mycelia sterilia displayed positive hydrolytic reactions to cellulose (Oses *et al*., 2006).

1.2.7.3. Biopesticides

Agricultural pests cause significant economic losses to a wide variety of crops. Chemical control is a widely used option for management of plant-parasitic nematodes. However, chemical pesticides are costly to produce and can have negative effects on the environment. Biopesticides are therefore of interest and can offer a clean environment. Webber (1981) was probably the first research worker to report an example of plant protection provided by an endophytic fungus, in which the endophyte *Phomopsis oblonga* protected elm trees against the beetle *Physocenemum brevilineum*. Endophytic fungi such as *Fusarium oxysporum* protect tomatoes from infections by *Meloidogyne incognata* (Hallman and Sikora, 1995), banana endophytic fungi from Central America could control the burrowing nematode *Rhadopholus similis* (Pocasangre *et al*., 2001), and *Colletotrichum gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis* from *Theobroma cacao* could protect *T. cacao* pod loss due to *Moniliophthora rosei* and *Phytophthora* spp. (Mejia *et al*., 2008).

1.2.7.4. Novel compounds

There has been considerable interest in screening endophytic fungi for novel compounds (Kumar and Hyde, 2004; Xia *et al.*, 2008). Schulz *et al.* (2002) isolated around 6,500 endophytic fungi and tested their biological potential. They analyzed 135 secondary metabolites and found that 51% of the bioactive compounds isolated from endophytic fungi were new natural products compared to 38% from soil fungi. This indicated that endophytic fungi are a good source of novel compounds. Two novel compounds xyloketol H and marinamide (Figure 2a-b) were isolated from *Xylaria* sp.2508 (Liu *et al.*, 2006). An endophytic fungus *Aspergillus fumigatus* from *Juniperus communis* produced the novel podophyllotoxin and deoxypodophyllotoxin (Figure 2c-d) (Kusari *et al.*, 2009).

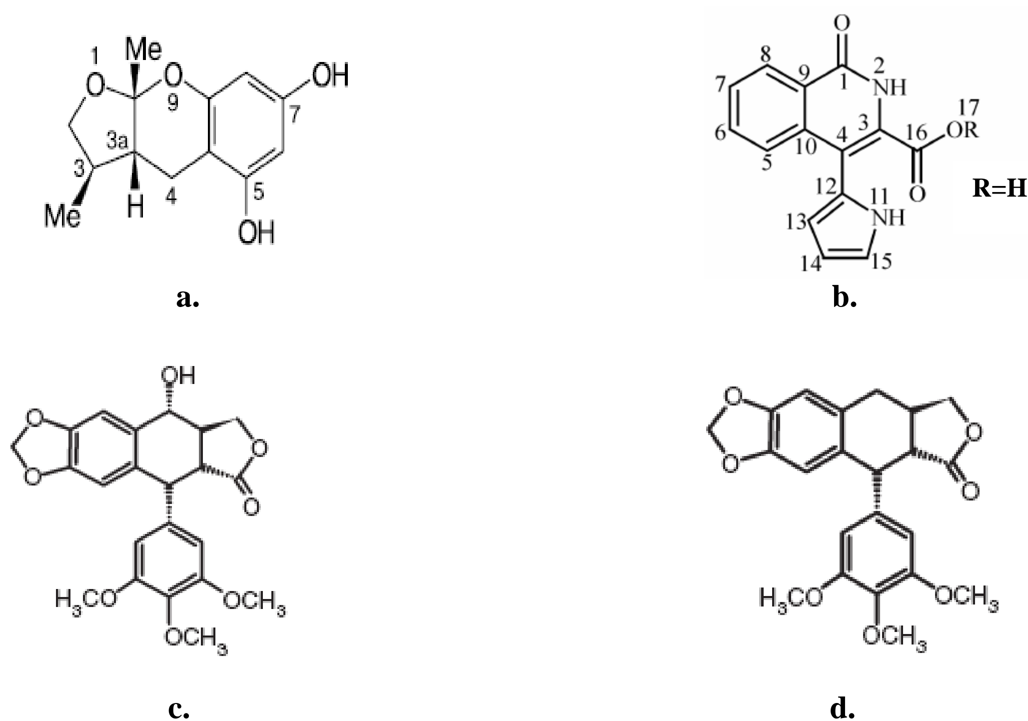


Figure 2 Novel compounds from endophytic fungi: (a.) xyloketol H, (b.) marinamide, (c.) podophyllotoxin, and (d.) deoxypodophyllotoxin

1.2.8 Mangrove endophytic fungi

Mangrove fungi are the second largest group among the marine endophytic fungi (Sridhar, 2004). The latest estimate of marine fungi is 1,500 species (Hyde *et al*., 1998). Endophytic fungi have also been isolated from the leaves of *Rhizophora apiculata* and *Rhizophora mucronata*, two typical mangrove plants grown in the Pichavaram mangroves of Tamil Nadu, Southern India (Suryanarayanan *et al*., 1998). More than 200 species of endophytic fungi were isolated and identified from mangroves, mainly *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, *Phoma*, *Phyllosticta* and *Trichoderma* (Liu *et al.,* 2007). Recently, research on the secondary metabolites of mangrove endophytic fungi has led to the isolation of many new compounds with different bioactivities (Chen *et al*., 2007). A novel 1-isoquinolone analog designated as marinamide and its methyl ester were produced by mangrove endophytic fungal strains 1920 and 3893 isolated from Hong Kong (Zhu and Lin, 2006). Chen *et al*. (2007) reported that a mangrove endophytic fungus *Penicillium thomi* isolated from the root of *Bruguiera gymnorrhiza* produced a new compound 4',5-dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl that had cytotoxicity against A549, HepG2 and HT29 cell lines with IC_{50} values in the range of 8.9-12.2 µM. The novel compounds phomopsin A, B, and C and cytosporone A, C from the mangrove endophytic fungus *Phomopsis* sp. ZSU-H70 obtained from the South China Sea inhibited two pathogenic fungi *Candida albicans* and *Fusarium oxysporum* with MICs that ranged from 32-64 µg/ml (Huang *et al*., 2008). Two new lactones 1893A and 1893B, together with other known compounds have been isolated from the mangrove endophytic fungus 1893 from *Kandelia candel* obtained from an estuarine mangrove on the South China Sea Coast (Chen *et al*., 2006).

Mangrove endophytic fungi have also been proven to be important sources of enzymes. Maria *et al*. (2005) tested seven mangrove endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum* for the production of extracellular enzymes (amylase, cellulase, chitinase, lacase, lipase, protease and tyrosinase). Cellulase and lipase activity were detected in all fungi, while amylase and protease were present in a few. No fungus exhibited chitinase, laccase or tyrosinase

activity. Chaowalit (2009) reported that 32% of mangrove endophytic fungi produced lipase, 24% produced cellulase and only 1% produced amylase on solid media. Of these, 20 isolates produced high amounts of lipase and 1 isolate produced high amounts of cellulase. These included *Cladosporium* sp., *Diaporthe* sp., *Guignardia* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Leptosphaerulina chartarum*, *Massarina corticola*, *Schizophyllum commune*, Botryosphaeriaceae sp., Diaporthaceace sp., Pleosporales sp. and Xylariaceae sp.

The antimicrobial activity of mangrove endophytic fungi isolated in Thailand has been rarely studied. Therefore, this study aimed to screen for fungal endophytes from mangrove plants in the south of Thailand coast that produced antimicrobial metabolites against human pathogens. To identify these isolates as far as possible and establish if the ability to become endophytes and produce antimicrobial activities was associated with any particular group of fungi.

1.3 Molecular characterization and phylogeny of fungi

In previous times, fungal phylogeny and taxonomy have been based almost entirely on morphology. Morphological characters still remain defining features for many fungal groups even though some characters can have one or more alternative character states. Therefore, the morphological characteristics of a fungus are often too limited for unquestionable identifications. Molecular systematics of fungi has recently increased our understanding of taxonomic groupings and evolutionary histories within different groups of fungi.

1.3.1 Molecular techniques

In the 1950s studies on molecular evolution were being dominated by protein sequencing. In the 1970s DNA sequencing was being used for molecular evolution studies by comparing small stretches of DNA of 20-25 bp in length. Nucleic acid sequencing then became a common procedure in molecular systematics in the 1980s (Ranghoo *et al*., 2000). Studies on the ribosomal RNA gene were first conducted by Woese and Olsen (1986); Lake (1987); Pace *et al*. (1986), with

investigations on the structure and function of the large ribosomal RNA gene (Hillis *et al*., 1996). Molecular techniques used for fungal systematics can be divided into two main areas: protein and DNA analyses. Protein analysis includes such procedures as isozyme electrophoresis. Isozyme data are analysed in such a way that changes in the mobility of enzymes in an electrical field reflect changes in the encoding sequence. With improvements in techniques for purifying genomic DNA and for determining nucleotide sequences together with PCR techniques many new processes were devised for using DNA analysis for systematic studies. The finding that the ribosomal genes of fungi and other eukaryotes differed from those of prokaryotes in that they contained type 1 intron that were less conserved than the genes for the RNA that formed the ribosomal RNA focused attention on the use of ribosomal genes to study fungal systematics.

Some of the other techniques that had been used included: comparisons of the guanine plus cytosine (G + C) content of nuclear DNA; DNA-DNA hybridization methods to measure the degree of genetic similarity between complete genomes by measuring the amount of heat required to melt (melting points) the hydrogen bonds between the base pairs that form the links between the two strands of the double helix of duplex DNA; restriction fragment length polymorphisms (RFLPs) using restriction enzymes to cut DNA at specific 4-6 bp recognition sites, random amplified polymorphic DNA (RAPD) is a technique in which organisms may be differentiated by analysis of patterns derived from varied primers, amplified fragment length polymorphisms (AFLPs) is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA, sequencing and analysis of the ribosomal RNA genes (Ranghoo *et al*., 2000). The molecular techniques for ecological studies such as temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) are forms of electrophoresis where there is a temperature or chemical gradient across the gel. TGGE and DGGE are useful for analyzing nucleic acids such as DNA and RNA, and sometimes for proteins and to detect fragments that have a mutation in the first melting domain (http://en.wikipedia.org/wiki/Temperature_gradient_gel_electrophoresis), and single strand conformation polymorphism (SSCP) is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequences that results in a

different secondary structure and a measurable difference in mobility through a gel (http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Parker/method.html).

1.3.2 Ribosomal RNA gene (rRNA) for fungal identification

The ribosomal RNA (rRNA) genes are widely used for identifying fungi and for constructing phylogenetic trees. Ribosomal RNA is needed by the cell in such large amounts to produce the cellular ribosomes that there are multiple copies of the rRNA genes, often arranged in tandem but separated from one another by untranscribed spacers. The ribosomal DNAs vary in length from about 3500–5000 nucleotides in most eukaryotes and contain regions of rapidly and slowly evolving regions. Each single rRNA gene has coding information for the three types of rRNA found in eukaryotic ribosome, 18S, 5.8S and 28S (Figure 3). The small subunit (SSU) and large subunit (LSU) rDNA have been useful in establishing relatedness between distantly related taxa or phyla. More recently, ribosomal internal transcribed spacer (ITS) regions have been used for fungal systematics and classification. The ITS region is a multi-copy, transcribed but non-coding and easily amplified region of the ribosomal DNA. The ITS regions are much more variable, but sequences can be aligned with confidence only between closely related taxa (Guarro *et al*., 1999). The 5.8S gene is much less conserved and may change more rapidly over time. The regions between the genes or intergenic regions (IGRs) are useful for elucidating differences between closely related taxa at the species or intraspecies level (Ranghoo *et al*., 2000).

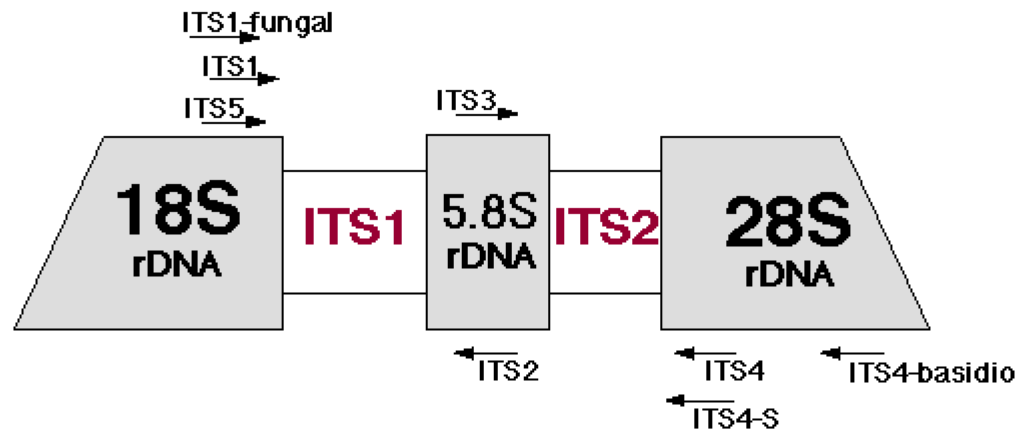


Figure 3 The ribosomal RNA gene region of eukaryotic cell

Source: http://plantbio.berkeley.edu/~bruns/picts/results /its-map.GIF

1.3.3 Phylogenetics

Phylogenetics is the study of genealogical relationships and historical patterns of evolutionary change among organisms. One of the underlying assumptions in phylogenetics is that patterns of change are recorded in the characters inherited by organisms from earlier generations. The record of change can be embedded in characters ranging from the morphological to genotypic features of DNA and protein sequences. Typically, phylogenetic relationships are diagrammed in the form of dichotomously branching trees generated, usually, by one of several computer algorithms. Incorporation of molecular data into the genealogical relationships of fungi has revolutionized our understanding of fungal phylogenetics. Phylogenetic trees show the ancestor descendant relationships among genus, species, or other taxonomic groups. Trees consist of terminal taxa, usually living species, connected by a network of internodes and nodes. The terminal taxa may be taxonomic groups other than species, such as genera or families, or they may be genes or proteins (Berbee and Taylor, 1999).

1.3.3.1 Phylogenetic analysis

A straightforward phylogenetic analysis consists of four steps (Baxwvanis and Ouellette, 2004):

1.3.3.1.1 DNA sequence alignment

Phylogenetic sequence data usually consist of multiple sequence alignments; the individual, aligned-base positions are commonly referred to as "sites". These sites are equivalent to "characters" in theoretical phylogenetic discussions, and the actual base (or gap) occupying a site is the "character state". Aligned sequence positions from phylogenetic analysis represent a priori phylogenetic conclusions because the sites themselves are effectively assumed to be homologous. Sites at which one is confident of homology and that contain changes in character states useful for the given phylogenetic analysis are often referred to as "informative sites". Steps in building the alignment include selection of the alignment procedure(s) and extraction of a phylogenetic data set from the alignment. The latter procedure requires determination of how ambiguously aligned regions and insertion/deletions will be treated in the tree-building procedure.

1.3.3.1.2. Analysis of data and generating phylogenetic trees

Methods of inferring trees fall into two classes, distance methods and discrete character methods (Hillis *et al*., 1996). Distance methods include UPGMA, and Neighbour-joining. Discrete character methods include maximum parsimony and maximum likelihood.

1.3.3.1.2.1 Distance methods

The distances can be calculated as the proportion of differences between pair of taxa relative to the total number of characters. Distances for DNA sequence data or protein data are usually corrected for multiple hits, or repeated substitutions at the same site. Some distance methods, like Neighbour-joining and UPGMA, use an exact algorithm to infer a tree from the distance matrix (Berbee and Taylor, 1999).

- **Neighbour-joining**: Neighbor-joining is based on the minimum-evolution criterion for phylogenetic trees, i.e. the topology that gives the least total branch length is preferred at each step of the algorithm. However, neighbor-joining may not find the true tree topology with least total branch length because it is a greedy algorithm that constructs the tree in a step-wise fashion. Even though it is sub-optimal in this sense, it has been extensively tested and usually finds a tree that is quite close

to the optimal tree. Nevertheless, it has been largely superseded in phylogenetics by methods that do not rely on distance measures and offer superior accuracy under most conditions (http://en.wikipedia.org/wiki/Neighbor-joining).

- UPGMA: UPGMA is the simplest of the distance methods and it clusters taxa successively based on similarities. UPGMA assumes that an evolutionary clock operated, or in other words, that changes had accumulated at a constant rate among all lineages. Averaging distances in the recalculation of the data matrix reflects this assumption of constant evolutionary rates. A side-effect of the clock assumption is polarization of the tree. The most similar taxa are assumed to have diverged most recently and are clustered together at the tips of the tree. The most divergent taxa originated earliest and emerge from the base of the tree. In this way, unlike most tree-building algorithms, UPGMA produces rooted trees (Berbee and Taylor, 1999).

1.3.3.1.2.2 Discrete character methods (Berbee and Taylor, 1999)

Discrete character methods use a data matrix as input. The data matrix can be a matrix of morphological character states or a DNA or amino acid sequence alignment. The discrete character methods superimpose each character state change on as many trees as possible. Two discrete character methods, maximum parsimony and maximum likelihood, both superimpose data on trees and choose the tree that best represents the data.

- Maximum parsimony: Maximum parsimony is a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length. The trees requiring the fewest character state changes are considered optimal. Individual characters are termed "informative" in maximum parsimony analysis when they require different numbers of changes on different trees. To be informative, an unpolarized character must have at least two character states and each state must be present in at least two taxa. Character state changes required for the tree is the length of the tree. As the number of taxa increases, the number of possible trees increases even faster, finding the most parsimonious trees (MPTs) for a given data set can be a computationally intensive task to choose the tree that result in fewest character state changes.

- Maximum likelihood: Maximum likelihood is concerned with the probability of a character changing state along a branch of a tree. Calculating maximum likelihood requires an initial estimate for the probability, for the given character, of changing from the state specified at the start of the segment to the state specified at the end of the segment above that of the probability of all possible character state changes. Applied to DNA sequences, maximum likelihood methods determine the probability of substitution in the data, the relative frequencies of the four nucleotides, and the different probabilities of transition and transversions.

1.3.3.2 Tree rooting

The methods described above produce unrooted trees. To evaluate evolutionary hypotheses, it is often necessary to locate the root of the tree. Rooting is generally evaluated by extrinsic evidence, that is, by means of determining where the tree would attach to an "outgroup", which can be any organism/sequence not descended from the nearest common ancestor of the organisms/sequences analyzed.

1.3.3.3 Tree evaluation

- Bootstrapping (Simpson, 2006)**:** Bootstrapping is a resampling tree evaluation method that works with distance, parsimony, likelihood and just about any other tree derivation method. The result of bootstrap analysis is typically a number associated with a particular branch in the phylogenetic tree that gives the proportion of bootstrap replicates that supports the monophyly of the clade. Bootstrap values greater than 70% correspond to a probability of greater than 95% that the true phylogeny has been found and greater than 50% will be an overestimate of accuracy. A high bootstrap value can make the right phylogeny.

- Consistency index (CI) (Simpson, 2006)**:** One measure of the relative amount of homoplasy in the cladogram is the consistency index. The consistency index is equal to the ratio of minimum changes of character state or minimum possible tree length that must occur and the actual number of changes or tree length that do occur. A consistency index close to 1 indicates little to no homoplasy; a CI close to 0 is indicative of considerable homoplasy.

$$CI = \frac{\text{Minimum possible tree length}}{\text{Tree length}}$$

- Retention index (RI) (Simpson, 2006)**:** The retention index is calculated as the ratio (g-s)/(g-m), where g is the maximum possible tree length that could occur on any conceivable tree, s and m are tree length and minimum possible tree length, respectively. Thus, the RI is influenced by the number of taxon in the study. A consistency index close to 1 indicates little to no homoplasy; a RI close to 0 is indicative of considerable homoplasy.

$$RI = \frac{\text{Maximum possible tree length - tree length}}{\text{Maximum possible tree length - minimum possible tree length}}$$

1.4 Objectives

1) To isolate endophytic fungi from mangrove plants.
2) To screen for antimicrobial activity of crude extracts from the endophytic fungal isolates.
3) To determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC) of crude extracts from the isolated endophytic fungi.
4) To identify the endophytic fungi which produce antimicrobial substances.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sampling of mangrove plants

Leaves and branches from 18 mangrove species were collected from 8 mangrove areas in the south of Thailand (Table 4).

Table 4 Mangrove plant species collected for endophytic fungal isolation and origins

Scientific name	Thai name	No. of plant	Original code
Aegiceras corniculatum (L.) Blanco	เล็บมือนาง	1	ST2
Avicennia alba Bl.	แสมขาว	1 1 1 1	ST1 ST2 SR1 TR1
Avicennia officinalis L.	แสมดำ	1 2 2	ST1 ST3 SK1
Bruguiera cylindrica Bl.	ถั่วขาว	1	SR2
Bruguiera gymnorrhiza (L.) Soavigny	พังกาหัวสุมดอกแดง	1	ST1
Bruguiera parviflora Wight & Arn. Ex Griff	ถั่วดำ	1 1 3	ST1 ST2 SR2

Table 4 (cont.) Mangrove plant species collected for endophytic fungal isolation and origins

Scientific name	Thai name	No. of plant	Original code
Ceriops decandra Ding Hon	โปรงขาว	2	ST3
Ceriops tagal (Perr.) C. B. Rob.	โปรงแดง	2	ST1
Heritiera littoralis Ait.	หงอนไก่ทะเล	1	ST2
Lumnitzera littorea Voigt.	ฝาดดอกแดง	1 1	ST1 ST2
Rhizophora apiculata Bl.	โกงกางใบเล็ก	1 3 3 3 2	ST1 ST2 SK1 SK2 TR1
Rhizophora mucronata Poir.	โกงกางใบใหญ่	1 3 1 1 3	ST1 ST2 SK1 SK2 SR1
Sonneratia caseolaris (L.) Engl.	ลำพู	4	SK2
Sonneratia griffithii Kurz.	ลำแพนหิน	1	SR1
Sonneratia ovata Back.	ลำแพน	1 1	ST1 SR2
Scyphiphora hydrophyllacea Graetn.f.	สีง้ำ	2 1	ST1 ST2
Xylocarpus granatum Koen.	ตะบูนขาว	3 1	ST1 ST2
Xylocarpus moluccensis Roem.	ตะบูนดำ	1 2 1	ST1 ST2 SR1
Total		63	

Province	Origin	Orginal code	Date
Songkhla	1) Khlongwong, Hatyai	SK1	11 July2006
	2) Ban-hlampho, Kutao, Hatyai	SK2	10 Jan 2007
Surathani	3) Donsak	SR1	18 Oct 2006
	4) Surat Coastal Fisheries Research and Development Center, Khanjanadit	SR2	18 Oct 2006
Satun	5) Ban-klonglidi, Thapae	ST1	23 Jan 2007
	6) Mangrove Forest Development Station 36, Thapae	ST2	23 Jan 2007
	7) Bangsila coast	ST3	23 Jan 2007
Trang	8) Pakmeang coast, Sikao	TR1	22 Oct 2006

2.1.2 Test microorganisms

Bacteria

- *Staphylococcus aureus* ATCC25923
- Methicillin-resistant *Staphylococcus aureus* SK1 (MRSA-SK1) isolated from patient by Pathology Department, Faculty of Medicine, Prince of Songkla University
- *Escherichia coli* ATCC25922
- *Pseudomonas aeruginosa* ATCC27853

Yeasts

- *Candida albicans* ATCC90028
- *Cryptococcus neoformans* ATCC90112

Filamentous fungus

- *Microsporum gypseum* MU-SH4 isolated from patient by Microbiology Department, Faculty of Medicine Siriraj Hospital, Mahidol University

2.1.3 Chemicals

- 5% Sodium hypochlorite (Clorox)
- 0.85% NaCl, normal saline solution (NSS)
- Ethanol (Commercial grade)
- Lacto phenol cotton blue
- Teepol
- 15% glycerol (MERCK)
- McFarland Standard (Appendix 2)

2.1.3.1 Media

- Potato dextrose broth (PDB) (Difco)
- Potato dextrose agar (PDA) (Difco)
- Corn meal agar (CMA) (Merck)
- Mueller-Hinton agar (MHA) (Difco)
- Sabouraud dextrose broth (SDB) (Difco)
- Sabouraud dextrose agar (SDA) (Difco)
- Nutrient broth (NB) (Difco)
- Nutrient agar (NA) (Difco)
- RPMI-1640 without phenol-red (pH7) (Sigma Chemical Co., USA)

2.1.3.2 Antibiotics

- Vancomycin (Fujisawa, USA)
- Gentamicin (Oxoid)
- Amphotericin B (Bristol-Myer Squibb Co., USA)
- Miconazole (Sigma Chemical Co., USA)
- Penosep (Penicillin + Streptomycin) (M&H manufacturing Co., Th)

2.1.3.3 Chemicals for molecular analysis

- Agarose (Vivantis)
- Phenol
- Chloroform (Lab-Scan Analytical Sciences)
- Isoamyl alcohol (Bio Basic)

- CTAB lysis buffer	(Appendix 2)
- Absolute ethanol	(Carlo Erba reagent)
- 6x Loading dye	(Appendix 2)
- Ethidium bromide	(Fluka)
- TAE buffer (Tris Acetate EDTA buffer, pH8)	(Appendix 2)
- Glacial acetic acid	(Merck)
- Ammonium acetate	(Bio Basic)
- EDTA (Ethylenediaminetetraacetic acid)	(Bio Basic)
- Tris (hydroxymethyl) aminomethane	(Research Organics)
- Tris Hydochloride	(Vivantis)
- 50 mM $MgCl_2$	(Fermentas)
- 10x PCR buffer	(Fermentas)
- 10mM dNTPs mix	(Fermentas)
- Taq DNA polymerase	(Fermentas)
- Lamda DNA/Eco91I (BstEII) Marker	(Fermentas)
- Nucleospin® Extract DNA purification kit	(Machery-Nagel)
- Bromophenol Blue	(Bio Basic)
- Xylene cyanol	(Research Organics)
- Glycerol	(Merck)
- Nanopure water	

2.1.3.4 Instruments

- Incubator	
- PCR Thermal Cycler	(Bio-Rad Thermal Cycler)
- Centrifuge	(Eppendorf 5417R)
- Gel electrophoresis machine	(Amersham Bioscience)
- Gel Documentation	(Model Syngene Gene Genius)
- Heat box	(Labnet)
- Light Microscope	(Olympus CX31)
- Stereo zoom microscope	(Olympus SZ-PT)

2.2 Methods

2.2.1 Sampling of mangrove plants

Leaves and branches from healthy mangrove plants were collected from 8 mangrove areas in the south of Thailand coast in Satun, Songkhla, Surathani and Trang provinces (Table 4) and brought back immediately to the laboratory. The eighteen mangrove species (Appendix 1) were *Rhizophora mucronata*, *Rhizophora apiculata*, *Sonneratia ovata*, *Sonneratia caseolaris*, *Bruguiera cylindrica*, *Bruguiera parviflora*, *Soneratia griffithii*, *Bruguiera gymnorrhiza*, *Heritiera littoralis*, *Avicennia alba*, *Avicennia officinalis*, *Xylocarpus granatum*, *Xylocarpus moluccensis*, *Ceriops decandra*, *Ceriops tagal*, *Lumnitzera littorea*, *Aegiceras corniculatum* and *Scyphiphora hydrophyllacea*.

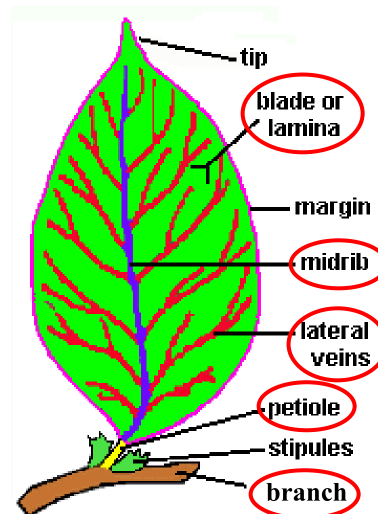


Figure 4 Plant segments

Source: http://generalhorticulture.tamu.edu/lectsupl/anatomy/P14f1.gif

2.2.2 Isolation of endophytic fungi

Leaves and branches were washed with detergent (Teepol) and tap water, cut into small, 1x1 cm^2, segments including midrib, lamina, vein, petiole, branch for 3, 3, 4, 4 and 6 segments respectively (Figure 4). The segments were

surface-sterilized by sequential washes in 95% (v/v) ethanol (30 sec), 5% (v/v) NaOCl (5 min), 95% (v/v) ethanol (30 sec) and rinsed with sterile water (Phongpaichit *et al*., 2006). The sample segments were dried under sterile conditions and then placed on corn meal agar medium (CMA) supplemented with antibiotics (penosep 50 mg/l). Plates were incubated at 25 °C for 1 week and observed every day. Pure cultures were isolated on potato dextrose agar (PDA) plates without antibiotics by hyphal tip isolation under stereo zoom microscope (Olympus SZ-PT) magnification (10X/22) and when found to be pure (Figure 5c), stored in 15% glycerol at -80 °C.

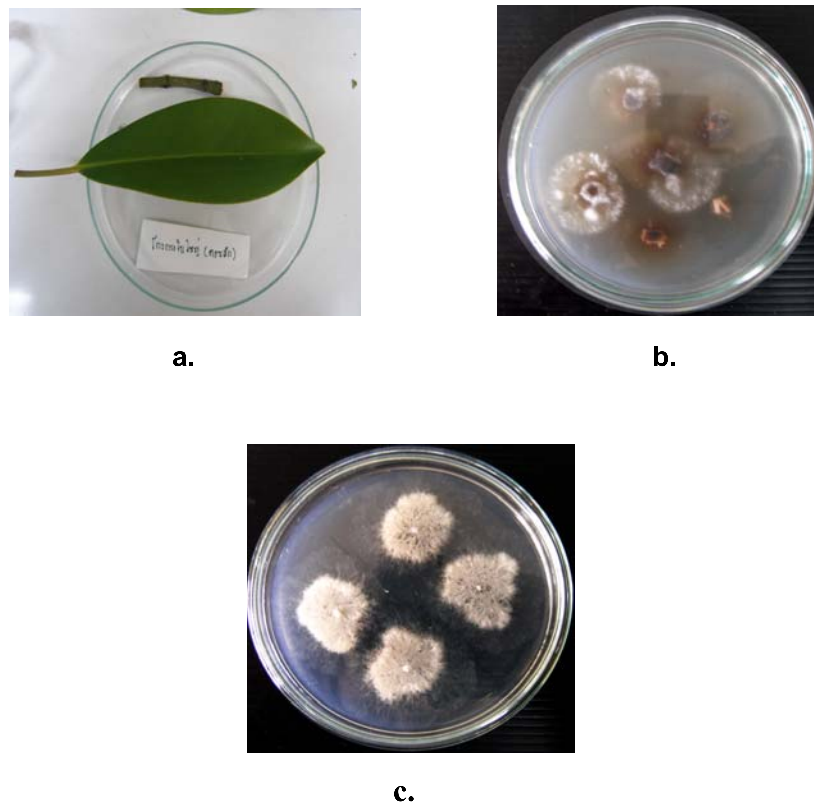


Figure 5 Endophytic fungal isolation on CMA with antibiotics

(a.) leaf and branch of healthy *Rhizophora mucronata*

(b.) mangrove plant segments on CMA after incubation at 25 °C

(c.) pure culture on PDA

2.2.3 The isolation rate calculation

The isolation rate was calculated as follows (Jordaan *et al.*, 2006):

$$\text{Isolation rate} = \frac{\text{total number of isolates yielded}}{\text{total number of sample segments}}$$

2.2.4 Fermentation and extraction of endophytic fungi

Collected endophytic fungal cultures of different morphologies were grown on PDA and incubated at 25 °C for 3-5 days. Six agar plugs (1 x 1cm^2) from the edge of the mycelium from an actively growing culture were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) and incubated for 3 weeks at RT for production of antimicrobials (Phongpaichit *et al*., 2006). The culture broth was filtered through Whatman #1 to separate the filtrate and mycelia. The filtrate was extracted three times with an equal volume of ethyl acetate (EtOAc, AR grade) in a separating funnel. The combined EtOAc extracts were dried over anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness under reduced pressure at 45 °C using a rotary vacuum evaporator to give the BE extract. The fungal mycelia were extracted with 500 ml of MeOH for 2 days. The aqueous MeOH layer was concentrated under reduced pressure. H_2O (50 ml) was added to the extract and the mixture was then mixed with hexane (100 ml). The aqueous layer was then extracted three times with an equal volume of EtOAc. The hexane extract and the combined EtOAc extracts were dried over Na_2SO_4 and evaporated to dryness under reduced pressure at 45 °C using a rotary vacuum evaporator to give CH and CE extracts respectively (Figure 6). All crude extracts were firstly subjected to thin-layer chromatography (TLC) and their H^1 NMR spectra recorded.

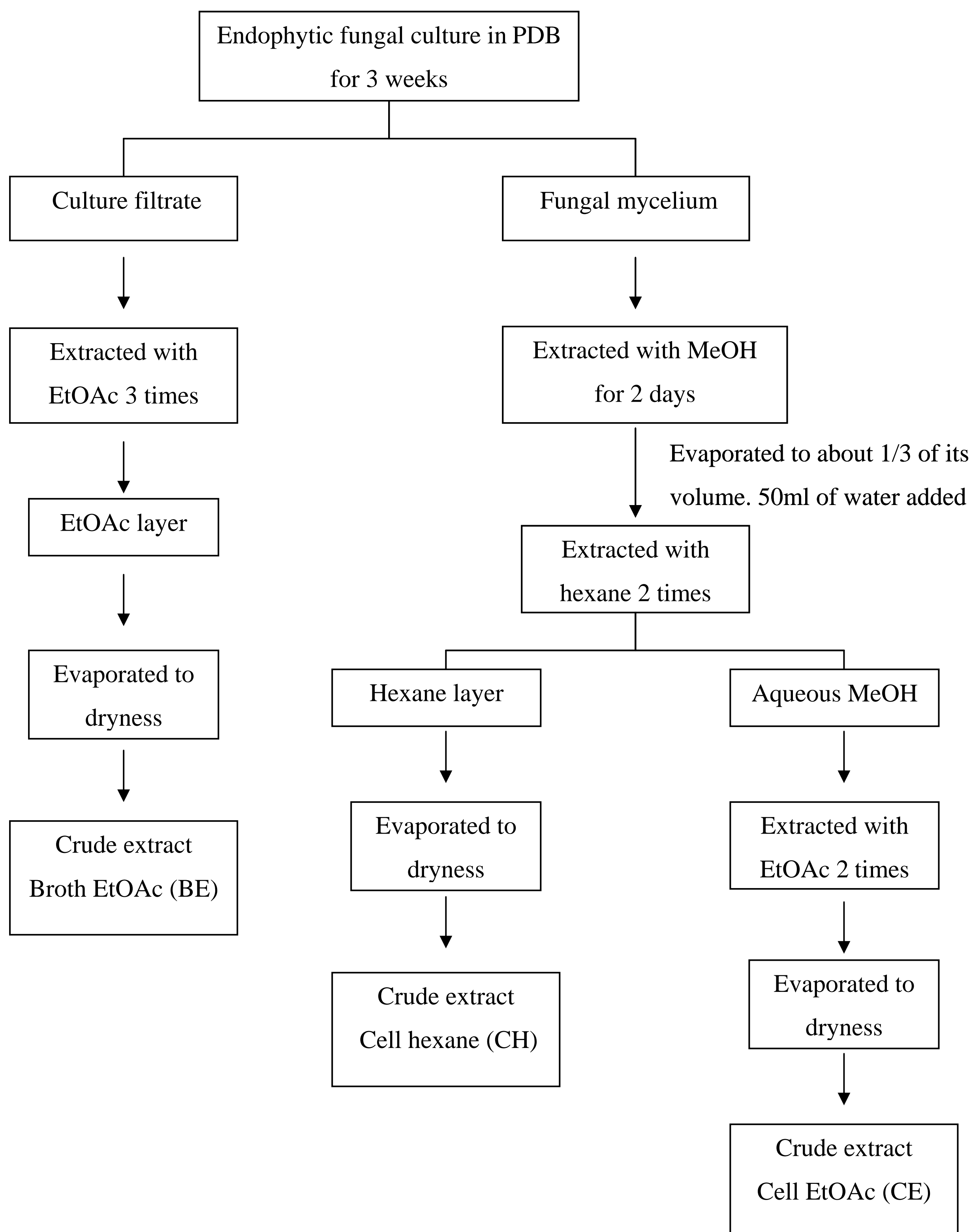


Figure 6 Extraction of chemicals produced by endophytic fungi

2.2.5 Screening for antimicrobial activities

2.2.5.1 Inoculum preparation

S. aureus ATCC25923, MRSA-SK1, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853 were streaked onto nutrient agar (NA) and incubated at 35 °C for 18-24 hours. *C. albicans* ATCC90028 was streaked on Sabouraud dextrose agar (SDA) and incubated at 35 °C for 18-24 hours. *C. neoformans* ATCC90112 was streaked on SDA and incubated at room temperature for 48 h. Three to five single colonies of bacteria were picked into nutrient broth (NB) of *C. albicans* and *C. neoformans* were picked into RPMI-1640, and both incubated at 35 °C for 3-5 hours while shaking at 150 rpm/min. After incubation, sterile normal saline (NSS) was used to adjust the turbidity to equal the 0.5 McFarland standard (MF) for bacteria and the 2.0 MF for yeasts. Agar plug of *M. gypseum* was placed on SDA and placed in an incubator at 25 °C for 2-3 weeks or until it produced spores. The spores were collected by scraping with a sterile glass beads and were suspended with NSS, the spore suspension was adjusted to 4×10^3 –5×10^4 CFU/ml using a hemacytometer.

2.2.5.2 Testing for antibacterial activity (modification of CLSI M7-A4, 2002a)

The endophytic fungal extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 100 mg/ml, and stored at -20 °C until used. DMSO was used to dilute to 1:10 and Mueller-Hinton broth (MHB) for further dilutions of 1:25 to obtain concentrations of 400 µg/ml. Triplicate 50 µl samples of crude extracts (400 µg/ml) were placed into sterile 96-well microtiter plates. The bacterial inocula (0.5 MF) were diluted 1:200 (~5×10^5 CFU/ml) using MHB and 50 µl added to the top well in each row so that the final concentration of crude extract was then 200 µg/ml. Plates were incubated at 35 °C for 15 hours, then 10 µl of resazurin indicator (0.18%) (Appendix 2) was added to each well and examined after incubation for 2-3 hours at 35 °C for the completed incubation time (Drummond and Waigh, 2000).

Vancomycin and gentamicin at a final concentration of 4 µg/ml were used as standard antibacterial agents for positive inhibitory controls and for comparisons with the extracts.

2.2.5.3 Testing for antifungal activity (yeasts) (modification of CLSI MA27-A2, 2002c)

Yeasts were tested in a similar way to bacteria but using RPMI-1640 medium. Microtiter plates were incubated at 35 ^{o}C for 24 hours for *C. albicans* and 48 hours at room temperature for *C. neoformans*, then 10 µl resazurin indicator (0.18%) was added to each well and examined after incubation for 5 hours at 35 ^{o}C (Drummond and Waigh, 2000).

Amphotericin B at a final concentration of 4 µg/ml was used as a positive inhibitory control and for comparison with the extract.

2.2.5.4 Testing for antifungal activity (filamentous fungus) (modification of CLSI MA38-A, 2002b)

Filamentous fungus (*M. gypseum*) was tested in a similar way to bacteria but using RPMI-1640 medium. Microtiter plates were incubated at 25 ^{o}C for 6 days, then 10 µl resazurin indicator (0.18%) was added to each well and incubated for one day at 25 ^{o}C.

Miconazole at final concentration 4 µg/ml was used as the standard fungicide control and for comparison with the extract.

After incubation, a blue or purple color of the wells indicated inhibition of growth (positive result) and a pink color meant growth had occurred (negative result). Crude extracts shown to have antimicrobial activity were assayed for their minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC) and minimal fungicidal concentrations (MFC).

2.2.6 Determination of minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) of the endophytic fungal crude extracts

The MICs of crude extracts were determined by a broth microdilution method according to a modification of CLSI MA7-A4 (CLSI, 2002a) against bacteria, a modification of the microbroth dilution CLSI MA27-A2 (CLSI, 2002c) against yeasts and a modification of the microbroth dilution CLSI MA38-A (CLSI, 2002b)

againsts *M. gypseum*. Crude extracts were diluted using the serial dilution method starting with 128, and diluting to 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml, each tested in triplicate.

After incubation under appropriate conditions, the lowest concentration of extract that inhibited growth (blue or purple color) was recorded as the MIC.

The MBCs and the MFCs of the endophytic fungal crude extracts were determined by the streaking method. Concentrations of crude extract less dilute than the MIC and the MIC were streaked onto NA plate for bacteria and SDA plates for yeasts and filamentous fungus. Plates were incubated under appropriate conditions and the lowest concentration of extract that killed organisms (no growth) was recorded as the MBC or MFC.

2.2.7 Identification of endophytic fungi

Only endophytic fungi that produced substances with satisfied antimicrobial activity and interested NMR profile were selected for identification.

2.2.7.1 Morphological identification

- Macroscopic morphology

Cultures were grown on PDA plates. The colour of colonies and growth rate were observed under stereo zoom microscope and recorded.

- Microscopic morphology

The microscopic morphology analyses were based on observations with an Olympus CX31 research microscope. Specimens for light microscopy were mounted in lactophenol cotton blue for observation of spores and other characteristics, and then identified. Identification of the fungi follows Barnett and Hunter (1998).

2.2.7.2 Molecular identification

-Genomic DNA extraction

Fungi used for the molecular study were grown in PDB at a volume of 50 ml per flask and incubated at room temperature for 1-2 weeks, or until enough mycelia for DNA extraction.

The fungal mycelia was harvested through cheesecloth and washed with sterile distilled water (~60 ^{o}C) several times and placed mycelia onto a large absorbent paper towel to absorb away as much liquid as possible, placed in sterile mortar. Mortar was frozen in -80 ^{o}C freezer for 1-2 hours. Genomic DNA was extracted using CTAB lysis buffer (O' Donnell *et al.*, 1997). The mycelia were ground into a fine powder with sterile sand and added extraction buffer about 200 µl. Semiliquid mycelial powder was transfered to microtube (1.5 µl) and added extraction buffer 300 µl. The microtube incubated at 70 ^{o}C for 30 minutes, then centrifuged at 14,000 rpm, 25 ^{o}C for 15 minutes. The supernatant was transferred to a new microtube, then an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, then centrifuged at 14,000 rpm for 15 minutes. Upper liquid phase was transferred to a new microtube, added twice with chloroform: isoamyl alcohol (24:1) for equal volume, then centrifuged at 14,000 rpm, 25 ^{o}C for 15 minutes. Upper liquid phase was transferred to a new microtube, added 7.5 M ammonium acetate for half of total volume and added chilled absolute ethanol for 2.5 of total volume. Mixture was kept at 20 ^{o}C for at least 30 minutes, or until required for DNA precipitation, then centrifuged at 14,000 rpm, 4 ^{o}C for 15 minutes. DNA pellet was washed twice with chilled 75% ethanol and air dried after removing of the ethanol. Finally, DNA was resuspended with 50 µl of sterile nanopure water. The DNA was stored at 4 ^{o}C for the polymerase chain reaction (PCR). The total genomic DNA were estimated quality and quantity by observing the intensity and purity of the bands in 1% agarose gel electrophoresis using TAE buffer (Appendix 2).

-PCR amplification

Target regions of the ITS1-5.8S-ITS2 rDNA regions and large subunit (28S) were amplified using universal primers listed in Table 5 and Figure 7. The PCR mixtures were amplified in a Bio-Rad Thermal Cycler (C1000 ™ Thermal Cycler #1 and #2).

Figure 7 ITS, LSU regions and universal primers

Source: http;//www.lutzonilab.net/primers/image247

Table 5 Primers used for the polymerase chain reaction (PCR) and DNA sequencing

Primers	Sequence (5´-3´)	References
	LSU	
LROR	ACCCGCTGAACTTAAGC	Bunyard *et al*., 1994
LR7	TACTACCACCAAGATCT	Landvik, 1996
JS1	CGCTGAACTTAAGCATAT	
JS8	CATCCATTTTCAGGGCTA	
	ITS1-5.8S-ITS2	
ITS1	TCCGTAGGTGAACCTGCGG	White *et al*., 1990
ITS4	TCCTCCGCTTATTGATATGC	
ITS5	GGAAGTAAAAGTCGTAACAAGG	

PCR mixtures (i-Taq^{TM} DNA Polymerase)

PCR Mixtures	Stock concentration	Volume (µl)	Final concentration of 50 µl
Nanopure water		35.8	
PCR buffer	10 X	5.0	1 X
$MgCl_2$	25 mM	5.0	1.25mM
dNTPs	10 mM	1.0	0.2mM
Forword primer	10 mM	1.0	0.2mM
Revers primer	10 mM	1.0	0.2mM
Taq polymerase	5 units/µl	0.2	0.02 units
DNA template	100-500 ng	1.0	2-10 ng

PCR profiles for different amplifications:

a) The PCR profile for primers ITS5/LR7, JS1/JS8, LROR/LR7, NS5/ITS4

94 ^{o}C	2 minutes	
94 ^{o}C	1 minutes	35 cycles
55 ^{o}C	1.5 minutes	
72 ^{o}C	2.5 minutes	
72 ^{o}C	10 minutes	

b) The PCR profile for primers ITS1/ITS4, ITS5/ITS4

94 ^{o}C	2 minutes	
94 ^{o}C	1 minutes	35 cycles
55 ^{o}C	1 minutes	
72 ^{o}C	2 minutes	
72 ^{o}C	10 minutes	

PCR product was checked for quantity and quality by observing of intensity of the band in a 1% agarose gel electrophoresis, then purified directly by Nucleospin® Extract DNA purification kit.

-DNA sequencing and sequence alignment

The purified PCR product was used directly for DNA sequencing by Macrogen in a Korean biotechnology company. Sequencing reactions for each primer were checked manually for base ambiguities and assembled by BioEdit 7.0.7 (Hall, 2007). Then the sequence was run by BLASTN program for comparison with the GenBank database (http://www.ncbi nlm.nih.gov).

Sequences of the selected fungi and other sequences obtained from the GenBank database were aligned by ClustalW (Thompson *et al*., 1994). Manual gap adjustments were made to improve the alignments. The tree construction procedure was performed in PAUP* 4.0b10 in Macintosh and Window versions (Swofford, 2002). Maximum Parsimony (MP) was conducted using heuristic searches as implemented in PAUP, with the default options method. Clade stability was assessed in a bootstrap analysis with 500 replicates, random sequence additions with maxtrees

set to 1000 and other default parameters as implemented in PAUP* 4.0b10. Descriptive tree statistics (Tree Length, CI, RI) were calculated for trees generated under different optimality criteria. Kishino-Hasegawa (KH) tests (Kishino and Hasegawa, 1989) were performed in order to determine whether tree were significantly different. The sequence aligments have been deposited in GenBank database.

CHAPTER 3

RESULTS

3.1 Isolation of endophytic fungi from mangrove plants

A total of 619 endophytic fungi were isolated from 63 plants of 18 mangrove species (Table 6). The average rate of endophytic fungal isolation was 9.8 isolates per plant or 0.49 isolates per sample disc. The highest rate was found in *B. cylindrica* (21 isolates/plant or 1.05 isolates/disc) and the lowest rate was from *B. gymnorrhiza* (2 isolates/plant or 0.1 isolate/disc).

The percentages of endophytic fungi isolated from different plant parts were as follows: branch 39.03%, vein 20.32%, midrib 15.00%, lamina 12.90% and petiole 12.74% (Figure 8).

One hundred and fifty isolates (24.23%) were randomly selected for antimicrobial assay. Sixty four of them were from *R. apiculata*, 17 from *R. mucronata*, 14 from *A. alba*, 11 from *B. parviflora* and 1-9 from the rest of the plants. There were no isolates selected from *S. ovata*, *S. griffithii*, *B. cylindrica*, *H. littoralis*, *C. decandra* and *C. tagal* because the fungi from these plants died during storage.

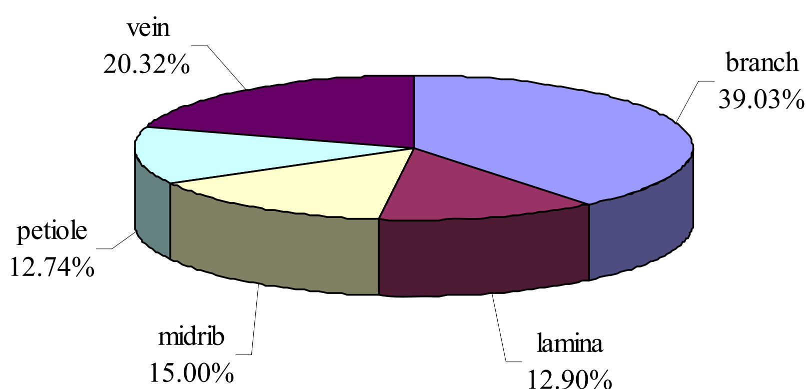


Figure 8 Percentage of endophytic fungi isolated from each mangrove plant part

Table 6 Endophytic fungi isolated from various mangrove host plants

No.	Thai name	Scientific name	No. of plant	No. of isolated fungi	Rate of isolation	
					Isolates /plant	Isolates /plant disc
1	เล็บมือนาง	*Aegiceras corniculatum* (L.) Blanco	1	4	4	0.2
2	แสมขาว	*Avicennia alba* Bl.	4	34	8.5	0.43
3	แสมดำ	*Avicennia officinalis* L.	5	17	3.5	0.17
4	ถั่วขาว	*Bruguiera cylindrica* Bl.	1	21	21	1.05
5	พังกาหัวสุมดอกแดง	*Bruguiera gymnorrhiza* (L.) Savigny	1	2	2	0.1
6	ถั่วดำ	*Bruguiera parviflora* Wight & Arn.ex Griff	5	48	9.6	0.48
7	โปรงขาว	*Ceriops decandra* Ding Hou	2	8	4	0.2
8	โปรงแดง	*Ceriops tagal* (Perr.) C. B. Rob.	2	5	2.5	0.13
9	หงอนไก่ทะเล	*Heritiera littoralis* Ait	1	11	11	0.55
10	ฝาดดอกแดง	*Lumnitzera littorea* Voigt	2	34	17	0.85
11	โกงกางใบเล็ก	*Rhizophora apiculata* Bl.	12	200	16.6	0.83
12	โกงกางใบใหญ่	*Rhizophora mucronata* Poir.	9	84	9.3	0.47
13	ลำพู	*Sonneratia caseolaris* (L.) Engl.	4	34	8.5	0.43
14	ลำแพนหิน	*Sonneratia griffithii* Kurz	1	4	4	0.2
15	ลำแพน	*Sonneratia ovata* Back	2	19	9.5	0.48
16	สีง้ำ	*Scyphiphora hydrophyllacea*	3	22	7.3	0.37
17	ตะบูนขาว	*Xylocarpus granatum* Koen.	4	29	7.3	0.36
18	ตะบูนดำ	*Xylocarpus moluccensis* Roem.	4	43	10.8	0.54
		Total/average	63	619	9.8	0.49

$$\text{Isolation rate (Jordaan } et\ al.\text{, 2006)} = \frac{\text{total number of isolates yielded}}{\text{total number of sample segments}}$$

3.2 Screening of fungal crude extracts for antimicrobial activity

The 150 selected fungal isolates fungi were grown in PDB for 3 weeks and their culture broths and mycelia were extracted. In total, 385 crude extracts comprising crude ethyl acetate extracts from culture broths (BE, 145), crude ethyl acetate extracts of mycelia (CE, 144) and crude hexane extracts of mycelia (CH, 96) were obtained for antimicrobial assay. The amount of materials in some extracts was too small to assay so were not further investigated.

The assays, for antimicrobial activity each of 200 µg/ml using the colorimetric microdilution method (Figure 9) revealed that 181 extracts (47.01%) from 92 endophytic fungal isolates (61.33%, Table 7) were inhibitory. The highest number of active isolates was from *R. apiculata* (34) followed by *R. mucronata* (9), *A. alba*, *A. officinalis* and *B. parviflora* (8 each), *S. hydrophyllacea* and *L. littorea* (6 each), and from *X. granatum*, *X. moluccensis*, *S. caseolaris* and *A. corniculatum* 5, 4, 3 and 1 isolates (Table 7), respectively. No activity was detected from those isolated from *B. gymnorrhiza*. All 3 selected isolates (100%) from *S. caseolaris* displayed antimicrobial activity. Although the highest number of selected endophytes was from *R. apiculata* only 53.13% of them were active.

Figure 9 Primary assay against *C. neoformans* by the colorimetric microdilution method at 200 µg/ml (blue or violet color indicates an inhibitory result and pink color indicates growth of the test strain)

According to the types of crude extract CH provided 49/96 (51.04%) of positives followed by CE (67/144, 46.53%) and BE (65/145, 44.83%) (Table 8 and Figure 10). CH extracts exhibited the most activity against MRSA (38) and *S. aureus* (38), followed by *M. gypseum* (27), *C. neoformans* (11) and *C. albicans* (9). CE extracts exhibited the most activity against MRSA (46), followed by *S. aureus* (40), *P. aeruginosa* (2), *C. albicans* (11), *C. neoformans* (16), and *M. gypseum* (33). BE extracts exhibited the most activity against MRSA (43) followed by *M. gypseum* (38), *S. aureus* (33), *C. neoformans* (18) and *C. albicans* (9) (Table 9).

Extracts of active endophytic fungi were highly active against both strains of *S. aureus* (65-70 isolates) followed by *M. gypseum* (59 isolates), *C. neoformans* (35 isolates) and *C. albicans* (21 isolates). Most of the active isolates were from *R. apiculata*. Only 2 isolates which had anti-*P. aeruginosa* activity were from *A. alba* and *X. granatum*. None of the selected endophytic fungi inhibited *E. coli* (Table 7).

Table 7 Selected endophytic fungi from various mangrove species and their antimicrobial activity against pathogenic microorganisms

	No. of endophytic fungi from mangrove species												
	Ac	Aa	Ao	Bg	Bp	Ll	Ra	Rm	Sc	Sh	Xg	Xm	Total
No. of selected fungi	2	14	9	1	11	7	64	17	3	8	9	5	150
No. of fungal isolates from each mangrove species (%) causing inhibition	1 (50.00)	8 (57.14)	8 (88.89)	0 (0.00)	8 (72.73)	6 (85.71)	34 (53.13)	9 (52.94)	3 (100)	6 (75)	5 (55.56)	4 (80)	92 (61.33)
% of active fungi (92 isolates)	1.09	8.70	8.70	0.00	8.70	6.52	36.96	9.78	3.26	6.52	5.43	4.35	100
Anti-*S. aureus* ATCC25923	1	3	5	0	7	6	24	6	3	4	3	3	65
Anti-MRSA-SK1	1	5	7	0	6	4	26	7	3	4	5	2	70
Anti-*E. coli* ATCC25922	0	0	0	0	0	0	0	0	0	0	0	0	0
Anti-*P. aeruginosa* ATCC27853	0	1	0	0	0	0	0	0	0	0	1	0	2
Anti-*C. albicans* ATCC90028	0	2	4	0	0	1	10	0	0	1	2	1	21
Anti-*C. neoformans* ATCC90112	1	5	5	0	0	2	13	2	1	4	1	1	35
Anti-*M. gypseum* MU-SH4	1	7	5	0	4	5	21	5	1	4	3	3	59

Ac = *Aegiceras corniculatum* Aa = *Avicennia alba* Ao = *Avicennia officinalis* Bg = *Bruguiera gymnorrhiza* Bp = *Bruguiera parviflora*
Ll = *Lumnitzera littorea* Ra = *Rhizophora apiculata* Rm = *Rhizophora mucronata* Sc = *Sonneratia caseolaris* Sh = *Scyphiphora hydrophyllacea*
Xg = *Xylocarpus granatum* Xm = *Xylocarpus moluccensis*

Table 8 Type of crude extracts of endophytic fungi having antimicrobial activity

Crude extract	BE	CE	CH	Total
No. of crude extracts tested	145	144	96	385
No. of active crude extracts (%)	65 (44.83)	67 (46.53)	49 (51.04)	181 (47.01)

BE = Broth EtOAc, CE = Cell EtOAc, CH = Cell Hexane

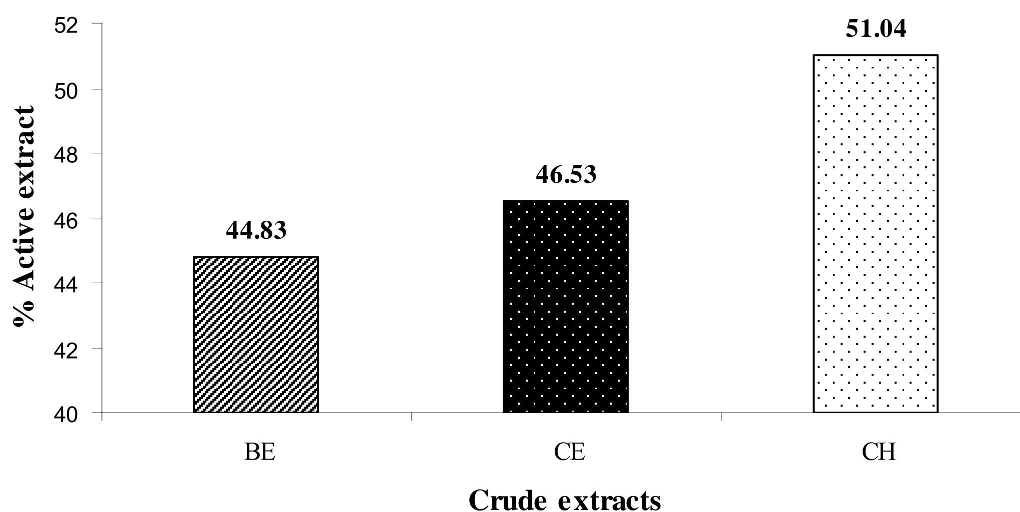
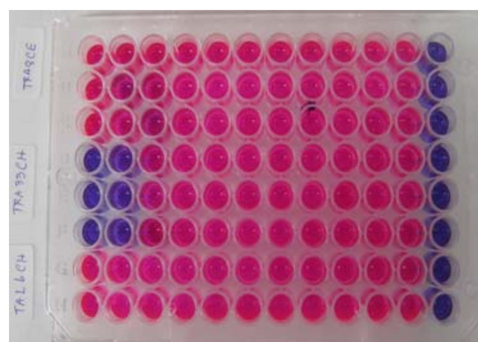


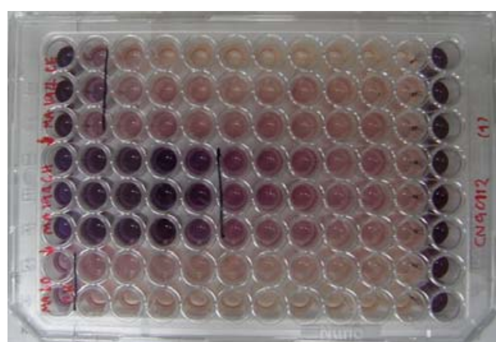
Figure 10 Percentage of types of active crude extracts from endophytic fungi tested at 200 µg/ml

BE = Broth EtOAc, CE = Cell EtOAc, CH = Cell Hexane

a.) S. *aureus* ATCC25923



b.) MRSA-SK1



c.) *C. neoformans* ATCC90112

Figure 11 Determination of MIC of crude extracts by colorimetric microdilution

3.3 Determination of MIC and MBC or MFC

All extracts that showed inhibitory activity at 200 µg/ml were further assayed for their MICs (Figure 11) MBCs or MFCs by the colorimetric microdilution method. The results are shown in Table 9-11.

3.3.1 Antibacterial activity

Of the 385 crude extracts, 134 (34.8%) extracts from 76 endophytic fungal isolates exhibited antibacterial activity. 28.31%, 31.95%, and 0.52% of extracts were active against *S. aureus*, MRSA-SK1 and *P. aeruginosa* (Figure 12, Table 9) with MIC/MBC values that ranged from 8-200/64-200, 4-200/64-200, and 200/>200

μg/ml, respectively. None of the extracts inhibited *E. coli* (MIC and MBC >200 μg/ml). Crude hexane extracts from the mycelia of MA34 (MA34CH) exhibited the strongest antibacterial activity against both strains of *S. aureus* (MIC 4-8 μg/ml) and this was comparable to vancomycin (MIC 0.5-1 μg/ml) followed by MA132CE (MIC 16 μg/ml). Only 2 extracts, MA82CE and MA125CE had anti-*P. aeruginosa* (MIC 200 μg/ml) while the control drug gentamicin had an MIC value of 1 μg/ml.

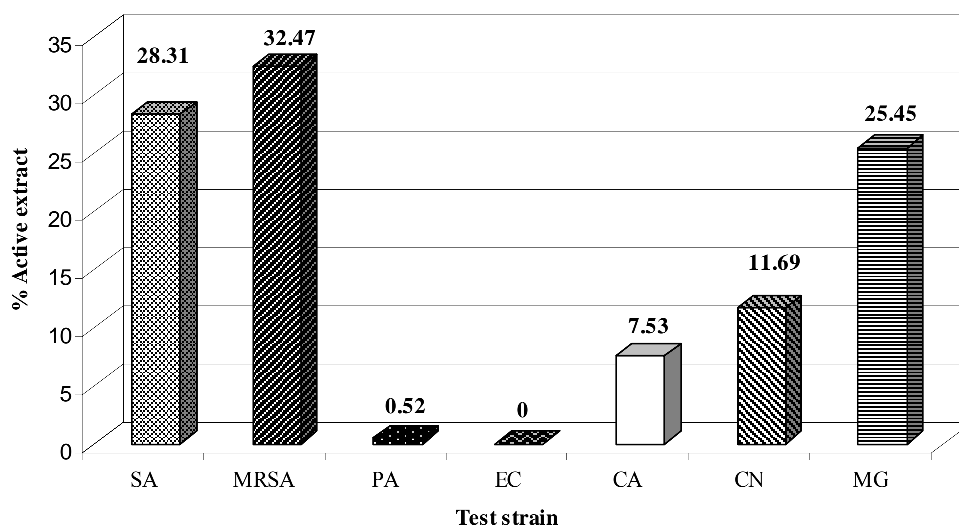


Figure 12 Antimicrobial activity of crude extracts against various microbes tested at 200 μg/ml

SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
PA = *Pseudomonas aeruginosa* ATCC27853
EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112
MG = *Microsporum gypseum* MU-SH4

3.3.2 Antifungal activity

One hundred and fourteen (29.61%) extracts from 65 isolates displayed antifungal activity. 25.45% of the extracts from 59 isolates were active against *M. gypseum* (Figure 12, Table 9) with MIC and MFC value of 4-200 and 8-200 μg/ml, respectively. The most active extract was MA96CH (MIC 4 μg/ml, MFC 8 μg/ml) and this was comparable to the antifungal drug miconazole (MIC 0.5 μg/ml, MFC 2 μg/ml) followed by MA148CE, MA156BE and MA194CH (MICs and MFCs

8 μg/ml). For yeasts, only 11.69 and 7.53% of the extracts were active against *C. neoformans* and *C. albicans* with MIC/MFC values that ranged from 8-200/8-200 μg/ml and 32-200/32-200 μg/ml, respectively. MA12CH and MA194CH showed the best anti-*C. neoformans* with MIC value of 8 μg/ml compared to amphotericin B (MIC 0.5 μg/ml) followed by MA148CE (MIC 16 μg/ml), whereas MA99CH and MA194CH were most active against *C. albicans* with MIC value of 32 μg/ml (amphotericin B, MIC 0.25 μg/ml).

3.3.3 Antimicrobial spectrum

Of the 181 active extracts that inhibited 1 to 5 test strains 35.36, 32.04, 16.57, 8.84 and 7.18% of the extracts inhibited 2, 1, 3, 4 and 5 strains, respectively (Figure 13). MA2CH, MA3CH, MA20BE, MA20CE, MA60CH, MA70CE, MA147BE, MA147CE, MA156BE, MA177CE, MA194CE, MA194CH and MA195BE exhibited the broadest antimicrobial spectrum against both strains of *S. aureus*, *C. albicans*, *C. neoformans* and *M. gypseum* (Table 10).

3.3.4 Antimicrobial potential

When considering the antimicrobial activity according to the MIC values, it was found that most of the extracts exhibited weak activity (MIC 128-200 μg/ml), except for the anti-*M. gypseum*, most extracts had moderate activity (MIC 16-64 μg/ml) (Table 11). Only six extracts (MA12CH, MA34CH, MA96CH, MA148CE, MA156BE, MA194CH) showed strong activity (MIC ≤ 8 μg/ml) (Table 10). Most of the extracts had MBC and MFC values > 200 μg/ml (Table 11).

Table 9 Antimicrobial activity of crude extracts from endophytic fungi

Activity	Anti-SA	Anti-MRSA	Anti-EC	Anti-PA	Anti-CA	Anti-CN	Anti-MG
Active isolates (%) (n=150)	65 (43.33)	70 (46.67)	0 (0.00)	2 (1.33)	21 (14.00)	35 (23.38)	59 (39.33)
Active extract (%) (n=385)	109 (28.31)	125 (32.47)	0 (0.00)	2 (0.52)	29 (7.53)	45 (11.69)	98 (25.45)
BE (%)	33 (30.28)	43 (34.96)	0 (0.00)	0 (0.00)	9 (31.03)	18 (40.00)	38 (38.78)
CE (%)	40 (36.70)	46 (37.39)	0 (0.00)	2 (100)	11 (37.93)	16 (35.56)	33 (33.67)
CH (%)	36 (33.03)	36 (29.27)	0 (0.00)	0 (0.00)	9 (31.03)	11 (24.44)	27 (27.55)
MIC (μg/ml)	8-200	4-200	>200	200	32-200	8-200	4-200
MBC/MFC (μg/ml)	64-200	64-200	>200	>200	32-200	8-200	8-200

SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
PA = *Pseudomonas aeruginosa* ATCC27853
EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112
MG = *Microsporum gypseum* MU-SH4

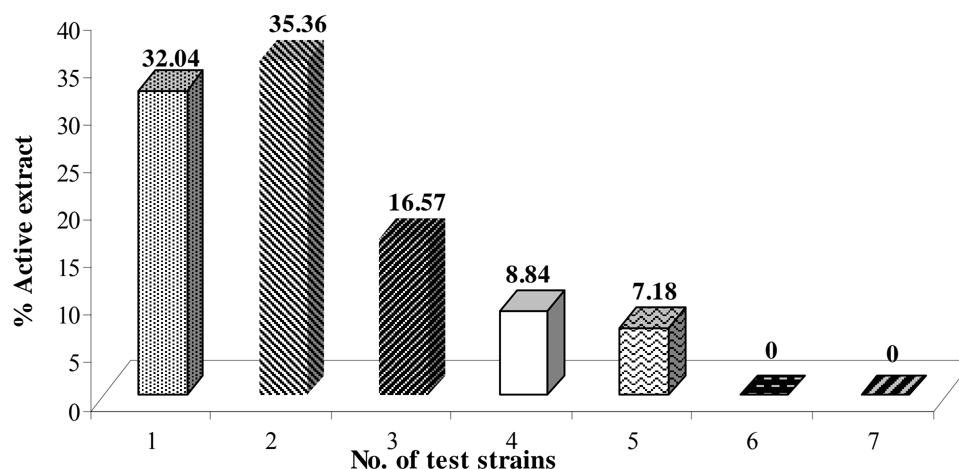


Figure 13 Number of test strains inhibited by crude extracts

Table 10 MICs, MBCs or MFCs of selected active crude extracts from endophytic fungi

Test organisms / Endophytic fungi and their extracts		Bacteria								Yeasts				Filamentous fungus	
Code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
		MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
MA2	CH	32	64	32	64					200	>200	200	200	32	32
MA3	CH	200	>200	200	>200					128	200	200	>200	32	32
MA12	CH			64	128							8	8	200	>200
MA20	BE	64	>200	64	>200					200	>200	200	>200	32	200
	CE	64	200	64	>200					128	200	200	>200	32	200
MA34	CH	8	64	4	64										
MA60	CH	128	200	128	200					200	200	200	200	16	>200
MA70	CE	200	>200	200	>200					200	>200	64	200	32	200
MA82	CE			200	>200	200	>200					200	>200	64	64

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 10 (cont.) MICs, MBCs or MFCs of selected active crude extracts from endophytic fungi

Test organisms / Endophytic fungi and their extracts		Bacteria								Yeasts				Filamentous fungus	
Code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
		MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
MA96	CH	64	128	128	128					128	>200			4	8
MA99	CH									32	128				
MA125	CE			200	>200	200	>200			200	>200				
MA132	CE	16	64	16	64							64	>200		
MA147	BE	64	64	64	64					200	>200	200	200	32	32
	CE	64	64	64	64					200	>200	200	200	32	32
MA148	CE											16	16	8	8
MA156	BE	128	>200	128	>200					128	128	128	128	8	8
MA177	CE	200	>200	200	>200					128	128	32	32	32	32

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml
BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 10 (cont.) MICs, MBCs or MFCs of selected active crude extracts from endophytic fungi

Test organisms / Endophytic fungi and their extracts		Bacteria								Yeasts				Filamentous fungus	
Code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
		MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
MA194	CE	128	>200	128	>200					200	200	128	200	16	16
	CH	32	>200	32	>20					32	32	8	200	8	8
MA195	BE	200	200	128	200					200	>200	128	>200	128	>200
Vancomycin		0.5	1	1	2										
Gentamicin						1	2	0.5	2						
Amphotericin B										0.25	1	0.5	1		
Miconazole														0.5	2

SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
PA = *Pseudomonas aeruginosa* ATCC27853
EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112
MG = *Microsporum gypseum* MU-SH4
* = µg/ml
BE = Broth EtOAc
CE = Cell EtOAc
CH = Cell Hexan
** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration
MBC = Minimal Bactericidal Concentration
MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 11 MIC, MBC or MFC values of active crude extracts against pathogenic microorganisms

Concentration (µg/ml)	No. of active extracts (%)													
	SA (n=107)		MRSA (n=126)		PA (n=2)		EC (n=0)		CA (n=29)		CN (n=45)		MG (n=98)	
	MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
4	0	0	1	0	0	0	0	0	0	0	0	0	1	0
8	1	0	0	0	0	0	0	0	0	0	2	2	3	4
16	1	0	2	0	0	0	0	0	0	0	1	1	5	4
32	4	0	2	0	0	0	0	0	2	1	3	1	**45**	32
64	**11**	5	**9**	5	0	0	0	0	2	0	5	0	**16**	11
128	**14**	1	**8**	3	0	0	0	0	**8**	3	6	2	21	19
200	**78**	8	**104**	5	2	0	0	0	**17**	6	**28**	12	7	14
>200		93		113		2		0		19		27		14

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4
BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan
MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration * = µg/ml
Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

3.4 Identification of endophytic fungi

Only 21 endophytic fungi from 150 isolates showed highly antimicrobial activity. Of these, eight fungal endophytes showed interesting NMR profiles (MA34, MA60, MA70, MA71, MA82, MA92, MA125 and MA194). They were further identified using morphological and molecular techniques.

3.4.1 Morphological identification

The morphological characteristics of all 21 active endophytic fungi are different as shown in Table 12 and Figures 14-16. All endophytes are septate, 13 strains possess hyaline hyphae and eight isolates have pigmented hyphae. The majority of the selected isolates are fast growing except MA2, MA12, MA82 and MA148 which are slow growers. Only nine isolates sporulate on PDA including, MA21, MA70, MA71, MA90, MA92, MA99, MA148, MA165 and MA177.

Five endophytic fungi (MA21, MA92, MA99, MA165 and MA177) are identified as *Pestalotiopsis* sp. based on morphological characterization. The conidia are spindle-shaped, 4-septate, hyaline apical cell, brown median cell, 2-3 apical appendages and a single basal appendage (Figure 15). Endophytic fungus MA90 could be identified as *Bipolaris bicolor* based on macroscopic and microscopic observations.

The conidia size of *Bipolaris bicolor* ranged from 30 to 78 µm long and from 12.1 to 17.3 µm wide. It presented 5 to 9 pseudosepta, generally six. They were straight, ellipsoid, with round edges, occasionally similar to an inverted club, rarely curve, from medium brown to dark brown in color. In more advanced developmental stages, cells from the edges tend to become more hyaline and pseudosepta in these cells seemed to be thicker, whereas the central part became almost black and made pseudosepta impossible to count.

Although, endophytic fungi MA70, MA71 and MA148 sporulated on media, however they could not be classified to generic and species levels.

Endophytic fungi MA34 and MA132 are the Xylariaceous taxa, because they produced the xylariaceous-like stromata on PDA plate (Figure 16) but

no spores or any conidia were found. The stromata of endophytic fungus MA34 have pale orange color, short and tapering and MA132 have short, thin and pale brown color stromata.

Table12 Antimicrobial (AM), interesting NMR profile (NMR) and morphological identification of 21 active isolates

Code	Growth rate	Colony character	Hyphae colour	spore	Identification	AM	NMR
MA2	S	dark brown and pale yellow, smooth	dark	X	Unidentified	√	
MA12	S	dark brown and smooth	dark	√	Unidentified	√	
MA21	F	colony whitish and turning pale pink, cottony	hyaline with pigmented conidia	√	*Pestalotiopsis* sp.	√	
MA34	F	colony whitish and cottony, develop stromata	hyaline	X	Xylariales sp.	√	√
MA60	F	colony at first white and becoming dark brown from the centre, develop conidiomata	dark	X	Unidentified	√	√
MA70	F	colony whitish and turning pale orange	hyaline	√	Unidentified	√	√
MA71	F	colony white to brown and turning dark brown	dark	√	Unidentified	√	√
MA81	F	colony at first white and becoming dark brown from the centre, develop conidiomata	dark	X	Unidentified	√	
MA82	S	colony greenish and turning black, roughened	dark	X	Unidentified	√	√
MA90	F	colony whitish-gray, cottony.	hyaline with dark conidia	√	*Bipolaris bicolor*	√	

Growth rate : F = growth full on plate in 5-7 days S = growth full on plate > 1 week

Septate : √ = septate X = non-septate

Table12 (cont.) Antimicrobial (AM), interesting NMR profile (NMR) and morphological identification of 21 active isolates

Code	Growth rate	Colony character	Hyphae colour	spore	Identification	AM	NMR
MA92	F	colony of whitish mycelia, cottony, small blackish acervular conidiomata	hyaline with pigmented conidia	√	*Pestalotiopsis* sp.	√	√
MA96	F	colony at first white, after 2–3 days becoming dark green from the centre, develop conidiomata	dark	X	Unidentified	√	
MA99	F	colony of whitish mycelia, small blackish acervular conidiomata	hyaline with pigmented conidia	√	*Pestalotiopsis* sp.	√	
MA125	F	colony at first pale brown, after 3-5 days becoming dark brown, smooth	dark	X	Unidentified	√	√
MA132	F	colony whitish and cottony, develop stromata	hyaline	X	Xylariales sp.	√	
MA148	S	colony whitish and smooth	hyaline	√	Unidentified	√	
MA156	F	colony at first pale brown and becoming dark brown, smooth	hyaline	X	Unidentified	√	
MA164	F	colony pale orange and smooth	hyaline	X	Unidentified	√	

Growth rate : F = growth full on plate in 5-7 days S = growth full on plate > 1 week

Septate : √ = septate X = non-septate

Table12 (cont.) Antimicrobial (AM), interesting NMR profile (NMR) and morphological identification of 21 active isolates

Code	Growth rate	Colony character	Hyphae colour	spore	Identification	AM	NMR
MA165	F	colony of whitish mycelia, small blackish acervular conidiomata	hyaline with pigmented conidia	√	*Pestalotiopsis* sp.	√	
MA177	F	colony of whitish mycelia, later developing into yellowish colony with small blackish acervular conidiomata	hyaline with pigmented conidia	√	*Pestalotiopsis* sp.	√	
MA194	F	colony at first white, after 2–3 days becoming dark green from the centre, develop conidiomata	hyaline	X	Unidentified	√	√

Growth rate : F = growth full on plate in 5-7 days S = growth full on plate > 1 week

Septate : √ = septate X = non-septate

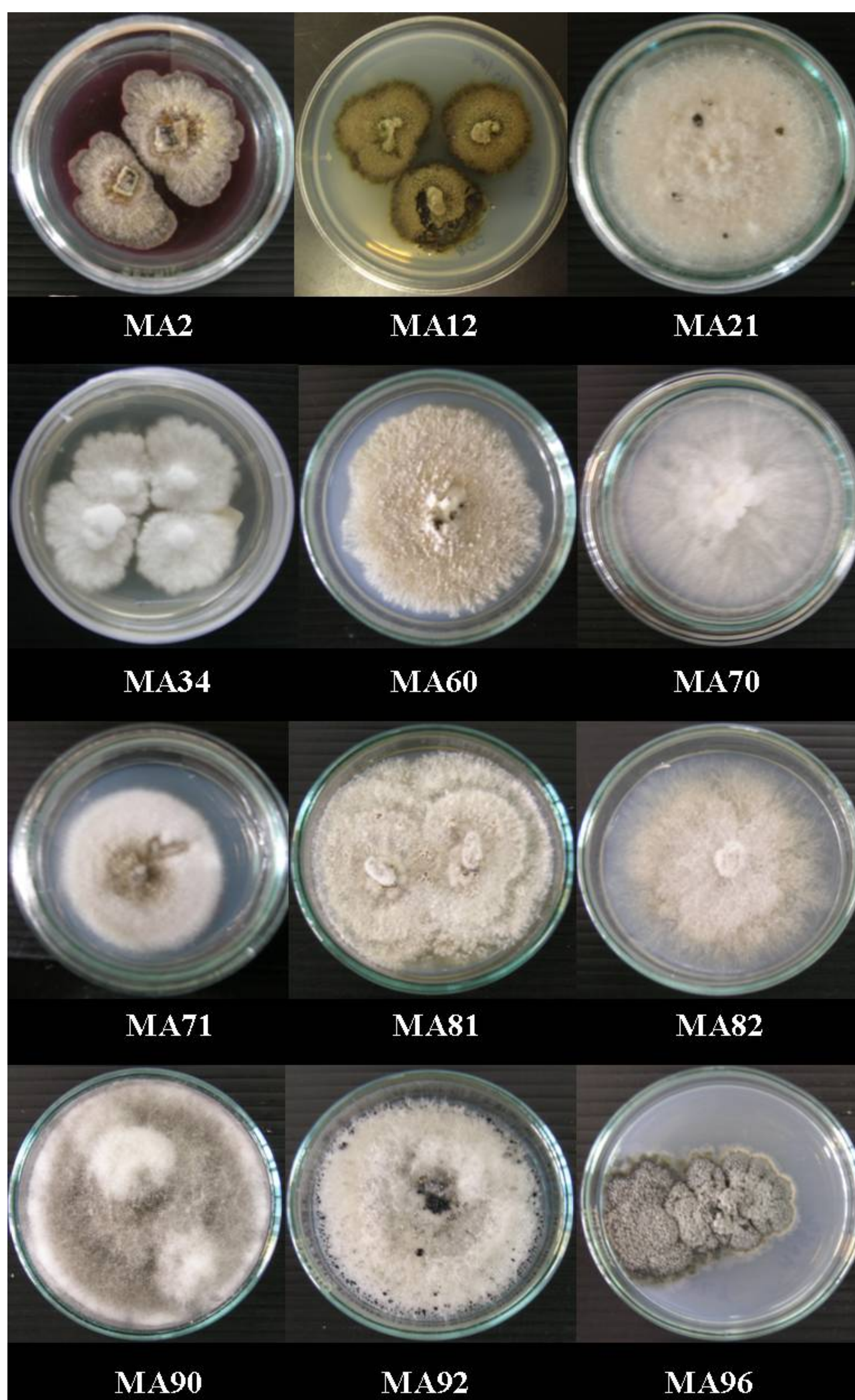


Figure 14 Endophytic fungi 21 isolates produced strong antimicrobial activity and/or interesting NMR profile

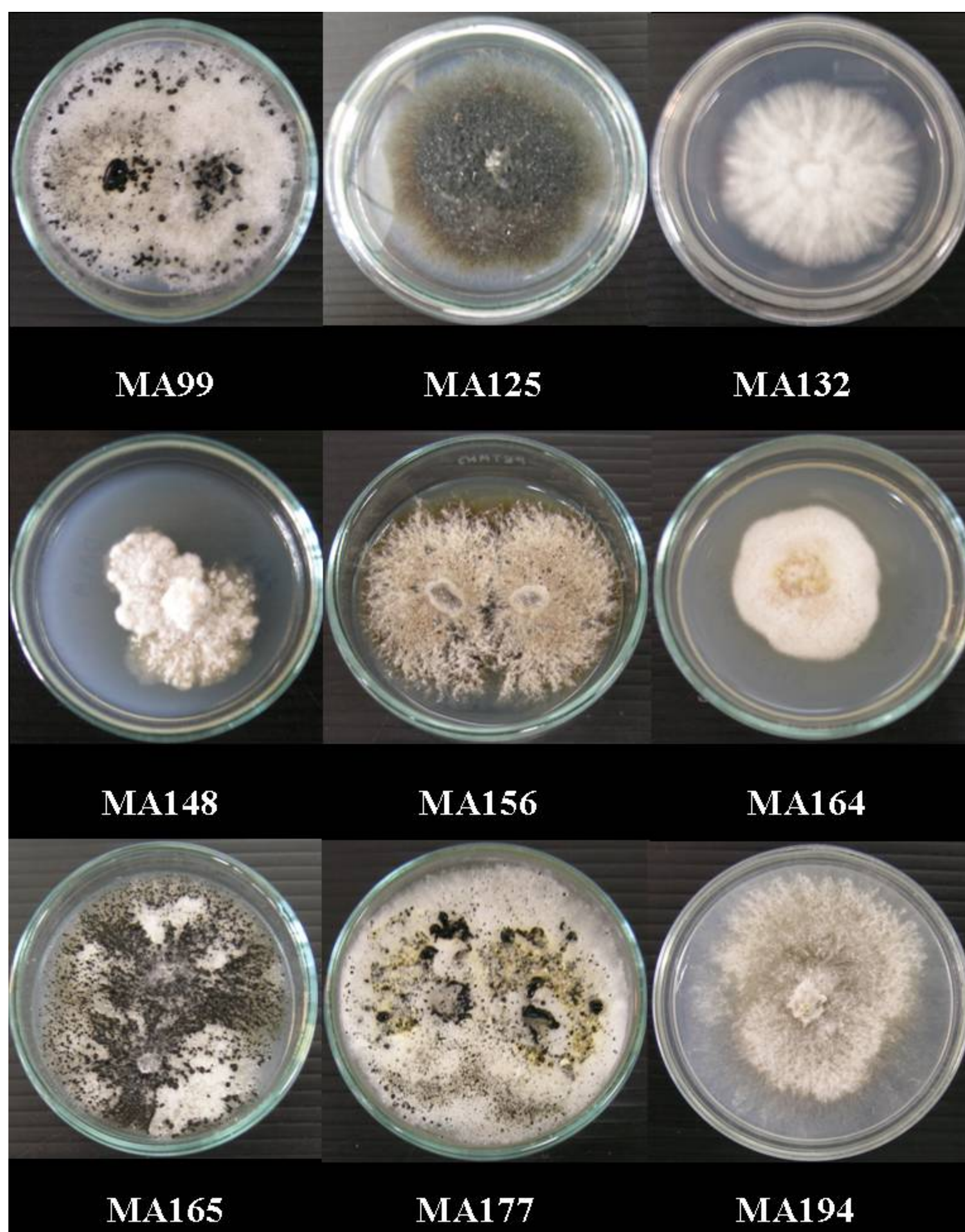
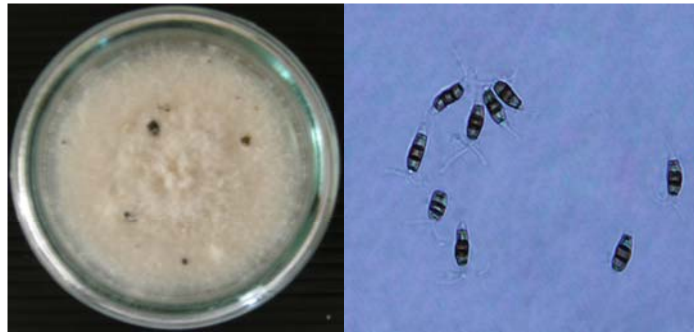
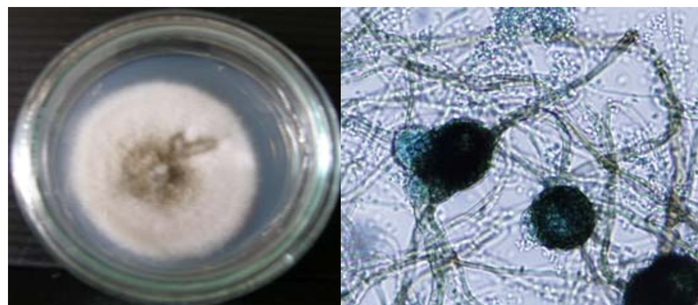


Figure 14 (cont.) Endophytic fungi 21 isolates produced strong antimicrobial activity and/or interesting NMR profile



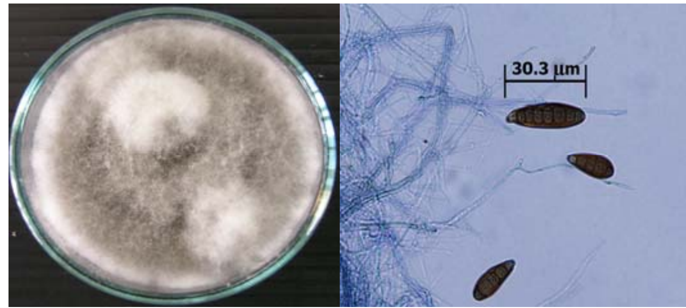
MA21: *Pestalotiopsis* sp.

MA70: unidentified fungus



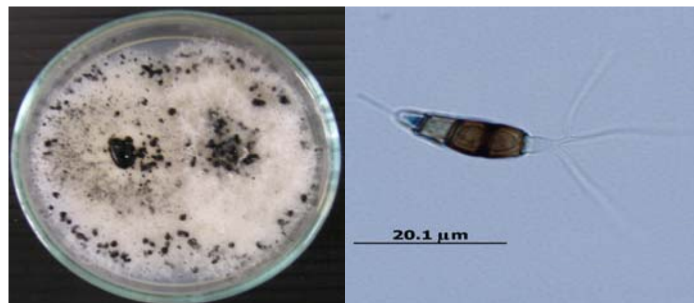
MA71: unidentified ascomycete

Figure 15 Colonies and conidia of nine active endophytic fungi and their identification



MA90: *Bipolaris bicolor*

MA92: *Pestalotiopsis* sp.

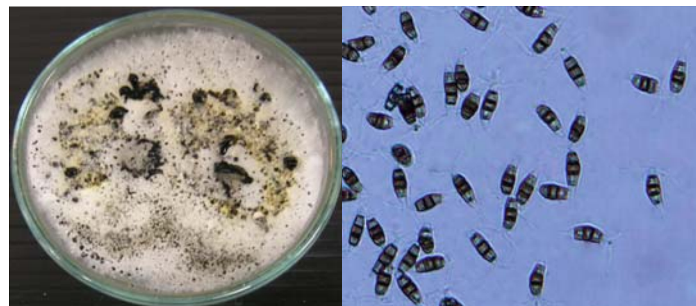


MA99: *Pestalotiopsis* sp.

Figure 15 (cont.) Colonies and conidia of nine active endophytic fungi and their identification

MA148: unidentified fungus

MA165: *Pestalotiopsis* sp.



MA177: *Pestalotiopsis* sp.

Figure 15 (cont.) Colonies and conidia of nine active endophytic fungi and their identification

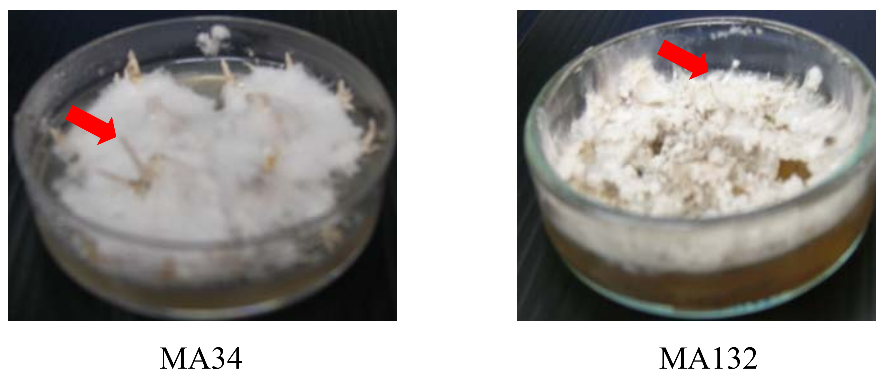


Figure 16 Stromata production on PDA media of MA34 and MA132

3.4.2 Molecular identification

Data from molecular and phylogenetic analyses were used to characterize the taxonomic position of the endophytic fungi isolated from mangrove plants. LSU and ITS1-5.8S-ITS2 rDNA sequences of 21 active strains were compared and analysed with sequences obtained from the GenBank database.

Results from BLAST search (Table 13) revealed that 15 endophytic fungi belong to phylum Ascomycota in five orders: Hypocreales, Xylariales, Diaporthales, Capnodiales and Pleosporales. LSU and ITS sequencing results of MA34 and MA132 were not satisfactory, therefore the data for them was not presented here. The rDNA region of endophytic fungi MA82, MA90, MA99 and MA148 could not be able to amplify.

Table 13 Summary of the molecular identification of active endophytic fungi using ribosomal DNA sequence analyses

Phylum	Class	Order	rDNA region	BCC code	Original code	Accession number	Morphological identification	Expected species	% ITS sequence identity
Ascomycota	Dothideomycetes	Capnodiales	ITS	35893	MA12*	GU591997	Unidentified fungus	Mycosphaerellaceae sp.	-
			LSU			GU592008			
		Pleosporales	ITS	35915	MA71*	GU592001	Unidentified ascomycota	*Didymella bryoniae*	98.7
			LSU			GU592011			
			ITS	35905	MA164	GQ254687	Unidentified fungus	Melanommataceae sp.	-
			LSU			GQ254669			
	Sordariomycetes	Diaporthales	ITS	35910	MA2	GQ254676	Unidentified fungus	*Diaporthe* sp.	95.8
			ITS	35913	MA60	GU591999	Unidentified fungus	*Phomopsis eucommii*	96.7
			LSU			GU592009			
			ITS	35916	MA81	GQ254678	Unidentified fungus	Diaporthaceae sp.	-
			LSU			GQ254662			
			ITS	35919	MA96	GU592003	Unidentified fungus	*Phomopsis* sp.	97.3
			LSU			GU592013			
			LSU	35895	MA125	GU592014	Unidentified fungus	*Phomopsis* sp.	-
			ITS	35909	MA194	GU592007	Unidentified fungus	*Phomopsis* sp.	99.4
			LSU			GU592018			

* Reproductive structure production

Table 13 (cont.) Summary of the molecular identification of active endophytic fungi using ribosomal DNA sequence analyses

Phylum	Class	Order	Gene Sequence	BCC code	Original code	Accession number	Morphological identification	Expected species	% ITS sequence identity
Ascomycota	Sordariomycetes	Hypocreales	ITS	35914	MA70	GU592000	Unidentified fungus	*Acremonium* sp.	-
			LSU			GU592010			
		Xylariales	ITS	35911	MA21*	GU591998	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	99.8
			ITS	35918	MA92*	GU592002	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	100
			LSU			GU592012			
			ITS	35906	MA165*	GU592005	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	100
			LSU			GU592016			
			ITS	35908	MA177*	GU592006	*Pestalotiopsis* sp.	*Pestalotiopsis* sp.	99.8
			LSU			GU592017			
			ITS	35903	MA156	GU592004	Unidentified fungus	*Hypoxylon* sp.	97
			LSU			GU592015			

* Reproductive structure production

Order Capnodiales

In this study, only one strain of endophytic fungus MA12 was placed in the Capnodiales. All of taxa retrieved from GenBank and MA12 were analysed using Maximum Parsimony (MP) and Distance Neighbor Joining (NJ). The LSU rDNA alignment consisted of 16 taxa, with *Paullicorticium ansatum* AY586693 from the Corticiales as an outgroup. The dataset comprised total 1301 characters, gaps were included in the analysis. Of these characters, 63 characters were parsimony informative, 177 characters were parsimony uninformative and 1061 characters were constant. MP analysis yielded eight most parsimonious trees (MPTs). They had identical topology and were not statistically different. The best tree estimated from K-H test is shown in Figure 17, with tree length, CI and RI of 303 steps, 0.8878 and 0.8583, respectively. Tree obtained from NJ analysis gave similar topology to the tree from MP analysis. Thus, only MPT is shown and discussed here.

Endophytic fungus MA12 grouped well in subclade A (Figure 17) containing taxa from the family Mycosphaerellaceae, the Capnodiales. This subclade comprised *Mycosphaerella marksii* DQ246249, *M. intermedia* DQ246248, *M. parkii* DQ246245, *M. madeirae* DQ204756 and *Pseudocercospora epispermogonia* DQ204758. This group received MP and NJ bootstrap values of 54% and 52%, respectively.

The result from LSU rDNA was consistent to the result of ITS rDNA analysis. The ITS rDNA dataset consisted of 14 taxa from family Mycosphaerellaceae, with *Dothidea hippophaeos* AF027763 and *Davidiella macrospora* EF679369 as the outgroups. MP analysis, with total character 585 characters resulted in 381 constant characters, 86 parsimony informative characters and 118 parsimony uninformative characters. This yielded four MPTs with tree length of 311 steps, CI of 0.8232 and RI of 0.7660 (Figure 18).

Endophytic fungus MA12 was well placed in subclade A (Figure 18) and closely related with *Stenella musae* EU514293, *Stenella musae* FJ441662, *Stenella musae* FJ441627 and Mycosphaerellaceae sp. EF694667. This group received highly bootstrap values from MP and NJ analyses of 93% and 99%, respectively. Nucleotide similarity between MA12 and taxa within subclade A varied from 97.5-

99.3%. While *Stenella musae* EU514293 and Mycosphaerellaceae sp. EF694667 were the most closely related taxa with 99.3% sequence similarity. ITS sequence of MA12 had the base insertion 1 and 2 bp at position site 358 and 362-363 respectively, compared with *Stenella musae* EU514293 and Mycosphaerellaceae sp. EF694667. Therefore, the data from molecular characteristics could identify MA12 as Mycosphaerellaceae sp.

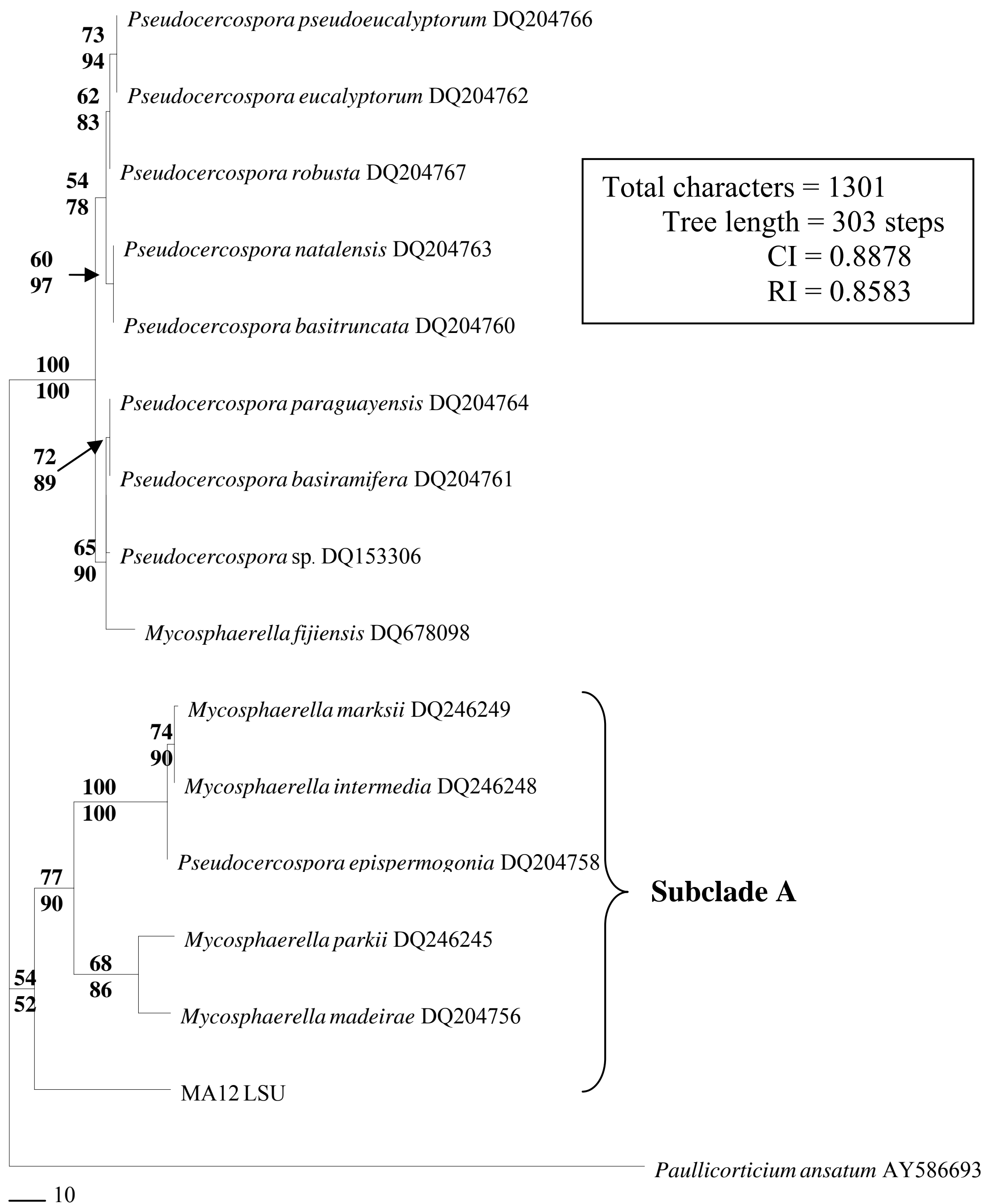


Figure 17 Phylogram obtained from LSU rDNA sequence analysis of endophytic fungus MA12 and fungi from the Capnodiales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

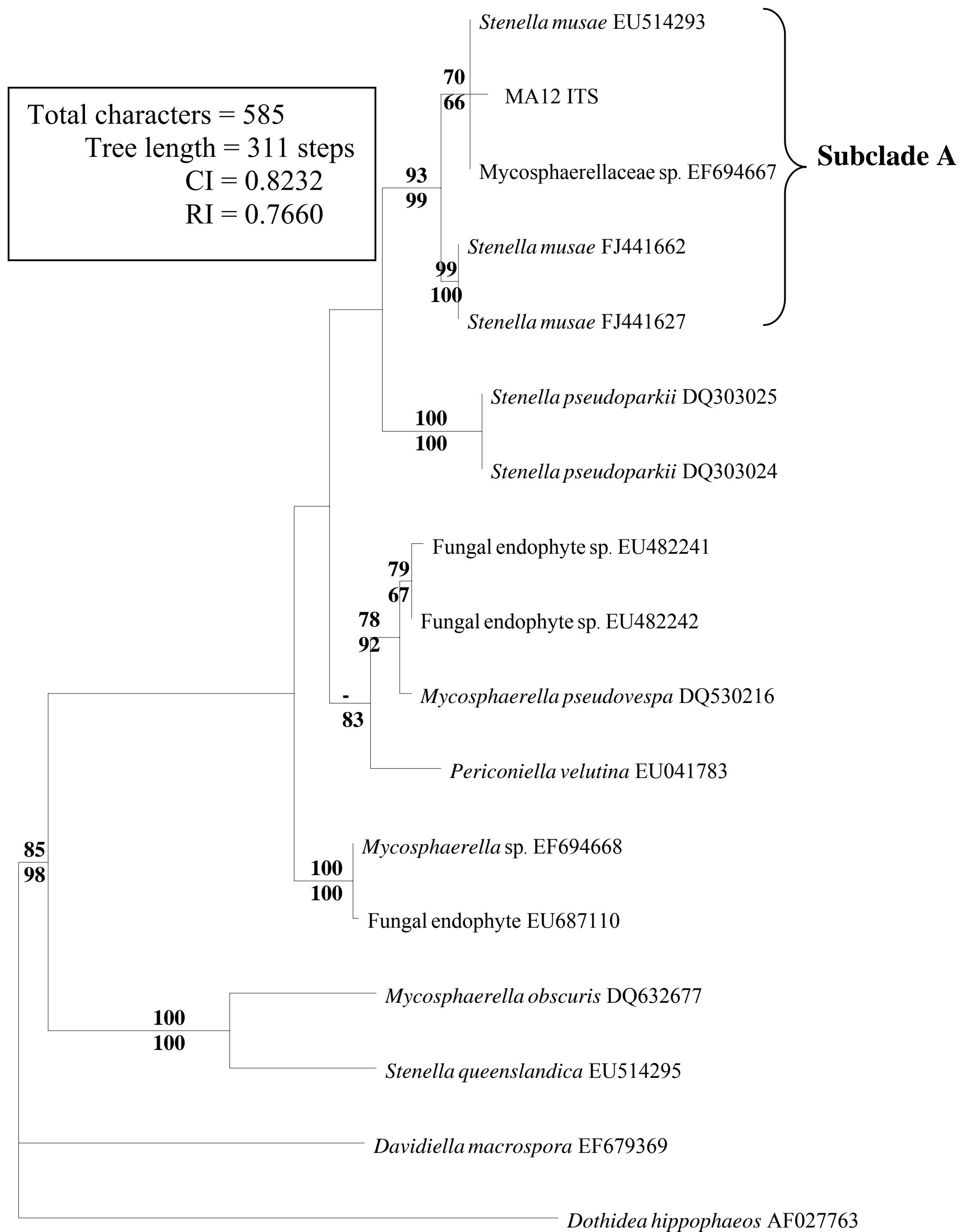


Figure 18 Phylogram obtained from ITS rDNA sequence analysis of endophytic fungus MA12 and fungi from the Capnodiales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

Order Pleosporales

The endophytic fungi MA71 and MA164 were placed in the Pleosporales. The LSU rDNA sequences of MA71 and MA164 were compared to sequences obtained from the GenBank database using MP and NJ analyses. The LSU dataset contained 24 taxa, with *Mytilinidion scolecosporum* FJ161186, *Mytilinidion resinicola* FJ161185 and *Mytilinidion rhenanum* FJ161175 as the outgroups. MP analysis, with a total of 1281 characters resulted in 1144 constant characters, 27 parsimony uninformative characters and 110 parsimony informative characters. The result yielded 2 MPTs with tree lengths, CI and RI of 203 steps, and 0.7340 and 0.9053, respectively. The best tree from the K-H test is shown in Figure 19. The tree from MP and NJ analysis showed identical topology. Thus, only the MPT tree is shown and discussed here.

The endophytic fungus MA71 was placed in subclade A (Figure 19) containing taxa from the Pleosporales incertae sedis. This subclade comprising *Didymella clematidia* (FJ515634, FJ515633, FJ515645) and *Didymella cucurbitacearum* AY293792 was supported with a 56% and 64% bootstrap obtained from the MP and NJ analyses, respectively.

The endophytic fungus MA164 was placed in subclade B (Figure 19) containing taxa from the Melanommataceae, order Pleosporales and was closely related to *Bimuria novae-zelandiae* with high bootstrap values; 91% from the MP and 88% from the NJ.

The result of the LSU rDNA analysis was consistent with the result of ITS rDNA analysis. The ITS rDNA dataset contained 30 taxa, with *Cochliobolus lunatus* FJ792584 and *Cochliobolus* sp. FJ235087 as the outgroups. Maximum parsimony analysis, with a total of 817 characters resulted in 517 constant characters, 237 parsimony informative characters and 63 parsimony uninformative characters. This yielded 1000 MPTs with tree lengths, CI, and RI of 515 steps, and 0.8252 and 0.9378, respectively (Figure 20).

The endophytic fungus MA71 was placed in subclade A (Figure 20) and was closely related to *Didymella bryoniae* with a short branch length. The nucleotide identity between the MA71 and other taxa within this subclade varied from

98.4%-98.7%. *Didymella bryoniae* AB266846 was closely related to the taxa with a 98.7% ITS sequence similarity. The MA71-ITS sequence showed a 1 bp insertion at position sites 8 and 398, and a 1 bp deletion at position site182.

The endophytic fungus MA164 was placed in subclade B (Figure 20) and was closely related with *Leptosphaerulina* sp. (EF694653, DQ092534) and *Leptosphaerulina chatarum* (DQ384571, EU272492, EU272493) with 100% bootstrapping from both the MP and NJ analyses. The nucleotide identity between the MA164 and taxa within this subclade varied from 74.7%-77.8%. *Leptosphaerulina chatarum* (DQ384571, EU272492, EU272493, FJ884104) were the most closely related taxa with a 77.8% ITS sequence similarity.

Therefore, the results from LSU and ITS rDNA analyses indicated that the endophytic fungus MA71 was identified as *Didymella bryoniae* and the endophytic fungus MA164 was identified as Melanommataceae sp.

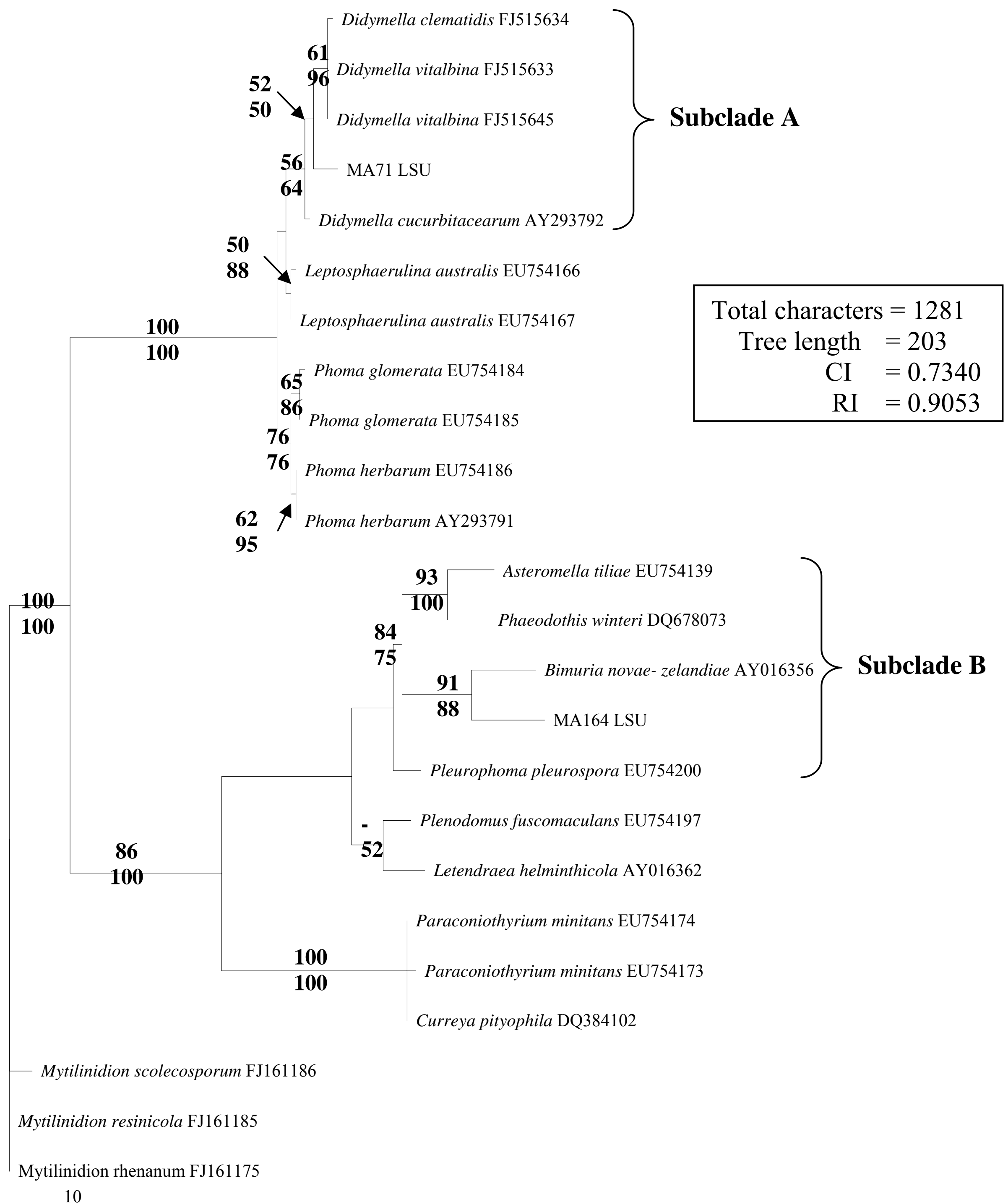


Figure 19 The phylogram obtained from LSU rDNA sequence analysis of the endophytic fungi MA71, MA164 and fungi from the Pleosporales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes.

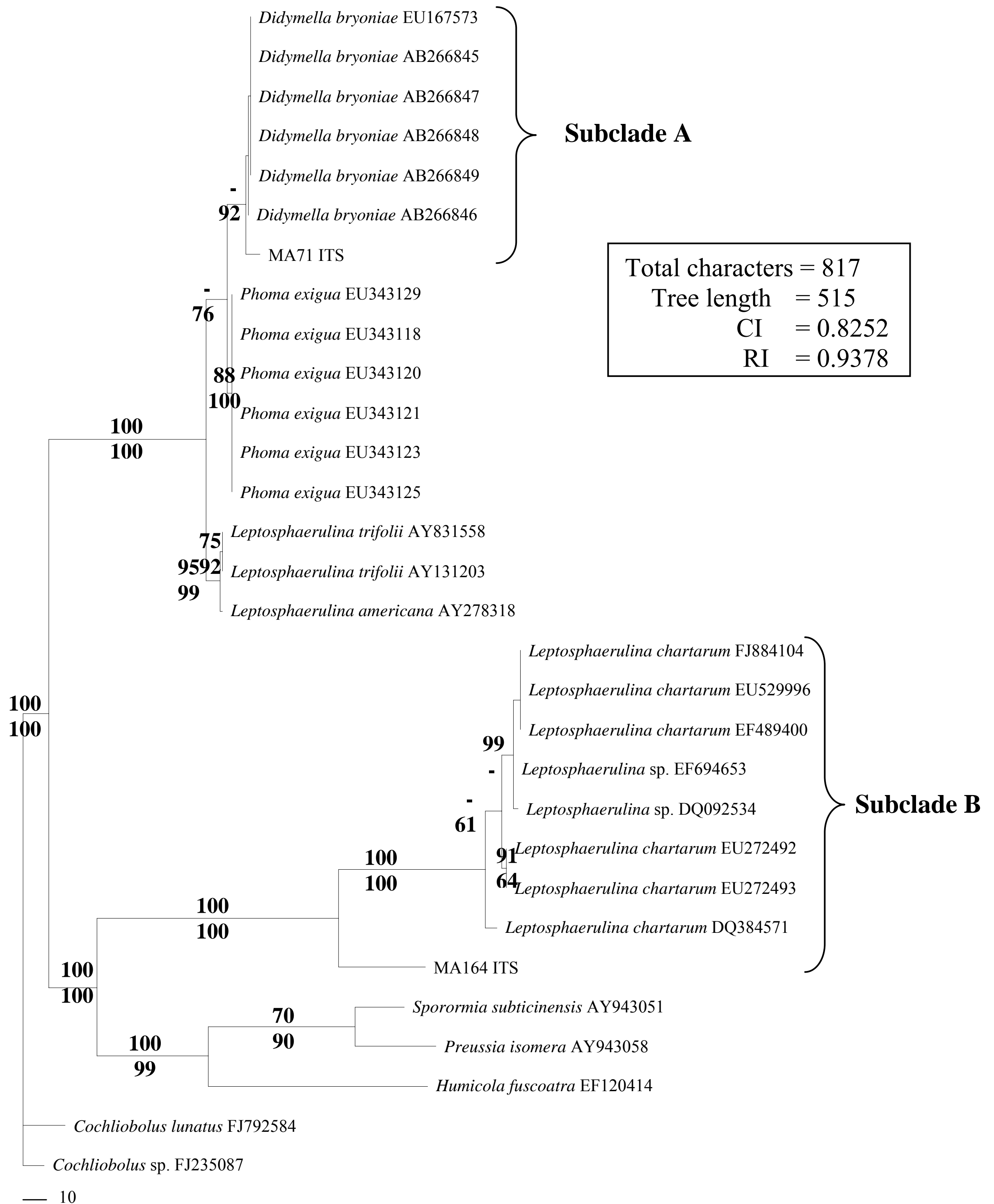


Figure 20 The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungi MA71, MA164 and fungi from the Pleosporales, generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes.

Order Diaporthales

Endophytic fungi MA2 (only ITS sequence was obtained), MA60, MA81, MA96, MA125 (only LSU sequences were obtained) and MA194 had an affinity with the Diaporthales. They clustered with different species of the genus *Phomopsis*. The LSU rDNA alignment consisted of 30 taxa, with *Apoharknessia insueta* AY720813, AY720814 as the outgroups. Maximum parsimony analysis, with total character 1319 characters resulted in 1199 constant characters, 61 parsimony informative characters and 59 parsimony uninformative characters. This yielded 1000 MPTs with tree length of 164 steps, CI of 0.7927 and RI of 0.8283, as shown the best tree from K-H test in Figure 21. Tree obtained from NJ analysis gave similar topology to the tree from MP analysis. Thus, only MPT is shown and discussed here.

Endophytic fungi MA60, MA96, MA81, MA125 and MA194 were placed in subclade A (Figure 21) and closely related with the fungi in the Diaporthaceae. This subclade comprised *Phomopsis asparagi* AF439634, AF439636, *Phomopsis sclerotioides* AF439631, *Phomopsis viticola* AF439635 and *Phomopsis* sp. EU219393 with low bootstrap support of 55% and 54% from MP and NJ analyses, respectively. Endophytic fungus MA125 was analysed only LSU rDNA sequence and closely related with *Phomopsis* sp. This was supported by 55% and 54% MP and NJ bootstrap support, respectively (Figure 21). Therefore, the result from LSU rDNA analysis could only be classified MA125 as *Phomopsis* sp.

The result from LSU rDNA analysis was consistent with the ITS rDNA analysis. The ITS rDNA dataset consisted of 33 taxa from the Diaporthaceae, with *Leucostoma persoonii* EF447375 and *Leucostoma cinctum* AF191169 as the outgroups. Maximum parsimony analysis was conducted with total character 643 characters resulted in 460 constant characters, 107 parsimony informative characters and 76 parsimony uninformative characters. This yielded 1000 MPTs with tree length of 370 steps, CI of 0.6189 and RI of 0.8169, as shown the best tree based on K-H test in Figure 22.

Endophytic fungus MA194 was placed in subclade A (Figure 22) and supported by 90% and 96% bootstrap value by MP and NJ analyses, respectively. MA194 formed a clade with *Phomopsis* sp. DQ235675 with 81% bootstrap by MP

analysis. MA194 sequence had relatively high similarity (97.8-99.4%) with four *Phomopsis* species (EU002931, AB247183, DQ235675, FJ037768). It was closely related to *Phomopsis* sp. DQ235675 with 99.4% sequence similarity, with 1 bp base substitution at position site 81, 83 and 1 bp base deletion at position site 141.

Endophytic fungi MA60 and MA96 were placed in subclade B as shown in Figure 22. MA96 and *Phomopsis* sp. EU002930 formed a terminal cluster with 55% MP bootstrap support (96.7% sequence similarity). Within this subclade MA60 clustered with *Phomopsis eucommii* AY601921 with low bootstrap supports (96.7% sequence similarity). MA81 grouped with *Phomopsis* sp. EU395772 and *Diaporthe* sp. EF488434 in subclade C but not supported by any bootstrap value (Figure 21). Endophytic fungus MA2 was placed in subclade D, formed a monophyletic clade with uncultured *Diaporthe* EF619687 with a strong bootstrap support of 85% by NJ (Figure 22) and showed 95.8% sequence similarity. Therefore, the result from ITS rDNA analysis could classify MA2 as *Diaporthe* sp.

According to the results from LSU rDNA and ITS rDNA analyses, MA60 could be identified as *Phomopsis eucommii* and MA96, MA194 as *Phomopsis* sp. MA81 could only be verified only to family level, the Diaporthaceae but not closely related with any species except for *Phomopsis* sp. and *Diaporthe* sp.

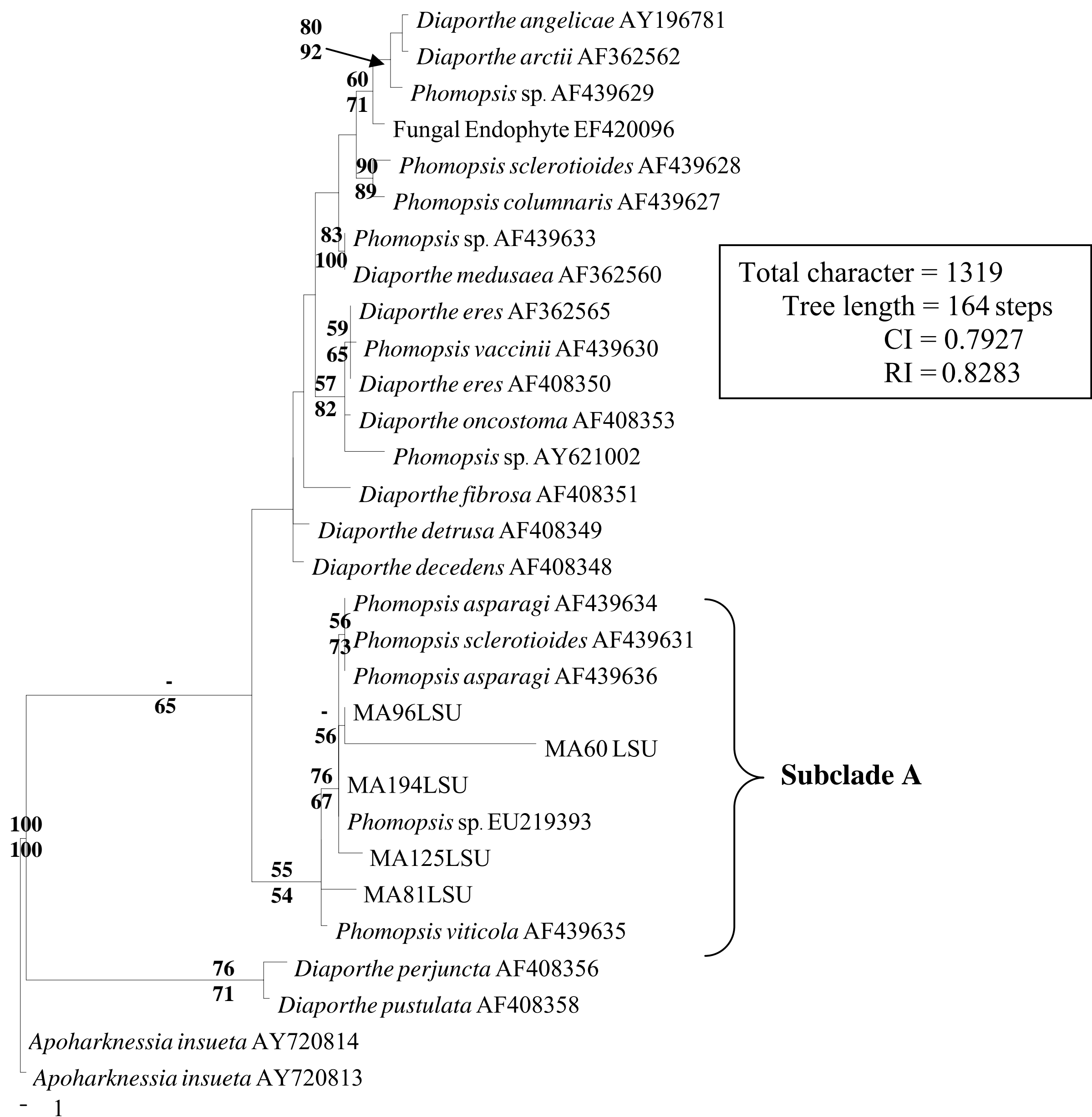


Figure 21 Phylogram obtained from LSU rDNA sequence analysis of endophytic fungi MA60, MA81, MA96, MA125, MA194 and fungi from the Diaportales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

Total characters = 646
Tree length = 349 steps
CI = 0.6304
RI = 0.8186

Phomopsis sp. EU219395
Phomopsis bougainvilleicola AY601920
Phomopsis sp. EU002920
Diaporthe phaseolorum EF488431
Phomopsis oryzae AF079777
Diaporthe sp. EF423521
Phomopsis sp. DQ235675
MA194 ITS
Phomopsis sp. FJ037768
Phomopsis sp. EU002931
Phomopsis sp AB247183
Subclade A
Phomopsis eucommii AY601921
MA60 ITS
Phomopsis sp. EU002930
MA96 ITS
Phomopsis phyllanthicola EF488375
Subclade B
Phomopsis sp. AB286211
Phomopsis loropetali AY601917
Diaporthales sp. AB255245
Phomopsis phyllanthicola AY620819
Phomopsis sp. AF103000
Diaporthe sp. DQ435767
Phomopsis sp. FJ037761
Phomopsis sp. DQ780461
Diaporthe phaseolorum EF488420
Diaporthe phaseolorum AF001017
Phomopsis sp. EU395772
Diaporthe sp. EF488434
MA81 ITS
Subclade C
Phomopsis glabrae AY601918
Phomopsis sp. DQ780436
Diaporthe sp. FJ799940
Diaporthe sp. EF423529
Fungal sp. EU563513
Fungal endophyte sp. EU977305
Uncultured *Diaporthe* EF619687
MA2 ITS
Subclade D
Leucostoma persoonii EF447375
Leucostoma cinctum AF191169
10

Figure 22 Phylogram obtained from ITS rDNA sequence analysis of endophytic fungi MA2, MA60, MA81, MA96, MA194 and fungi from the Diaportales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

Order Hypocreales

Endophytic fungus MA70 was placed in the Hypocreales. LSU rDNA sequence of MA70 was compared to sequences of fungi represented from the GenBank database. MP and NJ methods were used in this study. The LSU rDNA dataset contained 17 taxa of total 1275 characters, with *Fusarium oxysporum* EF590327 and *Cordyceps crassispora* AB067706 as the outgroups. Parsimony analysis yielded number of constant characters, parsimony informative, parsimony uninformative of 1108, 103 and 64 characters, respectively. The maximum parsimony analysis resulted in 15 most parsimonious trees. The best tree estimated from K-H test is shown in Figure 23, with tree length, CI and RI of 381 steps, 0.5354 and 0.4928, respectively. Tree obtained from NJ analysis gave similar topology to the tree from MP analysis. Thus, only MPT is shown and discussed here.

MA70 was related to *Acremonium alternatum* FJ176883, *Stilbocrea macrostoma* AY489725 and *Stephanonectria keithii* AY489727 in subclade A with 71% bootstrap value from MP and NJ analyses (Figure 23).

The result from LSU rDNA analysis was consistent to the result of ITS rDNA analysis. The ITS rDNA dataset consisted of 19 taxa from anamorphic Hypocreales and unclassified Hypocreales, with *Fusarium lateritium* AY781222 and *Fusarium lateritium* FJ228190 as the outgroups. Maximum parsimony analysis with total character 495 characters resulted in 288 constant characters, 177 parsimony informative characters and 30 parsimony uninformative characters. This yielded two MPTs with tree length of 488 steps, CI of 0.7131 and RI of 0.7738 (Figure 24). Tree obtained from NJ analysis gave similar topology to the tree from MP analysis. Thus, only MPT is shown and discussed here.

MA70 was placed in subclade A (Figure 24), with Hypocreales sp. EU164804, *Acremonium crotocinigenum* DQ882846 and *Acremonium crotocinigenum* AJ621773 as sister taxa (98% MP and 100% NJ bootstrap supports). Sequence similarity between MA70 and taxa within this subclade varied from 91-100%. Therefore, the results from LSU and ITS rDNA analyses suggested that endophytic fungus MA70 could be identified as *Acremonium* sp.

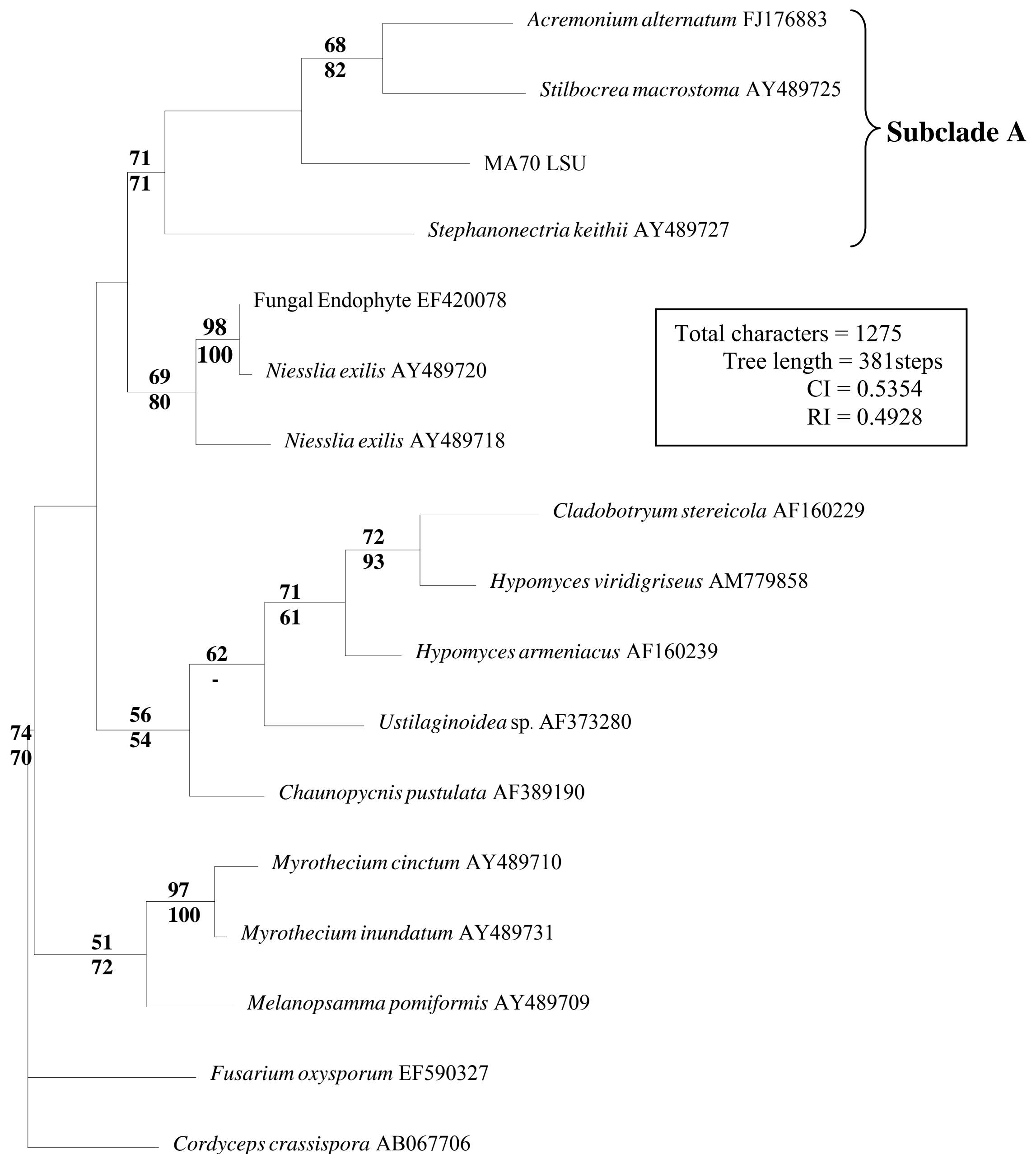


Figure 23 Phylogram obtained from LSU rDNA sequence analysis of endophytic fungus MA70 and fungi from the Hypocreales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

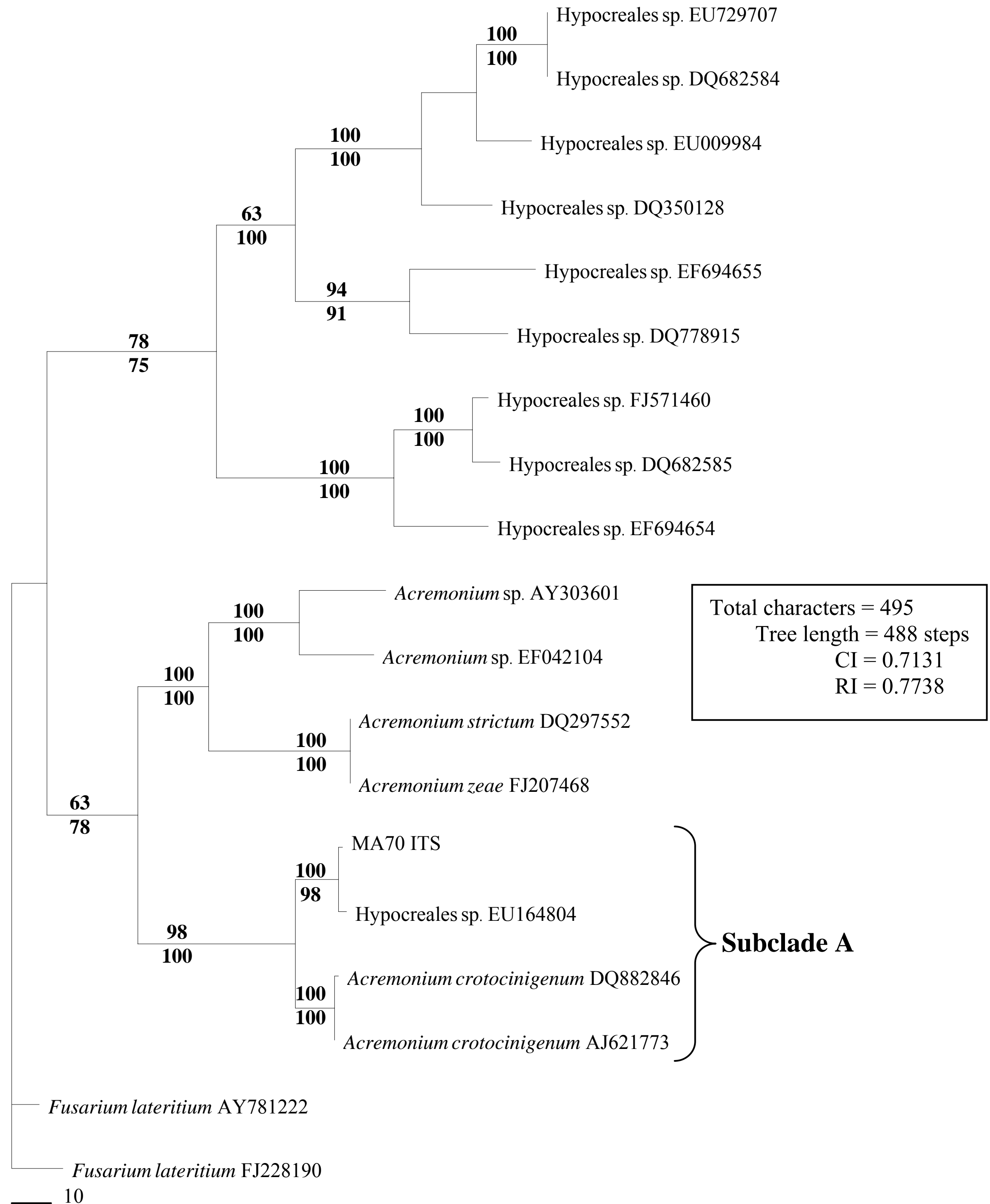


Figure 24 Phylogram obtained from ITS rDNA sequence analysis of endophytic fungus MA70 and fungi from the Hypocreales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below branches, respectively. Branch lengths are proportional to number of step changes.

Order Xylariales

Five endophytic fungi (MA21, MA92, MA156, MA165, MA177) were placed in the Xylariales. The endophytic fungus MA21 was studied using only the ITS rDNA.

The LSU rDNA alignment consisted of 13 taxa in the Xylariaceae, with *Nigrospora* sp. EU852533 and *Nigrospora oryzae* FJ176892 as an outgroup. The dataset comprised 1299 characters, 1128 of which were constant characters, 49 were uninformative characters and 122 were informative characters. Maximum parsimony analysis yielded 12 MPTs. The best tree as estimated by the K-H test is shown in Figure 25, with tree lengths CI and RI of 298 steps, and 0.6946 and 0.7874, respectively. The tree from the NJ analysis had an identical topology to the tree from the MP analysis. Thus, only the tree from the MP analysis is shown and discussed here.

The endophytic fungus MA156 was placed in subclade A (Figure 25) and was closely related to the fungi in the family Xylariaceae, comprising Xylariaceae sp. (DQ327625, DQ674832, DQ674834) with high bootstrap values of 99% and 100% by MP and NJ analyses, respectively.

The endophytic fungi MA92, MA165 and MA177 were placed in subclade B (Figure 25), and were closely related to *Xylaria* sp. AB073534 with 67% and 73% bootstrap values from the MP and NJ analyses, respectively.

The LSU rDNA analysis that resulted from the MP and NJ analyses was consistent with the result of the ITS rDNA analysis, as shown in Figures 25 and 26. The endophytic fungi MA21, MA92, MA165 and MA177 were closely related to different species in the genera *Pestalotiopsis*, with *Sarcostoma bisetulatum* EU552155 and *Sarcostoma restionis* DQ278923 as an outgroup (Figures 26). The dataset comprised 629 characters, with 533 constant characters, 15 uninformative characters and 81 informative characters. Maximum parsimony analysis yielded 1000 MPTs with tree lengths, CI and RI of 119 steps, and 0.9076 and 0.9771, respectively. The tree from NJ and MP analyses showed identical topologies. Thus, only the tree from the MP analysis is shown and discussed here.

The endophytic fungus MA92 was placed in subclade A (Figure 26) containing taxa from the genera *Pestalotiopsis*. This subclade comprised *Pestalotiopsis versicolor* AF405298, *Pestalotiopsis clavispora* AY682927, *Pestalotiopsis palmarum* AF409990 and *Pestalotiopsis* sp. AF409989. This group received a low bootstrap value of 61% from the MP analysis and was not supported with the bootstrap value from NJ analysis. The nucleotide identity between MA92 and taxa within this subclade varied from 99.8%-100%. *Pestalotiopsis versicolor* AF405298 and *Pestalotiopsis* sp. AF409989 were the most closely related with 100% ITS sequence similarity.

The endophytic fungi MA165 and MA177 were placed in subclade B (Figure 26). This subclade comprised *Pestalotiopsis diospyri* DQ417181, *Pestalotiopsis aquatica* AF409956, *Pestalotiopsis oxyanthi* AY687876, *Pestalotiopsis* sp. EU644754 and *Pestalotiopsis* sp. AF409963 and had a short branch length. The alignment result showed that the MA165 and MA177 sequences had a relatively high similarity of 99.2%. Furthermore, MA165 had high nucleotide similarities with taxa in this subclade, that varied from 99.6%-100%. MA165 was closely related to *Pestalotiopsis diospyri* DQ417181, *Pestalotiopsis* sp. EU644754 and *Pestalotiopsis* sp. AF409963 with a 100% ITS sequence similarity. The MA177 had high nucleotide similarities with other taxa in this subclade, that varied from 98.8%-99.2%. The MA177 was closely related to *Pestalotiopsis diospyri* DQ417181, *Pestalotiopsis oxyanthi* AY687876, *Pestalotiopsis* sp. EU644754 and *Pestalotiopsis* sp. AF409963 with 99.2% ITS sequence similarities. The ITS alignment result of the MA177 and related taxa showed that MA177 had a 1 bp insertion at position site 428 and a 2 bp insertion at position site 363-364.

The endophytic fungus MA21 was placed in subclade C (Figure 26). This subclade comprised *Pestalotiopsis adusta* AY687298, *Pestalotiopsis disseminata* AY687869, *Pestalotiopsis heterocomis* (AY681491, AY681492) *Pestalotiopsis olivacea* AY681486, *Pestalotiopsis neglecta* (AY682931, FJ037759) and *Pestalotiopsis microspora* (AY924273, EF488418) was supported with a bootstrap value of 100% from both the MP and NJ analysis. The nucleotide identity between MA21 and other taxa within this subclade varied from 99.6%-99.8%, with *Pestalotiopsis microspora* AY924273, *Pestalotiopsis neglecta* AY682931,

Pestalotiopsis disseminata AY687869 and *Pestalotiopsis olivacea* AY681486 being the most closely related taxa with a 99.8% ITS sequence similarity. The MA12-ITS sequence and related taxa alignment result showed that MA21 had a 1 bp insertion at position site 490.

The endophytic fungus MA156 was placed in subclade A (Figure 27) containing taxa from the family Xylariaceae. This subclade comprised *Hypoxylon* sp. DQ322113 and DQ322128 and was supported with 100 % bootstrapping from both the MP and NJ analyses. The endophytic fungus MA156 was closely related to *Hypoxylon* sp. DQ322113 with 99% and 86% bootstrapping and showed a 97% sequence similarity.

Therefore, the endophytic fungus MA92 was identified as *Pestalotiopsis vesicolor*. The endophytic fungi MA21, MA165 and MA177 were identified to the genus *Pestalotiopsis*. The endophytic fungus MA156 was identified as *Hypoxylon* sp.

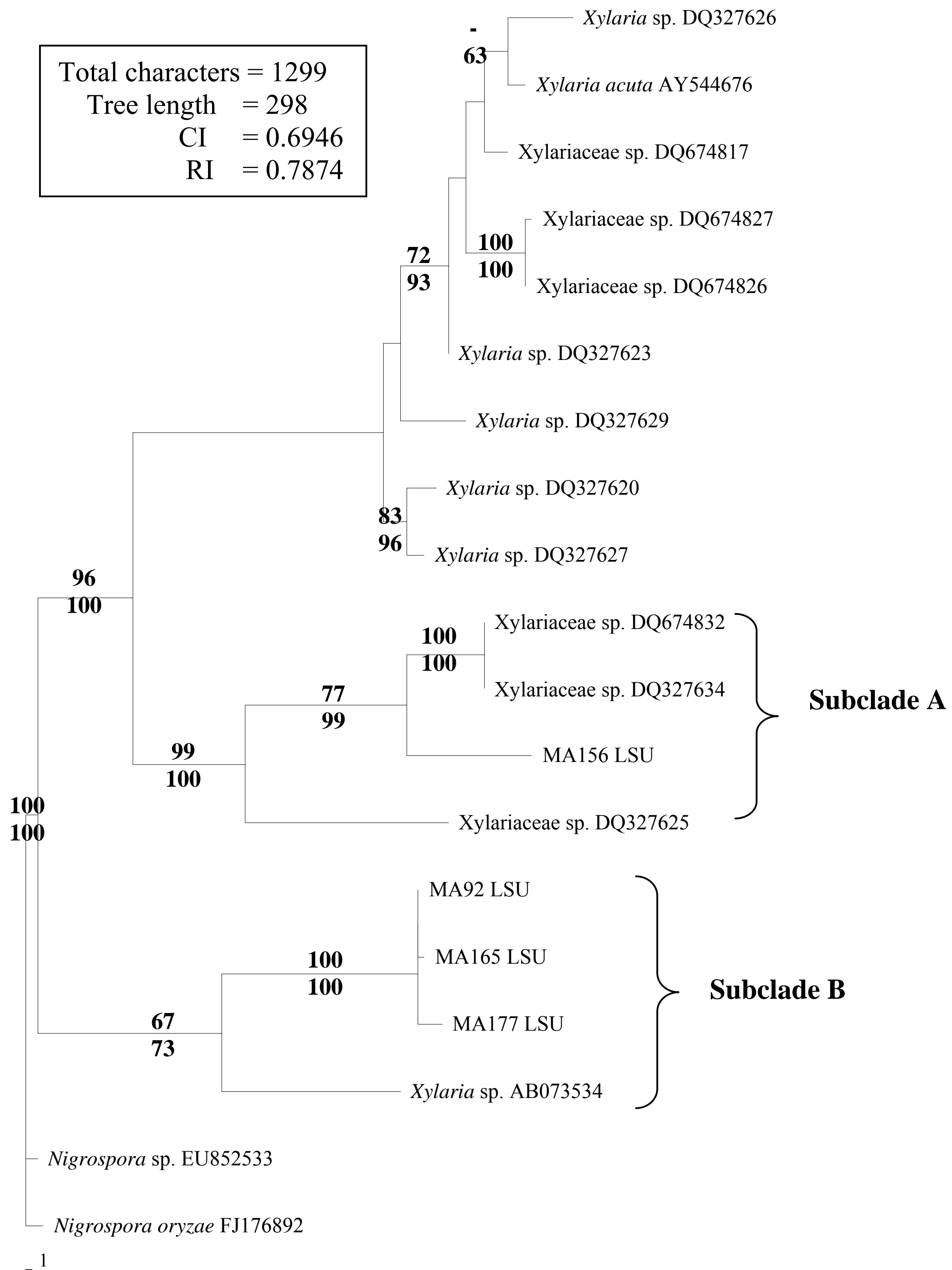


Figure 25 The phylogram obtained from LSU rDNA sequence analysis of the endophytic fungi MA92, MA156, MA165, MA177 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes.

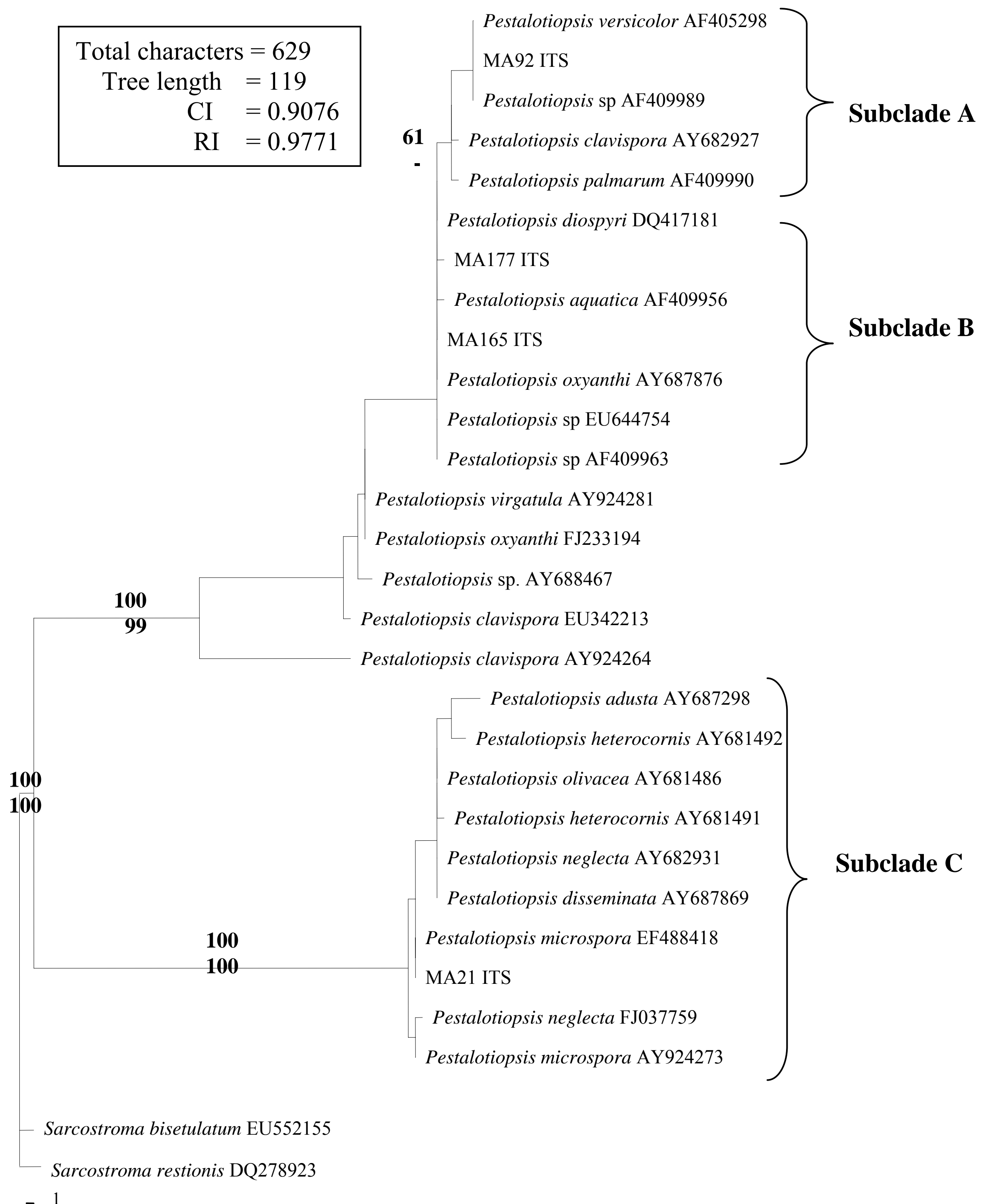


Figure 26 The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungi MA21, MA92, MA165, MA177 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes.

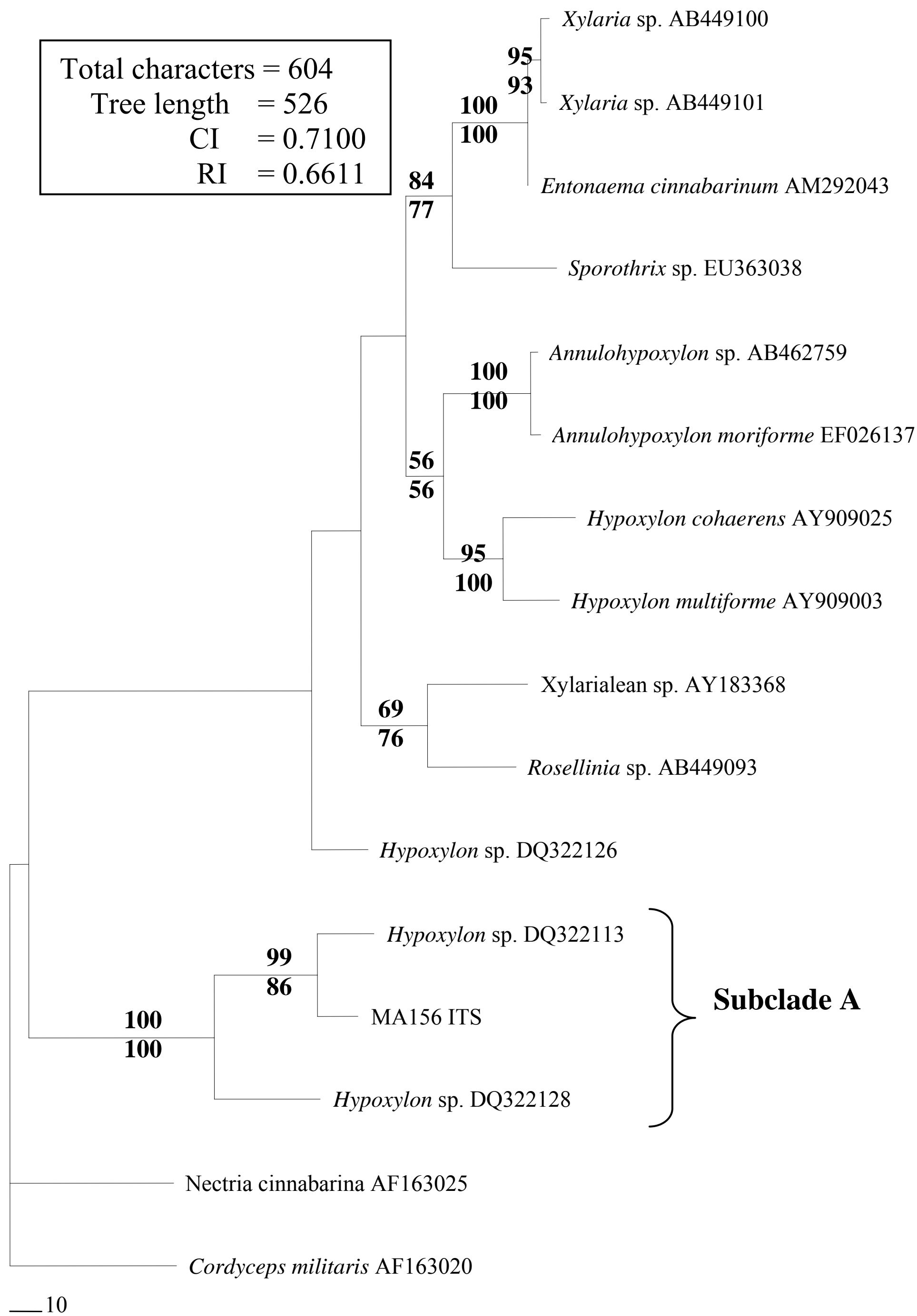


Figure 27 The phylogram obtained from ITS rDNA sequence analysis of the endophytic fungus-MA156 and fungi from the Xylariales generated from parsimony analysis. Parsimony bootstrap and Neighbor-joining bootstrap values (>50%) are shown above and below the branches, respectively. Branch lengths are proportional to the number of step changes.

CHAPTER 4

DISCUSSION

4.1 Endophytic fungal isolation from mangrove plants

Endophytic fungi are commonly found in various plants and in a variety of plant tissues. Endophytic fungi have been studied in terrestrial plants, especially in medicinal and rain forest plants. The isolation rate of endophytic fungi varies among terrestrial plants. Two hundred and seventy eight isolates of endophytic fungi were obtained from 2,400 segments (0.11 isolate/segment) of inner bark and twigs of the medicinal plant *Terminalia arjuna* (Combretaceae) (Tejesvi *et al*., 2005). Lin and coworkers (2007) isolated endophytic fungi from a pharmaceutical plant *Camptotheca acuminata* and found that 174 isolates were from 18 plants (9.7 isolate/plant) and 486 tissue samples (0.38 isolate/tissue sample). In this study, 619 endophytic fungi were isolated from 1260 segments of 63 plants of 18 mangrove species. The isolation rate of endophytic fungi was 9.8 isolates per plant or 0.49 isolate per sample disc and this was about the same rate as found by Lin *et al*. (2007). In addition, Chaowalit (2009) also isolated endophytic fungi from nine plants of seven mangrove species from the same region with the isolation rate of 8.4 isolates per plant or 0.42 isolate per sample disc. The endophytic fungal isolation rate may depend on various factors such as host plant species, plant parts and the isolation medium used. We have found endophytic fungi from all parts of the plants tested: branch, vein, midrib, lamina and petiole. The highest number of endophytic fungi (39%) was obtained from the branches and the lowest number (13%) was from the petiole and laminar**.** This result is similar to that of the endophytic fungi isolated from the inner branch and leaf of *Kandelia candel* when a higher number were isolated from the branch than from the leaf (Pang *et al*., 2008).

Among the mangrove species studied in this work, *B. cylindrica* produced the highest rate of endophytic fungal isolates (21 isolates/plant or 1.05 isolates/disc) and *B. gymnorrhiza* produced the lowest isolation rate (2 isolates/plant

or 0.1 isolate/disc). This may be because the *B. gymnorrhiz*a used in this study was very young. It had been grown in a greenhouse for less than a year. The young leaves of *Rhizophora apiculata* have been reported to have lower numbers of endophytic fungi than older leaves (Kumaresan and Suryanarayanan, 2002). In Japan, an investigation of the endophytic fungi from *B. gymnorrhiza* indicated that the number of isolates from young leaves was less than from mature leaves (Okane *et al*., 2001).

Various media have been used to isolate fungal endophytes such as potato dextrose agar (PDA), malt extract agar (MEA), water agar (WA) and corn meal agar (CMA). Mohanta and coworkers (2008) studied endophytic fungi from three medicinal plants using three different mycological media that included PDA, MEA and WA. They found that most endophytes were obtained using PDA and the least number of isolates was obtained on the WA medium. PDA and MEA are the principle media used for normal fungal isolation but perhaps are not the most appropriate ones to use for the isolation of endophytic fungi. We expensed the slow growing fungal species at the fast growing fungal isolation. CMA media that allows for a slow growing fungi could be present and isolation. Sun and coworkers (2008) isolated endophytic fungi from six medicinal plants using PDA and 973 endophytic fungi were isolated from 1144 segments or 0.85 isolates per segment. Theantana *et al*. (2009) reported that 194 isolates of endophytic fungi were isolated from 650 segments (0.29 isolates per segment) of medicinal plants in Thailand by using MEA media. Rungjindamai (2006) reported that the isolation rate of endophytic fungi from *Garcinia* plants in southern Thailand was 3.0 isolates per plant segment by using CMA media. The results of these studies also indicated that CMA is a good medium for isolating endophytic fungi with a high isolation rate and *B. cylindrica* was the most of fungal endophyte isolation rate.

4.2 Antimicrobial assay

At present, natural products still remain the most important sources for discovering new drugs. Large numbers of plants, microbial and marine sources have been tested for production of bioactive compounds (Firáková *et al*., 2007). Microorganisms are important sources of bioactive compounds. Many studies have

focused on them because they can grow and increase their cell mass in a short time. Previously, soil fungi and terrestrial plants have been major sources for new chemical structures, but these often led to previously isolated chemicals (Jones *et al*., 2008). Therefore, the search for new drugs and novel substances from other sources such as marine organisms (Schulz *et al*., 2008), marine fungi (Proksch *et al*., 2008) and endophytic fungi (Lin *et al*., 2001) have been investigated. In this study, we have searched for fungal endophytes and their crude extracts with interesting NMR profiles that exhibit antimicrobial activity. All crude extracts (385 extracts) from 150 endophytes was evaluated in antimicrobial screening tests. The results showed that 47% of extracts from 92 isolates (61.33%) inhibited at least one test microorganism. Of these, the endophytic fungi that produced secondary metabolites (present in culture media) were most highly active against Gram-positive bacteria (*S. aureus* ATCC25923 and MRSA-SK1) with 65 and 70 isolates followed by *M. gypseum* (59 isolates), *C. neoformans* (35 isolates) and *C. albicans* (21 isolates). Only two endophytic fungi produced inhibitors against *P. aeruginosa* and none of the selected endophytic fungi inhibited *E. coli*. The investigations by Rungjindamai (2006) on the antimicrobial activity of endophytic fungi isolated from *Garcinia* spp. also showed that none of the fungal crude extracts inhibited Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Gram-negative bacteria are usually less sensitive to antibacterial agents than Gram-positive microorganisms due to their outer membranes that play important roles as a permeability barrier and limit exposure of susceptible target sites to antimicrobial agents (Denyer *et al*., 2004).

A total of 181 extracts inhibited 1 to 5 strains of the test microorganisms. Most of them (35.36%) inhibited 2 strains. This result indicated that most crude extracts from mangrove fungal endophytes have a narrow antimicrobial spectrum. Only 7.18% had a broad antimicrobial spectrum that inhibited 5 strains of test microorganisms (*S. aureus*, MRSA-SK1, *C. albicans*, *C. neoformans* and *M. gypseum*). In contrast Maria and coworkers (2005), who reported that a crude extract and purified fractions from *Aspergillus* species isolated from mangrove plants had a wide spectrum of antimicrobial activity against *Bacillus subtilis, Enterococcus* sp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus faecalis, Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans*.

Among the active crude extracts, hexane extracts from fungal mycelia had the highest inhibitory activity in the screening tests with 51.04%, followed by ethyl acetate extracts from the mycelium (46.53%) and ethyl acetate extracts from the fermentation broths (44.83%). This result indicated that most of the active materials were present in the fungal mycelia. Active extracts may contain cell-bound components and low polarity substances. Ethyl acetate extracts from the mycelia were most active against *S. aureus* ATCC25923, MRSA-SK1, *P. aeruginosa* and *C. albicans*. In addition, ethyl acetate extracts from the fermentation broths had the most inhibitory activity against *C. neoformans* and *M. gypseum*. In previous reports, crude ethyl acetate extracts from *Prumnopitys andina* inhibited *M. gypseum* with an MIC value of 250 µg/ml (Schmeda-Hirschmann *et al*., 2005), ethyl acetate extracts from the fermentation broth of the mangrove endophytic fungus *Phomopsis* sp. inhibited *C. albicans* with an MIC that ranged from 32 to 64 µg/ml (Huang *et al*., 2008). Crude extracts using different organic solvents may contain different bioactive compounds that can inhibit different test microorganisms. Furthermore, different groups of fungi produce different compounds. Preedanon (2008) studied marine-derived fungal isolates from sea fans and found that most of the active extracts were ethyl acetate extracts from the fermentation broths (53%) followed by ethyl acetate extracts from the mycelia (24%) and hexane extracts from the mycelia (21%).

In this study, we found six endophytic fungi that produced extracts with low MIC values against test microorganisms. These isolates were MA12, MA34, MA96, MA148, MA156 and MA194. The MIC values of their extracts were comparable to the MIC values of standard drugs. A crude hexane extract of MA34 mycelium (MA34CH) had MIC values of 4 and 8 µg/ml, against MRSA-SK1 and *S. aureus* ATCC25923 respectively. These MICs were only four to eight folds higher than the MIC value for vancomycin however their MBC values of 64 µg/ml were much higher than the MBC value of vancomycin (1 and 2 µg/ml). This extract may therefore act only as a bacteriostatic agent. A hexane extract from the mycelia of MA96 (MA96CH) had MIC and MFC values of 4 and 8 µg/ml, against *M. gypseum*. This was comparable to the antifungal drug miconazole with MIC and MFC values of 0.5 and 2 µg/ml, respectively. A hexane extract from the mycelia of MA12 and MA194 had MIC values of 8 µg/ml against *C. neoformans* compared to an MIC of 0.5

μg/ml for amphotericin B. The most active extracts against *C. albicans* were hexane extracts from the mycelia of MA99 and MA194 with MIC values of 32 μg/ml compared to an MIC of 0.25 μg/ml for amphotericin B. Many antimicrobial compounds have been reported from fungal endophytes. Li and coworkers (2005) found that monomethylsulochrin from the endophytic fungus *Aspergillus* sp. CY72 isolated from a medicinal plant inhibited *C. albicans* with an MIC value of 30 μg/ml. 7-amino−α-methylcoumarin extracted from the endophytic fungus *Xylaria* sp YX-28 had inhibitory activity against *C. albicans* with an MIC value of 15 μg/ml (Liu *et al*., 2008). Primin from *Botryosphaeria mamane* PSU-M76 isolated from *Garcinia mangostana* inhibited MRSA with an MIC value of 8 μg/ml (Pongcharoen *et al*., 2008). Griseofulvin, a known antifungal drug was reported to be isolated from the endophytic fungus PSU-N24 from *Garcinia nigrolineata* (Sommart *et al*., 2008). The bioactive preparations with low MIC values isolated during this study are being further characterized.

In this study, only two isolates (MA82 and MA125) had inhibitory activity against *P. aeruginosa* ATCC27853 with an MIC value of 200 μg/ml and none of the extracts inhibited *E. coli*. This result is similar to that of Maria *et al*. (2005), who also found that metabolites of a mangrove fungal endophyte showed better anti-*P. aeruginosa* activity than for *E. coli*.

4.3 Identification of active endophytic fungi

Fungal taxonomy is traditionally based on comparative morphological features (Huang *et al*., 2009). However, these conventional methods for detection and identification are laborious and time-consuming (Wu *et al*., 2003) and non- or low-sporulating fungi are very difficult to identify (Sette *et al*., 2006). A large number of fungi do not sporulate in culture, therefore these isolates when examined by traditional methods can only be identified as sterilia mycelia. Many of the current plant fungal endophytes are sterilia mycelia as previously reported by Guo *et al*. (2000) and Chareprasert *et al*. (2006). In 2001, Kumaresan and Suryanarayanan reported that they had isolated sterilia mycelia from seven mangrove plants in southern India. Molecular techniques are now being used to identify sterilia mycelia

fungi and several recent studies have shown that genetic methods can be successfully used to identify and classify endophytic fungi.

In this study the LSU and ITS regions of rDNA have been used. Because the LSU region is highly conserved, this region is used for phylogenetic analysis at higher taxonomic levels (order, family, genera) (Landvik, 1996; Wang *et al*., 2008a). The highly variable ITS regions are used for analysis of lower taxonomic levels (species, strains) (Guo *et al*., 2000; Jeewon *et al*., 2003; Cheng *et al*., 2008). In this study, universal primers (Table 5) were used for the amplification of the LSU and ITS regions by the PCR reactions as suggested by Möller (2000). The universal primers have been used to amplify all, or at least the majority, of the existing ITS copies present and primers LROR and LR7 have been used to amplify the LSU regions (Möller, 2000; Lee *et al*., 2006; Woudenberg *et al*., 2009).

The basic methods for phylogenetic analysis consist of distance method (i.e. UPGMA, Neighbour-Joining (NJ)) and Discrete method (i.e. Maximum Parsimony (MP), Maximum Likelihood, Bayesian inference). NJ and MP analyses were used for phylogenetic characterization in this study. The topology of NJ tree obtained from the same dataset was essentially similar to the MP trees. This is supported by the recent studies by Lee *et al*. (2006) and Wang *et al*. (2008b). They found that the topology of the LSU and ITS trees obtained by NJ and MP were not different.

In our study, we are mainly interested in identifying endophytic fungi that produce antimicrobial substances and also produce compounds with an interesting NMR profile. The fungal endophytes were identified using a combination of morphological and molecular methods. Twenty one strains of fungal endophytes that produced high antimicrobial activities and compounds with an interesting NMR profile (Table 12) were selected for LSU and ITS sequence analyses. As the result of a combination of both morphological characters and DNA sequence data, we identified these endophytes to species, genera, familial and ordinal levels. Of these, ten isolates did sporulate on PDA media and eleven isolates were mycelia sterilia.

Fungal molecular identification based on LSU and ITS rDNA sequence analyses revealed that active isolates belonged to the phylum Ascomycota in five orders: Capnodiales, Pleosporales, Diaporthales, Hypocreales and Xylariales

(Table 13). This result showed that fungal endophytes isolated from mangrove plants and that produced antimicrobial compounds are found in various fungal groups. They can produce various secondary metabolites with antimicrobial activity such as the phomopsins obtained from the endophytic fungus *Phomopsis* sp. ZSU-H76 that also showed antifungal activity (Huang *et al*., 2008). Xyloketal H was obtained from the endophytic fungus *Xylaria* sp. 2508 but did not show biological activity (Liu *et al*., 2006).

In this study, only the endophytic fungus MA12 was placed in the Capnodiales and closely related to *Stenella musae* (EU514293) and Mycosphaerellaceae sp. (EF694667) with a short branch length (Figure 18). ITS sequences of *Stenella musae* (EU514293) and Mycosphaerellaceae sp. (EF694667) had a high sequence similarity of 99.3% with MA12, therefore MA12 should only be classified as Mycosphaerellaceae. *Stenella musae* EU514293 and Mycosphaerellaceae sp. EF694667 as the most closely related taxa with MA12 were previously reported as an fungal endophyte. *Stenella musae* is an anamorphic state of *Mycosphaerella* sp. in the Mycosphaerellaceae. *Mycosphaerella* sp. are among the most common plant pathogens with a wide host range including trees, herbaceous plants and plantation crops (Arzanlou *et al*., 2008). Furthermore, *Mycosphaerella pneumatophorae* (Mycosphaerellaceae) has been reported to be an endophytic fungus from the mangrove *Avicennia* spp. in India (Sarma *et al*., 2001). In this study, MA12 was isolated from a branch of *Sonneratia caseolaris* in Songkhla province and showed strong antimicrobial activity against *C. neoformans* ATCC90112 with MIC and MFC values of 8 µg/ml (Table 15).

The endophytic fungi MA71 and MA164 were placed in the Pleosporales. The endophytic fungus MA71 was most closely related to *Didymella bryoniae* AB266846 with a short branch length. ITS sequences of MA71 and *Didymella bryoniae* AB266846 had the highest similarity of 98.7%. The endophytic fungus MA71 was isolated from a branch of *Scyphiphora hydrophyllacea* in Satun province. Crude extracts from this fungus produced an interesting NMR profile and inhibited *S. aureus* ATCC25923 with moderate activity. The endophytic fungus MA164 was grouped with *Leptosphaerulina* sp. and *Leptosphaerulina chartarum* by a distinctly long branch length that was supported by 100% bootstrapping.

Leptosphaerulina chartarum was the most closely related taxa with 77.8% ITS sequence similarity. Due to the lack of sequences from the GenBank database for comparison with MA164, therefore we could only be identified the endophytic fungus MA164 as Melanommataceae. It was isolated from *Scyphiphora hydrophyllacea* in Satun province and crude extracts from this inhibited *C. neoformans* ATCC90112 and *M. gypseum* MU-SH4 with MIC values that ranged from 64-200 µg/ml. *Leptosphaeria* sp. and *Leptosphaeria australiensis* are in the Melanommataceae have been reported in marine fungi from *Avicennia marina* and *Bruguiera parviflora* wood (Alias and Jones, 2000). In 2008b, Wang and coworkers isolated *Leptosphaerulina chartarum* as a marine fungus from Hawaiian sponges, but there was no report of any antimicrobial activity.

Six fungal endophytes (MA2, MA60, MA81, MA96, MA125 and MA194) were placed in the Order Diaporthales using LSU and ITS rDNA sequence analyses. Morphological characteristics could not identify them because they did not sporulate on PDA media and did not produce any reproductive structures. For *Phomopsis* species identification, old *Phomopsis* cultures often lose their ability to produce pycnidia, which is important for providing taxonomically useful information (Farr *et al*., 1999). Based on ITS sequence analysis, the endophytic fungus MA2 supported with a high bootstrap value by NJ was closely related to an uncultured *Diaporthe* sp. EF619687 and had a high sequence similarity. Uncultured *Diaporthe* sp. EF619687 was an ectomycorrhizal fungus isolated from *Pinus taeda* root (Parrent and Vilgalys, 2007). Therefore, we could identify the endophytic fungus MA2 as a *Diaporthe* sp using only ITS sequence. It was isolated from a branch of *Avicennia officinalis* in Satun province. Crude extracts from MA2 inhibited both strains of *S. aureus* and *M. gypseum* with MIC and MBC or MFC values ranged 32-64 µg/ml (Table 15).

The endophytic fungus MA60 formed a monophyletic clade with *Phomopsis eucommii* AY601921, although supported by low bootstrap values by both MP and NJ analyses. *Phomopsis eucommii* AY601921 was isolated from a woody plant but there were no reports on it having antimicrobial activity. We identified the endophytic fungus MA60 as *Phomopsis eucommii* because there was high sequence similarity (96.7%). It was isolated from a branch of *Avicennia alba* in Satun province.

Crude extracts from this fungus showed moderate antifungal activity with MIC values that ranged 16-32 µg/ml (Table 15).

The endophytic fungus MA96 formed a monophyletic clade with *Phomopsis* sp. EU002930, an endophytic fungus isolated from a coffee plant. We could identify the endophytic fungus MA96 as a *Phomopsis* sp. because there was a high sequence similarity (96.7%) with *Phomopsis* sp. EU002930. The endophytic fungus MA96 was isolated from a branch of *Rhizophora apiculata* in Trang province. Crude extracts from MA96 had a broad spectrum of inhibitory activity, against the 5 microorganisms tested (*S. aureus*, MRSA-SK1, *C. albicans*, *C. neoformans*, *M. gypseum*) with MIC and MBC or MFC values that ranged 4-200 µg/ml (Table 15).

The endophytic fungus MA81 clustered with *Phomopsis* sp. EU395772 and *Diaporthe* sp. EF488434 (Figure 20). *Phomopsis* sp. EU395772 was an endophytic fungus and *Diaporthe* sp. EF488434 was an endophytic fungus isolated from the pharmaceutical plant (*Annona squamosa* L.). *Phomopsis* species are considered to be the asexual phase of *Diaporthe* species, a teleomorphic genus in the family Diaporthaceae (Girlanda *et al.*, 2002). Therefore, we could only identify the endophytic fungus MA81 as a member of the family Diaporthaceae. It was isolated from the midrib of *Avicennia alba* in Trang province. Crude extracts from this endophytic fungus MA81 showed moderate activity against *C. albicans* and *M. gypseum*.

The endophytic fungus MA194 formed a monophyletic clade with *Phomopsis* sp. DQ235675. There was a short branch length and this was supported with a high bootstrap value. This fungus had a 99.4% sequence similarity with *Phomopsis* sp. DQ235675. Therefore, we can conclude that the endophytic fungus MA194 should be named as a *Phomopsis* sp. It was isolated from the laminar of *Rhizophora apiculata* in Songkhla province. Crude extracts from this fungus had a broad spectrum of activity, against the 5 tested microorganisms (*S. aureus*, MRSA-SK1, *C. albicans*, *C. neoformans*, *M. gypseum*) with MIC and MBC or MFC values that ranged from 8-200 µg/ml (Table 15). The endophytic fungus MA125 was also identified as a *Phomopsis* sp. It was isolated from the branch of *Xylocarpus granatum* in Satun province. This fungus was one of the two isolates that produced substances active against *P. aeruginosa*. Our results also indicated that *Phomopsis* or *Diaporthe*

species have a broad spread of host range. In a recent study, Cheng and coworkers (2008) isolated *Diaporthe phaseolorum* var. *sojae* from *Kandelia candel* from an estuarine mangrove. A combination of morphological and molecular techniques was used for its identification. Huang and coworkers (2008) identified the endophytic fungus ZSU-H76 which was isolated from the stem of the mangrove tree *Excoecaria agallocha* in China as *Phomopsis* sp using ITS sequence analysis. Cytosporone B and C isolating from this fungus inhibited *Candida albicans* and *Fusarium oxysporum* with moderate activity.

The endophytic fungus MA70 was placed in the Order Hypocreales, and it was closely related to Hypocreales sp. EU164804, *Acremonium crotocinigenum* DQ882846 and *Acremonium crotocinigenum* AJ621773 with 91-100% ITS sequence similarity, a short branch length and supported by high bootstrap values from both analyses (Figure 16). Endophytic fungus MA70 produced *Acremonium*-like conidia on PDA plate. Conidia are one-celled, ellipsoidal-shaped, hyaline and smooth-walled (Figure 15). Therefore, the data from morphological and molecular characteristics could be classified MA70 as *Acremonium* sp. The endophytic fungus MA70 was isolated from a branch of *Rhizophora apiculata* in Satun province and showed moderate activity against *C. albicans* ATCC90028, *C. neoformans* ATCC90112 and *M. gypseum* MU-SH4. This agrees with a study by Sette and coworkers (2006). They isolated endophytic fungi from coffee plants and determined the antimicrobial activities of crude extracts from their fungi. They found that active endophytic fungi were placed in the order Hypocreales. Maria *et al*. (2005) also reported that *Acremonium* species isolated as fungal endophyte from mangrove plants exhibited antimicrobial activity.

The endophytic fungi MA21, MA92, MA156, MA165 and MA177 were placed in the Order Xylariales using LSU and ITS sequence analyses. This result corresponded to the morphological characterisics that could be used to identify these fungal endophytes as a *Pestalotiopsis* sp., except for the non-sporulating endophytic fungus MA156. Morphological characteristics are important for identifying *Pestalotiopsis* species (Lee *et al*., 2006; Hu *et al*., 2007). Recent studies employing rDNA sequence data have, however, clarified some of the confusion, and provided a more complete understanding of the generic circumscription for pestalotioid fungi

(Jeewon *et al*., 2003). In this study, the endophytic fungus MA21 was placed in the subclade C (Figure 22) and this was supported with 100% bootstrapping. The endophytic fungus MA21 formed a cluster with different species of *Pestalotiopsis* and had a high sequence similarity (99.6-99.8%) with taxa in subclade C that was supported by high bootstrap values. Therefore, we could only identify the endophytic fungus MA21 as a *Pestalotiopsis* sp. The endophytic fungus MA21 was isolated from a branch of *Sonneratia caseolaris* in Songkhla province. Crude extracts from MA21 inhibited *M. gypseum* and both strains of *S. aureus* with moderate activity. *Pestalotiopsis versicolor* AF405298 and *Pestalotiopsis* sp. AF409989 were the most closely related to the endophytic fungus MA92 with 100% ITS sequence similarity and had a short branch length. Therefore, we could identify the endophytic fungus MA92 as *Pestalotiopsis versicolor*. The endophytic fungus MA92 was isolated from a branch of *Rhizophora apiculata* in Trang province. Crude extracts from the endophytic fungus MA92 had an interesting NMR profile and inhibited *M. gypseum* and both strains of *S. aureus* with moderate activity. The endophytic fungi MA165 and MA177 were also closely related to different species of *Pestalotiopsis* with short branch lengths but there were no closely related taxa. Therefore, we could only classify them as *Pestalotiopsis* sp. The endophytic fungus MA165 was isolated from the midrib of *Aegiceras corniculatum* in Satun province. Crude extracts from MA165 showed activity against both strains of *S. aureus*, *C. neoformans* and *M. gypseum* with weak activity. The endophytic fungus MA177 was isolated from a vein of *Scyphiphora hydrophyllacea* in Satun province. Crude extracts from MA177 showed broad spectrum inhibitory activity against both strains of *S. aureus*, *C. albicans*, *C. neoformans* and *M. gypseum* with MIC values that ranged from 32-200 µg/ml. These results were in agreement with those from endophytic fungal isolates obtained from the root of *Acanthus ilicifolius* (mangrove plant) in the southwest coast of India. *Pestalotiopsis* sp. also was found to show a broad spectrum of antimicrobial activity (Maria *et al*., 2005).

Pestalotiopsis spp. represent the majority of taxa reported to be endophytic fungi as isolates from many plant species (Tejesvi *et al*., 2005). Furthermore, *Pestalotiopsis* was reported to be the main genus of endophytic fungi

from mangroves (Liu *et al*., 2007). Some research has reported that *Pestalotiopsis* species also act as pathogenic fungi of mangroves (Zhou and Huang, 2001).

The endophytic fungus MA156 formed a monophyletic clade with *Hypoxylon* sp. DQ322113 and this was supported with high bootstrap values by both MP and NJ analyses. The endophytic fungus MA156 showed 97% sequence similarity with *Hypoxylon* sp. DQ322113. Thus, we could identify the endophytic fungus MA156 as a *Hypoxylon* sp. It was isolated from the midrib of *Lumnitzera littorea* in Satun province. Crude extracts from this fungus had a broad spectrum of activity against both strains of *S. aureus*, *C. albicans*, *C. neoformans* and *M. gypseum* with MIC values that ranged from 8-128 µg/ml. Hyde and Lee (1995) isolated *Hypoxylon oceanicum* from mangrove wood with a non-specific host species. Furthermore, there have been reports antifungal polyesters from the marine fungus *Hypoxylon oceanicum* against *Neurospora crassa* OS-1 with an MIC range of 0.5 to 2 µg/ml (Schlingmann *et al*., 2002).

Some of the fungal endophytes such as MA34 and MA132 that belonged to the xylariaceous fungi were only identified to the ordinal level based on their morphological characteristics. Although, we were able to amplify the LSU and ITS regions of these two endophytes using the universal primers (Table 5), however the quality of the sequences was not satisfactory. Therefore, more details of their morphological characteristics were important to help with classification. The endophytic fungus MA34 was isolated from a branch of *Bruguiera parviflora* in Surathani province and had strong inhibitory activity against *S. aureus* ATCC25923 and MRSA-SK1 and moderate activity against *M. gypseum* .The endophytic fungus MA132 was isolated from *Rhizophora mucronata* in Surathani province and showed moderate activity against *S. aureus* ATCC25923, MRSA-SK1 and *C. neoformans*.

The LSU and ITS regions of the endophytic fungi MA82, MA90 and MA99 could not be amplified using the universal fungal primers. This is probably due to their highly species specific regions, therefore new specific primers will need to be designed. These fungi, except for MA82 were identified based on their morphological characteristics.

The endophytic fungus MA90 was isolated from the midrib of *Rhizophora apiculata* in Trang province and had inhibitory activity against

S. aureus ATCC25923 and moderate activity against MRSA-SK1. It was identified as *Bipolaris bicolor* in the Pleosporales, based on its morphological characteristics. The endophytic fungus MA99 was identified as *Pestalotiopsis* sp. in the Xylariales. It was isolated from *Rhizophora apiculata* in Trang province and showed activity against *C. albicans* with MIC and MFC values of 32 and 128 µg/ml (Table 15). The morphological characteristics alone could not identify the endophytic fungus MA82 to any genus or species. Hence we could only classify it as an Unidentified fungal endophyte. It was isolated from the midrib of *Avicennia alba* in Trang province. A crude ethyl acetate extract from MA82 inhibited *P. aeruginosa* ATCC27853 but only with weak activity. We were not able to isolate the genomic DNA of the endophytic fungus MA148. Its morphological characteristics were not sufficient to identifiy it to the level of any genus or species. Therefore we could only classify it as an Unidentified fungal endophyte. It was isolated from the midrib of *Avicennia alba* in Surathani province. A crude ethyl acetate extract of MA148 showed strong inhibitory activity against *M. gypseum.*

The use of both morphological and molecular characteristics have their limitations. Molecular identification cannot overcome the problem of ‘over-isolating’ fast growing fungal species at the expense of slow growing taxa, nor for identifying species that will not grow in culture. Therefore, identification of the endophytic fungi using a combination of morphological and molecular methods are more precise than using either method alone (Huang *et al*., 2009).

In this study, we identified fungal endophytes that had inhibitory activity against microorganisms as *Diaporthe* sp. (1 isolate), Diaporthaceae sp. (1 isolate), *Didymella bryoniae* (1 isolate), *Bipolaris bicolor* (1 isolate), *Pestalotiopsis* sp. (5 isolates), *Phomopsis* sp. (4 isolates), Melanommataceae (1 isolate), Mycosphaerellaceae (1 isolate), Xylariales sp. (2 isolates), *Hypoxylon* sp. (1 isolate), Hypocreales sp. (1 isolates), and Unidentified fungal endophytes (2 isolates).

The genera *Diaporthe, Phomopsis*, *Pestalotiopsis* and *Xylaria* have been reported to be the common endophytes on mangrove plants (Liu *et al*., 2007; Cheng *et al*.,2008; Huang *et al*., 2008; Pang *et al*., 2008; Schulz *et al*., 2008). Many mangrove endophytes have been reported to produce antimicrobial substances such as *Colletotrichum* sp., *Xylaria* sp., *Pestalotiopsis* sp., *Paecilomyces* sp., *Phomopsis* sp.

and *Phoma* sp. (Liu *et al*., 2007; Pang *et al.*, 2008; Schulz *et al*., 2008). This agrees with the results of this study as we have found *Diaporthe, Phomopsis*, *Pestalotiopsis* and*, Xylaria* as mangrove fungal endophytes that produce antimicrobial substances. Moreover, several genera including *Didymella bryoniae*, *Bipolaris bicolor*, Melanommataceae sp., Mycosphaerellaceae sp., *Hypoxylon* sp. and Hypocreales sp. found in this study have never been previously reported as mangrove fungal endophytes.

Most of the active endophytic fungi were isolated from branches (12 isolates) and these fungi have been frequently found in terrestrial plants (Gamboa and Bayman, 2001; Sette *et al*., 2006; Lin *et al*., 2007; Verma *et al*., 2007; Shankar Naik *et al*., 2008). This result is similar to a previous study by Kumaresan and Suryanarayanan in 2001 and Ananda and coworkers in 2002, as they both suggested that endophytic fungi of mangrove leaves, stems and roots were more closely related to terrestrial than marine fungi.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

In this study, 619 fungal endophytes were isolated from mangrove plants. The average endophytic fungal isolation rate was 9.8 isolates per plant or 0.49 isolates per sample disc and the highest number of isolates was from *Bruguiera cylindrical*. The biggest percentages of endophytic fungal isolates came from branches followed by veins, midribs, laminars and petioles. Crude extracts from 150 isolates were tested for antimicrobial activity. Many of them exhibited high antimicrobial activity. A crude extract from the endophytic fungus MA34 (Xylariales sp.) was the most active against *S. aureus* ATCC25923 (MIC 8 µg/ml) and MRSA-SK1 (MIC 4 µg/ml). A crude extract from the endophytic fungus MA96 (*Phomopsis* sp.) was the most active against *M. gypseum* MU-SH4 (MIC 4 µg/ml). Crude extracts from the endophytic fungi MA12 (Mycosphaerellaceae) and MA194 (*Phomopsis* sp.) were the most active against *C. neoformans* ATCC90112 (MIC 8 µg/ml). Crude extracts from the endophytic fungi MA99 (*Pestalotiopsis* sp.) and MA194 were the most active against *C. albicans* ATCC90028 (MIC 32 µg/ml). Crude extracts from the endophytic fungi MA82 (unidentified fungal endophyte) and MA125 (*Phomopsis* sp.) were the most active against *P. aeruginosa* ATCC27853 (MIC 200 µg/ml). None of the extracts was active against *E. coli* ATCC25922.

Twenty-one fungal endophytes that produced microbial inhibitors were identified based on their morphological and molecular characteristics using the data obtained by analysis of the sequences of the combined ITS and LSU regions. They were assigned to 5 orders: Hypocreales, Xylariales, Diaporthales, Capnodiales, and Pleosporales and 7 genera as follows:

1) Three isolates were identified to the species level, *Didymella bryoniae* (MA71), *Phomopsis eucommii* (MA60), and *Bipolaris bicolor* (MA90).
2) Eleven isolates were identified to the genus level, *Acemonium* sp. (MA70), *Diaporthe* sp. (MA2), *Phomopsis* sp. (MA96, MA125, MA194), *Pestalotiopsis* sp. (MA21, MA92, MA99, MA165, MA177) and *Hypoxylon* sp. (MA156).
3) Three isolates was identified to the family level, Diaporthaceae (MA81), Melanommataceae (MA164) and Mycosphaerellaceae (MA12).
4) Two isolates were identified to the order level Xylariales sp. (MA34, MA132).
5) Two isolates could not be identified to any level (unidentified fungal endophytes MA82, MA148).

5.2 Suggestion for future work

1) Further testing of the active extracts for anti-malarial, anti-cancer, anti-HIV, and antioxidant activities.

2) Purification and structural identification of the bioactive compounds that showed interesting NMR profiles such as from MA34 (Xylariales sp.), MA70 (*Acremonium* sp.), MA92 (*Pestalotiopsis* sp.), MA125 (*Phomopsis* sp.) and MA194 (*Phomopsis* sp.).

REFERENCES

Alias, S.A. and Jone, E.B.G. 2000. Colonization of mangrove wood by marine fungi at Kuala Selangor mamgrove stand, Malaysia. Fungal. Divers. 5, 9-21.

Ananda, K. and Sridhar, K.R. 2002. Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. Can. J. Microbiol. 48, 871-878.

Arzanlou, M., Groenewald, J.Z., Fullerton, R.A., Abeln, E.C.A., Carlier, J., Zapater, M.F., Buddenhagen, I.W., Viljoen, A. and Crous, P.W. 2008. Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. Persoonia. 20, 19-37.

Bamroongrugsa, N. 1999. Bioactive substances from the mangrove resource. Songklanakarin J. Sci. Technol. 21, 377-386.

Bandaranayake, W.M. 2002. Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands. Ecolo. Manage. 10, 421-452.

Barnett, H.L. and Hunter, B.B. 1998. Illustrated Genera of Imperfect Fungi. APS Press, Minnesota, USA.

Baxwvanis, A.D. and Ouellette, F. 2004. Bioinformatics: A Practical Guide to the Analysis of Gene and Proteins, New York.

Berbee, M.L. and Taylor, J.W. 1999. Fungal Phylogeny. Molecular Fungal Biology, Cambridge university press, UK.

Bunyard, B.A., Nicholson, M.S. and Royse, D.J. 1994. A systematic assessment of Morchella with RFLP analysis of the 28S ribosomal RNA gene. Mycologia. 86, 762–772.

Chaowalit, P. 2009. Screening of endophytic fungi from mangrove plants which produce lipase, cellulase, amylase or protease enzymes, Master of Science Thesis, Prince of Songkla University, Songkhla, Thailand.

Chareprasert, S., Piapukiew, J., Thienhirun, S., Whalley, A.J.S. and Sihanonth, P. 2006. Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World. J. Microbiol. Biotechnol. 22(5), 481-486.

Chen, G., Lin, Y., Vrijmoed, L. and Fong, W.F. 2006. A new isochroman from the marine endophytic fungus 1893#. Chem. Nat. Compd. 42(2), 138-141.

Chen, G., Zhu, Y., Wang, H.Z., Wang, S.J. and Zhang, R.Q. 2007. The metabolites of a mangrove endophytic fungus, *Penicillium thomii*. J. Asian. Nat. Prod. Res. 9, 159-164.

Cheng, Z.S., Tang, W.C., Su, Z.J., Cai, Y., Sun, S.F., Chen, Q.J., Wang, F-H., Lin, Y.C., She, Z-G. and Vrijmoed, L.L.P. 2008. Identification of mangrove endophytic fungus 1403 (*Fusarium proliferatum*) based on morphological and molecular evidence. J. Forest. Res. 19(3), 219-224.

Chinworrungsee, M., Wiyakrutta, S., Sriubolmas, N., Chuailua, P. and Suksamrarn, A. 2008. Cytotoxic activities of trichothecenes isolated from an endophytic fungus belonging to order hypocreales. Arch. Pharm. Res. 31(5), 611-616.

Choi, Y.W., Hodgkiss, I.J. and Hyde, K.D. 2005. Enzyme production by endophytes of *Brucea javanica*. J. Agri. Technol. 1, 55-66.

Chomcheon, P., Wiyakrutta, S., Sriubolmas, N., Ngamrojanavanich, N., Isarangkul, D. and Kittakoop, P. 2005. 3-Nitropropionic acid (3-NPA), a potent antimycobacterial agent from endophytic fungi: is 3-NPA in some plants produced by endophytes? J. Nat. Prod. 68(7), 1103-1105.

Clinical Laboratory Standards Institue (CLSI). 2002a. Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Clinical Laboratory Standards Institue, Pennsylvania, USA.

Clinical Laboratory Standards Institue (CLSI). 2002b. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard. CLSI documents M38-A. Clinical Laboratory Standards Institue, Pennsylvania, USA.

Clinical Laboratory Standards Institue (CLSI). 2002c. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard-second edition. CLSI documents M27-A2. Clinical Laboratory Standards Institue, Pennsylvania, USA.

Denyer, S.P., Hodges, N.A. and Gorman, S.P. 2004. Hogo and Russell's Pharmaceutical Microbiology. Blackwell Science Inc., Massachusetts, USA.

Dettrakul, S., Kittakoop, P., Isaka, M., Nopichai, S., Suyarnsestakorn, C., Tanticharoen, M. and Thebtaranonth, Y. 2003. Antimycobacterial pimarane diterpenes from the fungus *Diaporthe* sp. Bioorg. Med. Chem. Lett. 13(7), 1253-1255.

Drummond, A.J. and Waigh, R.D. 2000. Recent Research Developments in Phytochemistry. 4, 143–152.

Duke, N.C., Benzie, J.A.H., Goodall, J.A. and Ballmen, T.R. 1998. Genetic structure and evolution of species in the mangrove genus *Avicennia* (Avicenniaceae) in the indo-west pacific. Evolution. 52(6), 1612-1626.

Farr, D.F., Castlebury, L.A. and Pardo-Schultheiss, R.A. 1999. *Phomopsis amygdale* causes peach shoot blight of cultivated peach trees in the southeastern United States. Mycologia. 91, 1008–1015.

Firáková, S., Šturdíková, M. and Múčková, M. 2007. Bioactive secondary metabolites produced by microorganisms associated with plants. Biologia. 62(3), 251-257.

Gamboa, M.A. and Bayman, P. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). Biotropica. 33(2), 352-360.

Gangadevi, V. and Multhumary, J. 2008. Isolation of *Colletotrichum gloesporioides*, a novel endophytic taxol-producing fungus from the leaves of a medicinal plant, *Justicia gendarussa*. Mycol. Balcan. 5, 1-4.

Germaine, K., Keogh, E., Garcia-Cabellos, G., Borremans, B., van der Lelie, D., Barac, T., Oeyen, L., Vangronsveld, J., Moore, F.P., Moore, E.R.B., Campbell, C.D., Ryan, D. and Dowling, D.N. 2004. Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS. Microbiol. Ecol. 48(1), 109-118.

Girlanda, M., Ghignone, S. and Luppi, A.M. 2002. Diversity of sterile root-associated fungi of two Mediterranean plants. New Phytol. 155, 481-498.

Gong, L.J. and Guo, S.X. 2009. Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity. AJB. 8(5), 731–736.

Gopal, B. and Chauhan, M. 2006. Biodiversity and its conservation in the Sundarban mangrove ecosystem. Aquat. Sci. 68, 338-354.

Guarro, J., Gene, J. and Stchige, A.M. 1999. Developments in fungal taxonomy. Clin. Microbiol. Rev. 12(3), 454-500.

Gunatilaka, A.A.L. 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J. Nat. Prod. 69(3), 509-526.

Guo, L.D., Hyde, K.D. and Liew, E.C.Y. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytol, 147(3), 617-630.

Hall, T. 2007. Bioedit version 7.0.7. Department of Microbiology, North Carolina State University. http:// www.mbio.ncsu.edu/BioEdit/bioedit.html. (accessed 06/15/08).

Hallman, J. and Sikora, R. 1995. Influence of *Fusarium oxysporum*, a mutualistic fungal endophyte, on Meloidogyne incognita infection of tomato. J. Plant Dis. Protect. 101(5), 475-481.

Hillis, D.M., Moritz, C. and Mabel, B.K. 1996. Molecular Systematics. Sinauer Associates, Sunderland, Massachusetts.

Hirsch, G.U. and Braun, U. 1992. Communities of Parasitic Microfungi. In: Fungi in Vegetation Science. Kluwer Academic, Netherlands.

Hu, H., Jeewon, R., Zhou, D., Zhou, T. and Hyde, K.D. 2007. Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β- tubulin gene phylogenies. Fungal. Divers. 24, 1-22.

Huang, W.Y., Cai, Y.Z., Surveswaran, S., Hyde, K.D., Corke, H. and Sun, M. 2009. Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species. Fungal. Divers. 36, 69-88.

Huang, Y., Wang, J., Li, G., Zheng, Z. and Su, W. 2001. Antitumer and antifungal activities in endophytic fungi isolated from pharmaceutical plants. FEMS Immunol. Med. Micro. 31, 163-167.

Huang, Z., Cai, X., Shao, C., She, Z., Xia, X., Chen, Y., Yang, J., Zhou, S. and Lin, Y. 2008. Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. Phytochemistry. 69(7), 1604-1608.

Hyde, K.D., and Lee, S.Y. 1995. Ecology of mangrove fungi and their role in nutrient cycling: what gaps occur in our knowledge? Hydrobiologia. 295, 107-118.

Hyde, K.D., Jones, E. B. G., Leaño, E., Pointing, S.B., Poonyth, A.D. and Vrijmoed, L. L. P. 1998. Role of fungi in marine ecosystems. Biodivers. Conserv. 7(9), 1147-1161.

Jeewon, R., Liew, E.C.Y., Simpson, J.A., John Hodgkiss, I. and Hyde, K.D. 2003. Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. Mol. Phylogenet. Evol. 27(3), 372-383.

Jones, E.B.G., Stanley, S.J. and Pinruan, U. 2008. Marine endophyte sources of new chemical natural products. Bot. Mar. 51(3), 163-170.

Jordaan, A., Taylor, J.E. and Rossenkhan, R. 2006. Occurrence and possible role of endophytic fungi associated with seed pods of *Colophospermum mopane* (Fabaceae) in Botswana. S. Afr. J. Bot. 72(2), 245-255.

Kishino, H. and Hasegawa, M. 1989. Evolution of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Molec. Evol. 29, 170-179.

Kumar, D.S.S. and Hyde, K.D. 2004. Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal. Divers. 17, 69-90.

Kumaresan, V. and Suryanarayanan, T.S. 2001. Occurrence and distribution of endophytic fungi in a mangrove community. Mycol. Res. 105(11), 1388-1391.

Kumaresan, V. and Suryanarayanan, T.S. 2002. Endophytic assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal. Divers. 9, 81-91.

Kusari, S., Lamshöft, M. and Spiteller, M. 2009. *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J. Appl. Microbiol. 107(3), 1019-1030.

Lake, J.A. 1987. Prokaryotes and archaebacteria are not monophyletic. Rate invariants analysis of rRNA genes indicates that eukaryotes and eocytes form a monophyletic taxon. Cold Spring Harbour Symposium. Quantitative. Biology. 52, 839-846.

Landvik, S. 1996. *Neolecta*, a fruit-body-producing genus of the basal ascomycetes, as shown by SSU and LSU rDNA sequences. Mycol. Res. 100(2), 199-202.

Lee, S., Crous, P.W. and Wingfield, M.J. 2006. *Pestalotiopsis* fungi from *Restionaceae* in the Cape Floral Kingdom. Stud. Myco. 55, 175-187.

Li, H.J., Lin, Y.C., Vrijmoed, L.L.P. and Jone, E.B.G. 2004. A new cytotoxic sterol produced by an endophytic fungus from *Castaniopsis fissa* at the south China sea coast, Chinese Chem. Lett. 15(4), 419-422.

Li, Y., Song, Y.C., Liu, J.Y., Ma, Y.M. and Tan, R.X. 2005. Anti-helicobacter pylori substances from endophytic fungal cultures. World. J. Microbiol. Biotecnol. 21(4), 553-558.

Lin, X., Lu, C., Huang, Y., Zheng, Z., Su, W. and Shen, Y. 2007. Endophytic fungi from a pharmaceutical plant, Camptotheca acuminata: isolation, identification and bioactivity. World. J. Microbiol. Biotechnol. 23(7), 1037-1040.

Lin, Y.C., Wanng, J., Zhou, S.N. and Jones, E. B.G. 2001. New isocoumarins from the mangrove endophytic fungus #2533. Chem. J. Internet. 3, 406-410.

Liu, A.R., Wu, X.P. and Xu, T. 2007. Research advances in endophytic fungi of mangrove. Chin. J. Appl. Ecol. 18(4), 912-918.

Liu, X., Xu, F., Zhang, Y., Liu, L., Huang, H., Cai, X., Lin, Y. and Chan, W. 2006. Xyloketal H from the mangrove endophytic fungus *Xylaria* sp. 2508. Russ. Chem. Bull. 55(6), 1091-1092.

Liu, X., Dong, M., Chen, X., Jiang, M., Lv, Xin. and Zhou, J. 2008. Antimicrobial activity of an endophytic *Xylaria* sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin. Appl. Microbiol. Biotechnol. 78(2), 241-247.

Maria, G.L., Sridhar, K.R. and Raviraja, N.S. 2005. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. J. Agric. Technol. 1(1), 67-80.

Mejía, L.C., Rojas, E. I., Maynard, Z., Bael, S.V., Arnold, A.E., Hebbar, P., Samuels, G.J., Robbins, N. and Herre, E.A. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol. Control. 46(1), 4-14.

Mohanta, J., Tayung, K. and Mohapatra, U. 2008. Antimicrobial potentials of endophytic fungi inhibiting three Ethno-medicinal plants of Similipal Biosphere Reserve, India. Inter. J. Micro. 5(2), (http://www.ispub.com/ostia /index.php?xmlFilePath=journals/ijmb/vol5n2/fungi.xml).
(accessed 02/10/09).

Möller, M. 2000. How universal are universal rDNA primers? A cautionary note for plant systematists and phylogeneticists. Edinburgh J. Bot. 57(2), 151-156.

O'Donnell, K., Cigelink, E., Weber, N.S. and Trappe, J.M. 1997. Phylogenetic relationship among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia. 89, 48-65.

Okane, I., Nakagiri, A. and Ito, I. 2001. Assemblages of endophytic fungi on *Bruguiera gymnorrhiza* in the Shira River Basin, Iriomote Is. IFO Res. Comm. 20, 41-49.

Oses, R., Valenzuela, S., Freer, J., Baeza, J. and Rodríguez, J. 2006. Evaluation of fungal endophytes for lignocellulolytic enzyme production and wood biodegradation. Int. Biodeter. Biodegr. 57(2), 129-135.

Pace, N.R., Olsen, G.J. and Woese, C.R. 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell. 45, 325-326.

Pang, K.L., Vrijmoed, L.L.P., Goh, T.K., Plaingam, N. and Jones, E.B.G. 2008. Fungal endophytes associated with *Kandelia candel* (Rhizophoraceae) in Mai Po Nature Reserve, Hong Kong. Bot. Mar. 51, 171-178.

Parrent, J.L. and Vilgalys, R. 2007. Biomass and compositional responses of ectomycorrhizal fungal hyphae to elevated CO_2 and nitrogen fertilization. New Phytol. 176(1), 164-174.

Phongpaichit, S., Rungjindamai, N., Rukachaisirikul, V. and Sakayaroj, J. 2006. Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol. Med. Microbiol. 48, 367-372.

Phongpaichit, S., Nikom, J., Rungjindama, N., Sakayaroj, J., Hutadilok-Towatana, N., Rukachaisirikul, V. and Kirtikara, K. 2007. Biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. FEMS Immunol. Med. Microbiol. 51(3), 517-525.

Pocasangre, L., Sikora, R.A., Vilich, V. and Schuster, R-P. 2001. Survey of banana evdophytic fungi from Central America and screening for biological control of burrowing nematode (*Rhadopholus simillis*). Acta. Horticulturae. 531, 283-289.

Pongcharoen, W., Rukachaisirikul, V., Phongpaichit, S., Kühn, T., Pelzing, M., Sakayaroj, J. and Taylor, W.C. 2008. Metabolites from the endophytic fungus *Xylaria* sp. PSU-D14. Phytochemistry. 69(9), 1900-1902.

Preedanon, S. 2008. Screening and identification of sea fan-derived fungi that produce antimicrobial substances, Master of Science, Prince of Songkla University, Songkhla, Thailand.

Proksch, P., Ebel. R., Edrada, R-A., Riebe, F., Liu, H., Diesel, A., Bayer, M., Li, X., Lin, W.H., Grebenyuk, V., Muller, W.E.G., Draeger, S., Zuccaro, A. and Schulz, B. 2008. Spong-associated fungi and their bioactive compounds: the *Suberites case*. Bot. Mar. 51, 209-218.

Ranghoo, V.M., Hyde, K.D. and Liew, E.C.Y. 2000. Molecular techniques and phylogeny. Fungal. Divers. 2, 308-336.

Rukachaisirikul, V., Kaeobamrung, J., Panwiriyarat, W., Saitai, P., Sukpondma, Y., Phongpaichit, S. and Sakayaroj, J. 2007. A new pyrone derivative from the endophytic fungus *Penicillium paxilli* PSU-A71. Chem. Pharm. Bull. 55(9), 1383-1384.

Rungjindamai, N. 2006. Endophytic fungi from *Garcinia* spp. with produced antimicrobial substances. Master of Science, Prince of Songkla University, Songkhla, Thailand.

Sarma, V.V., Hyde, K.D. and Vittal, B.P.R. 2001. Frequency of occurrence of mangrove fungi from the east coast of India. Hydrobiologia. 455, 41-53.

Schardl, C.L. and Phillips, T.D. 1997. Protective grass endophytes: Where are they from and where are they going? Plant. Disease. 81(5), 430-438.

Schlingmann, G., Milne, L. and Carter, G.T. 2002. Isolation and identification of antifungal polyesters from the marine fungus *Hypoxylon oceanicum* LL-15G256. Tetrahedron. 58(34), 6825-6835.

Schmeda-Hirschmann, G., Hormazabal, E., Astudillo, L., Rodriguez, J. and Theoduloz, C. 2005. Secondary metabolites from endophytic fungi isolated from the Chilean gymnosperm *Prumnopitys andina* (Lleuque). World. J. Microbiol. Biotechnol. 21(1), 27-32.

Schulz, B., Draeger, S., dela Cruz, T.E., Rheinheimer, J., Siems, K., Loesgen, S., Bitzer, J., Schloerke, O., Zeeck, A., Kock, I., Hussain, H., Dai, J. and Krohn, K. 2008. Screening strtegies for obtaining novel, biologically active, fungal secondary metabolites from marine habitats. Bot. Mar. 51, 219-234.

Schulz, B., Boyle, C., Draeger, S., Römmert, Anne-Katrin and Krohn, K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res. 106(9), 996-1004.

Schwarz, M., Köpcke, B., Weber, R., Sterner, O. and Anke, H. 2004. 3-Hydroxypropionic acid as a nematicidal principle in endophytic fungi. Phytochemistry. 65(15), 2239-2245.

Selosse, M.A., Faccio, A., Scappaticci, G. and Bonfante, P. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb. Ecol. 47(4), 416-426.

Sette, L., Passarini, M., Delarmelina, C., Salati, F. and Duarte, M. 2006. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World. J. Microbiol. Biotechnol. 22(11), 1185-1195.

Shankar Naik, B., Shashikala, J. and Krishnamurthy, Y.L. 2008. Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India. Fungal. Ecol. 1, 89-93.

Simpson, M.G. 2006. Phylogenetic Systematics. Plant Systematics. Elsevier academic press, Cannada.

Sommart, U., Rukachaisirikul, V., Sukpondma, Y., Phongpaichit, S., Sakayaroj, J. and Kirtikara, K. 2008. Hydronaphthalenones and a dihydroramulosin from the endophytic fungus PSU-N24. Chem. Pharm. Bull. 56(12), 1687-1690.

Sridhar, K.R. 2004. Mangrove fungi in India. India. Curr. Sci. 86(12), 1586-1587.

Stierle, A., Strobel, G. and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science. 260(5105), 214-216.

Strobel, G.A. 2003. Endophytes as sources of bioactive products. Microbe. Infect. 5(6), 535-544.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R.S. and Hess, W.M. 1996. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallichiana*. Microbiology. 142, 435-440.

Sun, J.Q., Guo, L.D., Zang, W., Ping, W.X. and Chi, D.F. 2008. Diversity and ecological distribution of endophytic fungi associated with medicinal plants. Sci China C-Life Sci. 51(8), 751-759.

Suryanarayanan, T.S., Kumaresan, V. and Johnson, J.A. 1998. Foliar fungal endophytes from two species of the mangrove *Rhizophora*. Can. J. Microbiol. 44(10), 1003–1006.

Swofford, D.L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Tan, R.X. and Zou, W.X. 2001. Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18, 448-459.

Tejesvi, M., Mahesh, B., Nalini, M., Prakash, H., Kini, K., Subbiah, V. and Shetty, H. 2005. Endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* W. & A. (Combretaceae). World. J. Microbiol. Biotechnol. 21(8), 1535-1540.

Theantana, T., Hyde, K.D. and Lumyong, S. 2009. Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties. IJIB. 7(1), 1-8.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22), 4673-4680.

Tomlinson, P.B. 1986. The Botany of Mangroves. Cambridge University Press, Cambridge: Cambridge.

Verma, V., Gond, S., Kumar, A., Kharwar, R. and Strobel, G. 2007. The endophytic mycoflora of bark, leaf and stem tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India). Microb. Ecol. 54(1), 119-125.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., Ditervon, W., Franken, P. and Kogel, K.H. 2005. The endophytic fungus piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proceedings of the National Academy of Sciences of the United States of America, 102(38), 13386-13391.

Wang, A., Lin, W., Chen, X., Lu, G., Zhou, J. and Wang, Z. 2008a. Isolation and identification of Sclerotinia stem rot causal pathogen in *Arabidopsis thaliana*. JZUS-B. 9(10), 818-822.

Wang, G., Li, Q. and Zhu, P. 2008b. Phylogenetic diversity of culturable fungi associated with the Hawaiian sponges *Suberites zeteki* and *Gelliodes fibrosa*. Antonie. van. Leeuwenhoek. 93, 163-174.

Webber, J. 1981. A natural biological control of Dutch elm disease. Nature, 292(5822), 449-451.

Weishampel, P. and Bedford, B. 2006. Wetland dicots and monocots differ in colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza. 16(7), 495-502.

White, T.J., Bruns, T., Lee, S. and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., New York. pp. 315-322.

Woese, C.R. and Olsen, G.J. 1986. Archaebacterial phylogeny: perspectives on the urkingdoms. Syst Appl Microbiol. 7, 161-177.

Wu, Z., Tsumura, T., Blonquist, G. and Wang, X. 2003. 18 rRNA genevariation among common airborne fungi and development of specific oligonucleotide

probe for the detection of fungal isolates. Appl. Environ. Microbiol 69, 5389–5397.

Xia, X.K., Yang, L.G., She, Z.G., Shao, C.L., Huang, Z.J., Liu, F., Lin, Y.C. and Zhou, S.N. 2008. Two new acids from mamgrove endophytic fungus (No. ZZF13). Chem. Nat. Compd. 44(4), 416-418.

Xu, Q.Y., Huang, Y.J., Zheng, Z.H. and Song, S.Y. 2005. Purification, elucidation and activities study of cytosporone B. J. Xiamen Univ., Nat. Sci. 44(3), 425–428.

Yang, X., Strobel, G.A., Stierle, A., Hess, W.M., Lee, J. and Clardy, J. 1994. A fungal endophyte-tree relationship; *Phoma* sp. in *Taxus wallichiana*. Plant. Sci. 102, 1-9.

Zhang, H.W., Song, Y.C. and Tan, R.X. 2006. Biology and chemistry of endophytes. Nat. Prod. Rep. 23(5), 753-771.

Zhou, Z.Q. and Huang, Z.Y. 2001. Study on the species and ecological character of mangrove pathogenic fungi in Guangxi. Guihaia. 21(2), 157-162.

Zhu, F. and Lin, Y. 2006. Marinamide, a novel alkaloid and its methyl ester produced by the application of mixed fermentation technique to two mangrove endophytic fungi from the South China Sea, Chinese. Sci. Bull. 51(12), 1426-1430.

http://cyberlab.lh1.ku.ac.th/elearn/faculty/fisher/fi16/page_3.htm. (accessed 10/10/09).

http://en.wikipedia.org/wiki/Neighbor-joining. (accessed 08/11/09).

http://en.wikipedia.org/wiki/Temperature_gradient_gel_electrophoresis.(accessed 09/10/09).

http://generalhorticulture.tamu.edu/lectsupl/anatomy/P14f1.gif. (accessed 08/25/09).

http://plantbio.berkeley.edu/~bruns/picts/results /its-map.GIF. (accessed 08/25/09).

http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Parker /method.html. (accessed 11/20/09).

http://www.forest.go.th/stat/stat50/TAB6.htm. (accessed 09/10/09).

http://www.geocities.com/~nesst/mangrove.htm. (accessed 11/20/09).

http;//www.lutzonilab.net/primers/image247. (accessed 09/10/09).

http://www.ncbi nlm.nih.gov. (accessed 11/8/09).

http://www.wrm.org.uy/deforestation/mangroves/forest.html. (accessed 09/15/09)

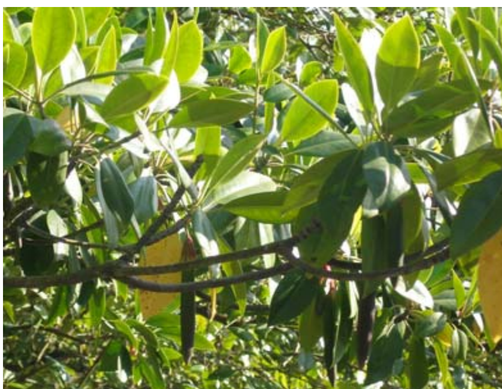
APPENDIX 1

a.) *Aegiceras corniculatum* (L.) Blanco

b.) *Avicennia alba* Bl.

c.) *Avicennia officinalis* L.

d.) *Bruguiera cylindrical* Bl.



e.) *Bruguiera gymnorrhiza* (L.) Savigny

f.) *Bruguiera parviflora* Wight & Arn.ex Griff

Figure 28 Mangrove plants were collected for endophytic fungi isolation

g.) *Ceriops decandra* Ding Hou

h.) *Ceriops tagal* (Perr.) C. B. Rob.

i.) *Heritiera littoralis* Ait.

j.) *Lumnitzera littorea* Voigt.

k.) *Rhizophora apiculata* Bl.

l.) *Rhizophora mucronata* Poir.

Figure 28 (cont.) Mangrove plants were collected for endophytic fungi isolation

m.) *Sonneratia caseolaris* (L.) Engl.

n.) *Sonneratia griffithii* Kurz.

o.) *Sonneratia ovata* Back.

p.) *Scyphiphora hydrophyllacea* Graetn.f.

q.) *Xylocarpus granatum* Koen

r.) *Xylocarpus moluccensis* Roem.

Figure 28 (cont.) Mangrove plants were collected for endophytic fungi isolation

APPENDIX 2

6XLoading Dye (250 ml)

Add 93.6 ml of 80% glycerol to a 250 ml Pyrex bottle and then add 3 ml of 0.5 M EDTA (give a final concentration of 6 mM), 0.3 g Bromophenol Blue and 0.3 g Xylene cyanol FF. Adjust with dH_2O for final volume 250 ml and mix thoroughly. Store at room temperature.

0.5 EDTA (pH8.0, 1000 ml)

Add 148 g of EDTA to 800 ml of dH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets). Adjust with dH_2O for final volume 1000 ml. Dispense into aliquots and sterilize by autoclaving.

Note: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

50XTAE buffer (Tris acetate EDTA buffer, 1000 ml)

Add Tris base 242.0 g, 0.5 M EDTA 100 ml and Glacial acetic acid 57.1 ml to 500 ml dH_2O in bigger. Adjust with dH_2O for final volume 1000 ml and mix thoroughly.

7.5 M Ammonium acetate (20 ml)

Add Ammonium acetate 11.562 g to 20 ml of MQH_2O. Sterilize by autoclaving.

Phenol: Choloroform: Isoamyl alcohol (25: 24: 1)

Add phenol 25 ml, chloroform 24 ml and isoamyl alcohol 1 ml in Pyrex bottle wrap with foil and shake. Store in freezer.

Cetyl Trimethyl Ammonium Bromide (CTAB) lysis buffer (O' Donnel *et al.*, 1997)

- 5 M NaCl 28 ml
- 10% CTAB 20 ml
- 5M Tris HCl 2 ml
- 0.5 M EDTA (pH 8.0) 5 ml

Addjust with H_2O for final volume 100 ml and autoclave at 121 °C, 15 minute, 15 pond/inch^2

DNA Determination

The total genomic DNA and PCR product are check for quantity and quality by observing of intensity of the band in a 1% agarose gel electrophoresis after stain with ethidium bromide (final concentration 2.5×10^{-5} mg/ml) for 10-15 minutes, check band with UV light transilluminator and take a picture with Gel Documentation.

1.8% resazurin

Add 1.8 g of resazurin dye to 100 ml of dH_2O and mix thoroughly. Resazurin dye solution is filtrate with membrane 0.45 µm and store in eppendrof wrap with foil at 4 °C. Dilute 1.8% resazurin with sterilize dH_2O for 1:10 and mix thoroughly befor using for antimicrobial test.

Table 14 Mixture of McFarland Standard

McFarland Standard	0.5	1	2	3	4	5	6	7	8	9	10
$BaCl_2$*	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
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
Approx. Cell density (x 10^8 cfu/ml)	1.5	3	6	9	12	15	18	21	24	27	30

* 0.048 $BaCl_2$ (1.175% W/V $BaCl_2H_2O$)

** 0.36 N H_2SO_4 (1% V/V)

Table 15 MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
BAO4	MA1	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
		CH	200	>200	200	>200							200	>200	32	32
BAO5	MA2	BE	200	>200	200	>200										
		CE	200	>200	200	>200					200	>200			32	32
		CH**	32	64	32	64					200	>200	200	200	32	32
BAO6	MA3	CH**	200	>200	200	>200					128	200	200	>200	32	32
BAO8	MA4	BE	200	200	200	200					200	>200			32	32
		CH	200	>200	200	>200							200	>200	32	32
DAO1	MA8	CE			200	>200										

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Endophytic fungi and their extracts / Test organisms			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
DAO4	MA11	BE									128	>200				
		CE									128	>200				
DAO5	MA12	BE													64	64
		CE			200	>200							64	128	64	200
		CH			64	128							**8**	**8**	200	>200
DSC1	MA15	BE			200	>200										
		CE	200	>200	200	>200										
DSC2	MA16	CE	200	>200	200	>200										
		CH	200	>200												

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Endophytic fungi and their extracts \ Test organisms			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
DXM1	MA18	BE													32	200
DXM2	MA19	BE													128	128
DXM5	MA20	BE**	64	>200	64	>200					200	>200	200	>200	32	200
		CE**	64	200	64	>200					128	200	200	>200	32	200
HSC11	MA21	BE	64	>200	64	>200							200	>200	32	200
		CE	200	>200	200	>200										
		CH	200	>200	200	>200										
KBP11	MA25	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
KBP3	MA29	BE	128	>200	128	>200									32	>200

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml
BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
KBP3	MA29	CE			128	>200										
KBP8	MA34	BE													32	>200
		CE	200	>200	200	>200										
		CH	**8**	64	**4**	64										
KRA13	MA36	BE			200	>200										
		CE	200	>200	200	>200										
		CH	200	>200	200	>200										
KRA15	MA38	CE	200	>200	200	>200										
KRA26	MA40	CH	200	>200												
KRA30	MA42	CE	200	>200	200	>200										

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
KRA30	MA42	CH	200	>200	200	>200										
KRA35	MA43	BE	200	>200	200	>200										
KRA4	MA45	BE			200	>200										
		CE			200	>200										
		CH													128	128
KRM5	MA53	BE													64	128
LAL6	MA60	BE	200	>200	200	>200									32	32
		CE	200	>200							200	>200			32	>200
		CH**	128	200	128	200					200	200	200	200	16	>200
LBP7	MA63	BE													32	>200

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
LBP8	MA64	BE	200	>200												
		CH													128	128
LLT16	MA66	CH	200	>200											128	128
LLT17	MA67	BE													128	128
		CE	200	>200											128	128
LRA8	MA69	BE	200	>200	200	>200					128	200	128	200		
		CE											200	>200	64	>200
		CH			200	>200										
LRA9	MA70	BE									64	200	32	>200	32	32
		CE**	200	>200	200	>200					200	>200	64	200	32	200

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml
BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
LSH3	MA71	CE	64	200	200	>200										
		CH	200	>200	200	>200										
LSH8	MA73	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
LXG8	MA75	CE	200	>200	200	>200										
		CH	200	>200	200	>200									32	200
NRA11	MA78	BE			200	>200										
		CE			200	>200							200	>200		
TAL6	MA81	BE													32	200
		CE									200	>200			128	128

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
TAL6	MA81	CH	200	>200	200	>200					64	>200			32	32
TAL8	MA82	CE			200	>200	200	>200					200	>200	64	64
TRA1	MA83	CH	200	>200	200	>200										
TRA11	MA84	BE													32	64
		CE													32	32
		CH	200	>200	200	>200									64	64
TRA13	MA86	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
TRA17	MA89	BE											200	>200		
		CE													128	>200

SA = *Staphylococcus aureus* ATCC25923　MRSA = methicillin-resistant *S. aureus* SK1　PA = *Pseudomonas aeruginosa* ATCC27853　EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028　CN = *Cryptococcus neoformans* ATCC90112　MG = *Microsporum gypseum* MU-SH4　* = µg/ml

BE = Broth EtOAc　CE = Cell EtOAc　CH = Cell Hexan　** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration　MBC = Minimal Bactericidal Concentration　MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
TRA19	MA90	BE	32	>200	64	>200							200	>200		
		CE	128	>200	200	>200										
		CH	128	>200	128	>200										
TRA2	MA91	BE			200	>200							200	>200	32	200
		CE	200	>200	200	>200									128	128
		CH	200	>200	200	>200							200	>200	128	32
TRA20	MA92	BE	32	>200	16	>200					200	>200			128	128
		CE	200	>200	200	>200									32	32
TRA26	MA95	BE													200	>200
TRA28	MA96	BE	128	>200	200	>200									32	32

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
TRA28	MA96	CE	64	>200	200	>200									16	16
		CH	64	128	128	128					128	>200			**4**	**8**
TRA30	MA98	BE													64	200
TRA32	MA99	BE	200	>200	200	>200							200	>200	200	>200
		CE	200	>200	200	>200										
		CH									32	128				
TRA33	MA100	CE	200	>200	200	>200										
		CH	200	>200	64	128					200	>200				
TRA8	MA103	BE	200	>200	200	>200										
		CE	200	>200	200	>200									128	>200

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4 * = µg/ml
BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
TRA8	MA103	CH	200	>200	200	>200										
BAO7	MA104	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
KBP1	MA105	CE	200	>200	200	>200										
KRA28	MA106	BE			200	>200										
HRA65	MA112	BE			200	>200									32	>200
		CH													128	128
LLT6	MA116	BE	200	>200	200	>200										
		CE	200	>200	200	>200										
LRM16	MA118	CE	200	>200	200	>200										

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
LRM24	MA119	BE	200	>200	200	>200									128	128
		CE													64	>200
		CH			200	>200									64	64
NRM37	MA120	BE	200	>200	200	>200							128	200	32	32
NRM37	MA120	CE	200	>200	200	>200									32	32
NRM19	MA121	CE	128	>200	200	>200									64	64
		CH	200	>200	200	>200										
NRM32	MA123	BE			200	>200										
		CE	200	>200	200	>200										
NXG11	MA124	BE			200	>200							200	>200	200	200

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
NXG11	MA124	CE	200	>200	200	>200					200	>200			32	32
		CH			200	>200										
NXG17	MA125	BE			200	>200										
		CE			200	>200	200	>200			200	>200				
NRM17	MA126	BE													32	32
NBP8	MA129	BE	200	>200	200	>200										
DRM3	MA132	BE	64	200	64	>200							200	>200		
		CE	16	64	16	64							64	>200		
DAL5	MA136	BE			200	>200									32	32
		CE			200	>200									32	32

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4 * = µg/ml
BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
LRM22	MA139	BE			200	>200										
NXG13	MA141	BE			200	>200										
TRA9	MA147	BE**	64	64	64	64					200	>200	200	200	32	32
		CE**	64	64	64	64					200	>200	200	200	32	32
		CH	200	200	200	200									128	128
DAL9	MA148	BE													128	128
		CE											16	16	**8**	**8**
		CH													32	32
DAL12	MA149	CE											32	>200	16	16
TAL1	MA151	CE											200	>200	64	64

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
TAL4	MA152	CH	200	>200	200	>200										
NLT21	MA156	BE**	128	>200	128	>200					128	128	128	128	**8**	**8**
		CE													32	32
		CH													32	32
NLT10	MA157	BE	200	>200	200	>200							200	>200	32	32
		CE													16	16
		CH	128	>200	200	>200									128	128
NLT4	MA160	CH	128	>200	200	>200									64	64
NSH8	MA164	BE											64	>200	128	128
		CE											128	>200		

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = µg/ml

BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
NSH8	MA164	CH													200	200
NAC2	MA165	CE													64	128
		CH	64	>200	200	>200							200	>200	64	64
NSH13	MA167	BE													64	64
		CE											200	200		
NXG7	MA168	CE													32	32
		CH	200	>200	200	>200										
TRA24	MA173	CE	128	>200	200	>200										
TRA24	MA173	CH	64	>200	200	>200							64	>200		
LXM29	MA174	CH	200	>200	200	>200										

SA = *Staphylococcus aureus* ATCC25923; MRSA = methicillin-resistant *S. aureus* SK1; PA = *Pseudomonas aeruginosa* ATCC27853; EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028; CN = *Cryptococcus neoformans* ATCC90112; MG = *Microsporum gypseum* MU-SH4; * = μg/ml
BE = Broth EtOAc; CE = Cell EtOAc; CH = Cell Hexan; ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 μg/ml; Moderate activity : MIC 16-64 μg/ml; Weak activity : MIC 128-200 μg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
NSH16	MA177	BE	200	>200	200	>200							200	200		
		CE**	200	>200	200	>200					128	128	32	32	32	32
		CH	200	>200	200	>200										
NSH14	MA178	BE	128	>200	200	>200									32	32
		CE													64	64
		CH											200	>200	128	128
LBP3	MA182	CE	128	>200	200	>200										
		CH	128	>200	200	>200										
KRA6	MA186	CH	200	>200												
KRA18	MA190	CE											200	200		

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4 * = µg/ml
BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration

Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
KRA18	MA190	CH	200	200	200	200					200	>200			200	200
KRA33	MA193	BE	200	>200	200	>200							200	>200	32	32
		CE													32	32
KRA34	MA194	BE	200	>200	200	>200										
		CE**	128	>200	128	>200					200	200	128	200	16	16
		CH**	32	>200	32	>20					32	32	**8**	200	**8**	**8**
KRA37	MA195	BE**	200	200	128	200					200	>200	128	>200	128	>200
		CE	200	>200	200	>200										
		CH													32	32
KRA42	MA198	BE													200	200

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922
CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4 * = µg/ml
BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan ** = Active extracts that inhibit 5 test strains
MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration
Strong activity : MIC ≤ 8 µg/ml; Moderate activity : MIC 16-64 µg/ml; Weak activity : MIC 128-200 µg/ml

Table 15 (cont.) MICs, MBCs or MFCs of active crude extracts compared to standard antibiotics

Test organisms / Endophytic fungi and their extracts/Drug control			Bacteria								Yeasts				Filamentous fungus	
Old code	New code	Extract	SA		MRSA		PA		EC		CA		CN		MG	
			MIC*	MBC*	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC*	MIC	MFC	MIC	MFC
KRA42	MA198	CE													128	128
KRA48	MA201	BE	200	>200											32	32
Vancomycin			0.5	1	1	2										
Gentamicin							1	2	0.5	2						
Amphotericin B											0.25	1	0.5	1		
Miconazole															0.5	2

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 PA = *Pseudomonas aeruginosa* ATCC27853 EC = *Escherichia coli* ATCC25922

CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4 * = µg/ml

BE = Broth EtOAc CE = Cell EtOAc CH = Cell Hexan ** = Active extracts that inhibit 5 test strains

MIC = Minimal Inhibitory Concentration MBC = Minimal Bactericidal Concentration MFC = Minimal Fungicidal Concentration

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

VITAE

Name Miss Jirayu Buatong

Student ID 5010220028

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology)	Prince of Songkla University	2005

Scholarship Award during Enrolment

1. Teaching Assistant Scholarship, Microbiology Department, Prince of Songkla University
2. Center of Excellence for Innovation in Chemistry (PERCH-CIC) Scholarship

List of Proceedings

Buatong, J., Phongpaichit S. and Sakayaroj J. 2009. Antimicrobial activities of secondary metabolite produced by endophytic fungus MA194 from *Rhizophora apiculata*. Proceeding of 4th BUU Grad. Research Conference. Burapha University, March, 13, 2009. pp. 102.

Buatong, J., Phongpaichit, S., Sakayaroj, J. and Rukachaisirikul, V. 2009. Antimicrobial activities of endophytic fungi isolated from *Rhizophora apiculata* and *Rhizophora mucronata*. Poster presentation on PERCH-CIC congress VI. Jomtien Palm Beach Hotel and Resort Pattaya, Chonburi, May, 3-6, 2009. pp. 279.