ราเอนโดไฟต์คัดเลือกจากพืชป่าชายเลนในจังหวัดจันทบุรี ประจวบคีรีขันธ์ และระนอง ประเทศไทย

นางสาวสุกัญญานี แซประเสริฐ



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# ENDOPHYTIC FUNGI FROM SELECTED MANGROVE PLANTS IN CHANTHABURI, PRACHUAP KHIRI KHAN AND RANONG PROVINCES, THAILAND

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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สุกัญญานี แซ่ประเสริฐ : ราเอนโดไฟต์คัดเลือกจากพืชป่าชายเลนในจังหวัดจันทบุรี ประจวบคีรีขันธ์ และระนอง ประเทศไทย. (ENDOPHYTIC FUNGI FROM SELECTED MANGROVE PLANTS IN CHANTHABURI, PRACHUAP KHIRI KHAN AND RANONG PROVINCES, THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ประกิตติ์สิน สีหนนทน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : Professor Anthony J. S. Whalley, จำนวนหน้า 135 หน้า.

เปรียบเทียบการแพร่กระจายราเอนโดไฟต์ในใบพืชป่าชายเลนที่เจริญในพื้นที่ 3 แหล่งของประเทศไทยได้แก่ อ่าวคุ้งกระเบน จังหวัดจันทบุรี วนอุทยานแห่งชาติปราณบุรี จังหวัดประจวบคีรีขันธ์ และศูนย์ชีวมณฑล จังหวัดระนอง โดยเก็บใบพืชป่าชายเลน 7 วงศ์ได้แก่ วงศ์โกงกาง (โกงกางใบใหญ่ โกงกางใบเล็ก โปรงขาว) วงศ์ลำพู (ลำพูทะเล) วงศ์ฝาด (ฝาดดอกขาว) วงศ์แสม (แสมขาว) วงศ์เหงือกปลาหมอ (เหงือกปลาหมอดอกม่วง) วงศ์ตะบูน (ตะบูนขาว และตะบูนดำ) และวงศ์โพ (โพทะเล) แยกราเอนโดไฟต์ได้จำนวนราเอนโดไฟต์ทั้งหมด 1,965 ไอโซเลท พบราเอนไฟต์ในใบพืชทุกชนิด ราสกุลเด่นที่พบในพืชทุกชนิดซึ่งเก็บจากจังหวัดจันทบุรีคือราในสกุล *Phyllosticta* และพบราชนิดนี้เป็นราสกุลเด่นในโกงกางใบเล็กและโปรงขาวที่เก็บจากจังหวัดประจวบคีรีขันธ์ และยังเป็นราสกุลเด่นในเหงือกปลาหมอดอกม่วงและตะบูนดำในจังหวัดระนอง ในขณะที่ราสกุลเด่นที่พบในใบโพทะเลคือราในสกุล *Phoma* ราเอนโดไฟต์สกุลเด่นที่พบในพืชที่เก็บจากจังหวัดระนองจะแตกต่างกันในพืชแต่ละชนิดโดยพบราสกุล *cladosporium* เป็นราสกุลเด่นในโกงกางใบเล็ก ราสกุล *Pestalotiopsis* เป็นราสกุลเด่นในโกงกางใบใหญ่และราสกุล *Glomerella* เป็นราสกุลเด่นในตะบูนขาว ราเอนโดไฟต์ที่พบในพืชป่าชายเลนส่วนใหญ่ ได้แก่ ราในสกุล *Cladosporium* สกุล *Colletotrichum* สกุล *Daldinia* สกุล *Pestalotiopsis* สกุล *Phomopsis* และสกุล *Xylaria* เมื่อนำราเอนโดไฟต์จำนวน 191 ตัวอย่างมาสกัดดีเอ็นเอและเพิ่มจำนวนดีเอ็นเอโดยใช้ไพร์เมอร์ ITS3 และ ITS4 สามารถเพิ่มจำนวนดีเอ็นเอได้สำเร็จจำนวน 168 ตัวอย่าง จากนั้นจัดกลุ่มของราเอนโดไฟต์ตามขนาดของดีเอนเอ็นได้จำนวน 11 กลุ่ม นำตัวแทนราแต่ละกลุ่มมาหาลำดับเบสที่ตำแหน่ง ITS เปรียบเทียบกับข้อมูลลำดับเบสที่อยู่ใน GenBank และวิเคราะห์ความสัมพันธ์เชิงวิวัฒนาการพบว่าราเอนโดไฟต์ที่แยกได้ส่วนใหญ่อยู่ใน phylum Ascomycota เมื่อเทียบผลที่ได้กับการจัดจำแนกสกุลราโดยใช้คุณลักษณะสัณฐานทางวิทยามีความสอดคล้องกัน ทำให้ทราบชนิดของ sterile mycelium ได้ จากการศึกษาผลการสร้างสารยับยั้งการเจริญของแบคทีเรียแกรมบวก (*Bacillus subtilis* ATCC 6633 และ *Staphylococcus aureus* ATCC 25923) แบคทีเรียแกรมลบ (*Pseudomonas aeruginosa* ATCC 27853 และ *Escherichia coli* ATCC 25922) และยีสต์ (*Saccharomyces cerevisiae* ATCC 5169 และ *Candida albicans* ATCC 10231) ในสารสกัดที่ได้จากอาหารเลี้ยงเชื้อราเอนโดไฟต์พบว่า ราสกุล *Cladosporium* sp. P1 ที่แยกจากใบโพทะเลสามารถสร้างสารยับยั้งการเจริญของจุลินทรีย์ที่ใช้ทดสอบได้ทุกชนิด นอกจากนี้ยังทำการศึกษาผลการสร้างสารยับยั้งเซลล์มะเร็ง 5 ชนิดได้แก่ เซลล์มะเร็งผิวหนัง (A375 ATCC CRL-1619) เซลล์มะเร็งลำไส้ (SW620 ATCC CCL-227) เซลล์มะเร็งกระเพาะอาหาร (Kato III ATCC HTB-103) เซลล์มะเร็งตับ (HepG2 ATCC HB-8065) และเซลล์มะเร็งเม็ดเลือดขาว (Jurkat ATCC CRL-2063) พบราเอนโดไฟต์จำนวน 7 สายพันธุ์ที่สามารถสร้างสารยับยั้งอย่างจำเพาะกับเซลล์มะเร็งเม็ดเลือดขาวโดยส่งผลให้มีอัตราการอยู่รอดของเซลล์น้อยกว่า 40 เปอร์เซ็นต์และราเอนโดไฟต์ในสกุล *Phomopsis* sp. R2 ที่แยกจากใบเหงือกปลาหมอสามารถสร้างสารยับยั้งอย่างจำเพาะกับเซลล์มะเร็งลำไส้โดยมีอัตราการอยู่รอดของเซลล์ที่ 9.82%

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา....2552..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : ENDOPHYTIC FUNGI / MANGROVE PLANTS / LEAF / TREES / THAILAND

SUKUNYANEE CHAEPRASERT: ENDOPHYTIC FUNGI FROM SELECTED MANGROVE PLANTS IN CHANTHABURI, PRACHUAP KHIRI KHAN AND RANONG PROVINCES, THAILAND. THESIS ADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D. , THESIS CO-ADVISOR : PROF. ANTHONY J. S. WHALLEY, 135 pp.

Compare endophytic fungi distribution in the mangrove leaf forest trees growing in three different locations (Kung krabaen bay; Chanthaburi Province, Pranburi forest park; Prachuapkhirikhan Province and Ranong Biosphere Reserve; Ranong Province) in Thailand. Three thousand and nine hundred leaf segments from ten different hosts belonging to seven families, Rhizophoraceae (*Rhizophora apiculata*, *R. mucronata*, *Ceriops decandra*), Sonneratiaceae (*Sonneratia alba*), Combretaceae (*Lumnitzera littorea*) Avicenniaceae (*Avicennia alba*), Acanthaceae (*Acanthus ilicifotius*), Meliaceae (*Xylocarpus granatum* and *Xylocarpus moluccensis*) and Malvaceae (*Thespesia populneoides*), were screened for the presence of fungal endophytes. One thousand nine hundred and sixty five fungal endophyte isolates were isolated. Dominant species isolated from Chanthaburi were the genus *Phyllosticta* and it was also the dominant genus in *R. apicula* and *C. decandra* growing in the Prachuap Khiri Khan site. Morever this species was found the dominant isolates in *A. Ilicifotius* and *X. moluccensis* The dominant endophytic species of *T. populneoides* was *Phoma*. The dominant endophytic species in mangrove plants from Ranong Province were different for each host plant in the mangrove community. Species of *Cladosporium* was the dominant in *R. apiculata* whereas *Pestalotiopsis* was the dominant in *R. mucronata*. It was found the species of *Glomerella* as the dominant fungal endophyte in *X. granatum*. The endophytic assemblages of the mangrove trees were composed of a number of species such as *Cladosporium Colletotrichum Daldinia Pestalotiopsis Phomopsis* and *Xylaria*. One hundred and sixty eight endophytic fungal isolates were successfully amplified by using primer ITS3 and ITS 4 and then were grouped based on ITS region size. Each representative was selected for DNA sequence analysis using the whole ITS region, including ITS1, 5.8S, and ITS2. BLAST search in GenBank showed that all the rDNA ITS sequences could be grouped within the fungi domain. Our study also showed that molecular identification based on ITS sequences and phylogenetic relationships analysis can be used to complement or verify morphological identification of unknown endophytes and mycelium sterilia. The antimicrobial potential activity of 71 endophytic fungi were tested with selected bacteria (*Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Saccharomyces cerevisiae* ATCC 5169 and *Candida albicans* ATCC 10231) by using ethyl acetate crude extracts fungal mycelia cultivated under static conditions. All test bacteria and yeast were inhibited by a *Cladosporium* sp. P1 isolated from the leaves of *T. populneoides*. Additionally eighty four endophytic fungal crude extracts were tested for anticancer activities by the MTT assay against A375 ATCC CRL-1619 (Human malignant melanoma), SW620 ATCC CCL-227 (Human colorectal adenocarcinoma), Kato III ATCC HTB-103 (Human gastric carcinoma), HepG2 ATCC HB-8065 (Human liver hepatoblastoma) and Jurkat ATCC CRL-2063 (Human acute T cell leukemia). Seven endophyte isolates acted as specific anticancer activities against Jurkat cells with cell viability below 40%. *Phomopsis* sp. R2 was isolated from *A. ilicifotius*[R] displayed specific inhibition with Human colorectal adenocarcinoma (9.82%).

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Field of Study : Biotechnology Advisor's Signature Prakitsin Sihanonth

Academic Year : 2009 Co-Advisor's Signature A.J.S. Whalley

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# List of Abbreviations

| | | |
|---|---|---|
| °C | = | degree celsius |
| cm | = | centrimeter |
| diam. | = | diameter |
| e.g. | = | for example |
| *et al.* | = | et alii (and others) |
| g | = | gram |
| ha | = | hectare |
| km | = | kilometer |
| l | = | liter |
| m | = | meter |
| M | = | molar |
| mins | = | minutes |
| ml | = | milliliter |
| mm | = | millimeter |
| mM | = | millimolar |
| mg | = | milligram |
| rpm | = | revolutions per minute |
| s | = | second |
| sp. | = | species |
| v/v | = | volume by volume |
| w/v | = | weight by volume |
| µg | = | microgram |
| µl | = | microlitre |
| µm | = | micrometer |

# CHAPTER 1
# INTRODUCTION

## 1.1 Significance of the study

Mangroves are defined as woody trees and shrubs that grow in places where river water mixes with seawater (Sridhar, 2004). Approximately one-fourth of the world's coastline is an dominated by mangroves and these are distributed in 112 countries and territories comprising a total area of about 181,000 $km^2$. Mangrove plants have morphologically and physiologically adapted to habitats with high salinity, tidal inundation, high wind velocity, high temperature and anaerobic clayey soils (Sridhar, 2004). Mangroves are well known for several by-products of medicinal value and economic importance. Numerous mangrove plants have been used in folklore medicine and extracts from mangroves have proven activity against human, animal and plant pathogens (Bandaranayake, 2002). Most of the marine fungi described from Malaysia are from mangrove substrate, either drift or attached mangrove wood (Jones, 2007) as is the case for records around the world (Schmit and Sheares, 2003; 2004). Asia has been the four of intensive studies including Brunei (Hyde, 1988); Malaysia (Jones and Kuthubuthen 1990; Alias *et al*. ,1995, 1996; Alias and Jones 2000); the Philippine (Jones *et al*., 1988) and Singapore (Tan *et al*., 1989; Leong *et al*., 1991). Ecologically vertical distribution, host specificity or preference and period of submersion of test bloscks in relation to colonization have all been investigated. The major aspects of these studies have been recently reviewed by Jones (2007). In spite of these detailed investigations of mangrove fungi there were few reports on the endophytic fungi of mangrove plants with the exception of a number of Indian studied.

Endophytic fungi are microorganisms that colonize internal plant tissue. They can live there for all or part of their life cycle without causing any apparent damage or disease (Petrini *et al*., 1992). These endophytes can be transmitted from one generation to the next through tissue of host seed, vegetative propagules, or horizonally, external to host tissue, by spores (Carroll, 1988). The mangrove habitat has proved to be a rich

source of new fungal species and these now form the second largest ecological sub-group of marine fungi (Li *et al*., 2003). As stated there have been few studies on the endophytic fungi of mangrove plant species, and they are confined to foliar endophytes. Some of the mangrove plants that have been studied for their endophyte associations include *Rhizophora apiculata* and *R. mucronata* (Suryanarayanan *et al*., 1998), *Aegiceras corniculatum*, *Avicennia marina, A. officinalis*, *Bruguiera cylindrical*, *Ceriops decandra*, *Excoecaria agallocha* and *Lumnitzera racemosa* (Kumaresan and Suryanarayanan, 2001), *Acanthus ilicifolius*, *Arthrocnemum indicum*, *Suaeda maritime* and *Sesuvium portulacastrum* (Suryanarayanan and Kumaresan, 2000). The potential role of endophytes and their potentially interesting biologically active metabolites in their association with the host has been investigated (Schulz *et al*., 2002; Strobel, 2003; Strobel and Daisy, 2003; Fiáková *et al*., 2007; Guo *et al*., 2008). Recently, research on the secondary metabolites of mangrove endophytic fungi had led to the isolation of a number of new compounds with different bioactivities (Chen *et al*., 2007, 2009; Guo *et al*., 2007; Haung *et al*., 2007, 2008; Li *et al*., 2008a, b; Lin *et al*., 2008a, b; Shao *et al*, 2007, 2008; Tan *et al*., 2008; Wang *et al*., 2008).

Due to its geographical position in tropics and climatic variation between north and south, Thailand is a country of high biological diversity ,which is reflected in the number of species reported (Tanicharoen, 2004). Watling (1988) compared the fungal biodiversity of Thailand with surrounding countries and concluded that it will be richer due to the convergence of elements. Endophytes of tropical plants have been widely researched in Thailand mainly concentrated on biodiversity, ranging from studies on banana plants (Photita *et al*., 2000), teak leaves (Chareprasert *et al*., 2006; Mekkamol, 1998), rain tree leaves(Chareprasert *et al*., 2006) and *Amomum siamense* (Bussaban *et al*., 2001). In Thailand, the estimated mangrove area in 2000 was 2,062 $km^2$ (RFD, 2004) or approximately 1.2% of the world mangroves with the dominant species belonging to the genera *Rhizophora*, *Ceriops* and *Bruguiera* in the family Rhizophoraceae, *Sonneratia* in the family Sonneratiaceae and *Avicennia* in the family Avicenniaceae (Aksornkoae 1993). Major mangrove forests stand on the Western shoreline of Thailand

facing Andaman Sea. The composition of mangrove forest encompasses a variety of plants including large trees, epiphytes, the liaras and algae, Thailand is home to 74 species of trees and shrubs in 35 different families. There were no reports on endophytic fungi in mangrove plants of Thailand. This study, therefore, provides the first information on the isolation of endophytic fungi from mangrove plants of Thailand. Due to the special living environment of tidal mudflats, mangrove plants have been considered as a source of compounds possessing physiological activities (Lin *et al*., 2008). Investigation of the secondary metabolites of microorganisms isolated from mangrove plants residing in niche environment may increase the chance of finding novel active compound.

## 1.2 Research Objectives

1.2.1 To study the distribution of endophytic fungi of different mangrove hosts in mangrove community at same location and different locations.

1.2.2 To screen for biological activities of the extracts from endophytic fungi culture broth including anticancer, antibacterial and antifungal activities.

# CHAPTER 2
# LITERATURE REVIEW

Fungi play the roles in all terrestrial environments. They are a major component of global biodiversity and control the rates of key ecosystem processes. Fungi directly shape the community of plants, animals and bacteria through interactions that span the mutualism-parasitism continuum. While some fungi are clearly parasites, the nature of many fungal interactions is uncertain and may change depending on the environment in which the interactions occure (Kabir *et al*., 2008). It now appears that an enormous, relatively untapped source of microbial diversity is represented by the microbial endophytes (Strobel, 2003). The word endophyte could refer to the location of the organism: endo means within and phyte means plant. Thus, an endophyte is an organism which lives inside a plant (Wilson, 1995). This is contrasted to epiphyte which refers to organisms living on the outer surface of host plant.

The term endophyte was originally defined by De Bary and referred to any organism occuring within plant tissues. In the1980's, the term became restricted to include only those organisms that cause asymptomatic infections in a host and excluded pathogenic fungi and mutualists such as mycorrhizae (Carroll, 1986). Various authors later narrowed the definition on accordance with particular research interests, leading to much confusion with application of the term. In 1991, Petrini provided a working definition for endophytes that has since been widely accepted: endophytes comprise " microorganisms inhabiting plant organ which at some time in their life colonize internal plant tissues without causing apparent harm to their host". So defined, endophytes include microorganisms that have an epiphytic phase prior to internal infection, mutualistic microbes, benign commensals, and latent or quiescent pathogens that may live symptomless in their hosts for some time before manifesting themselves (Petrini, 1991).

In 1995, Wilson who re-defined the term endophyte described not only the location, but the type of infection strategy of a particular fungus without regard to the taxonomy or infection cycle and it would exclude mycorrhizal fungi but include the following: 1) fungi which have dormant or latent phases within plant tissue prior to causing disease symptoms on the plant host. Although these fungi are clearly pathogenic, they can still have an endophytic phase to their life cycle. 2) fungi which are known pathogens of a plant host but never exhibit disease symptoms after infection. Brungrett (2004) distinguished mycorrhizal from endophytic interactions, mycorrhizas having synchronized plant-fungus development and nutrient transfer at specialized interfaces.

Endophytic fungi appear to be ubiquitous, every plant species examined to date harbors at least one fungal endophyte species (Arnold *et al*., 2000) and many plant species may be associated with tens to hundreds of endophyte species (Stone *et al*., 2000). Within hosts, fungal endophytes may inhibit all available tissue, including leaves, petioles, stems, twigs, bark, xylem, roots, fruit, flower, and seeds (Arnold, 2007; Rakotoniriana *et al*., 2008). Despite the broad occurrence of endophytes in plant organs, many endophytic fungi appear specialized to particular host tissues (e.g. twigs, petioles, leaves). In particular, leaves and leaf-inhabiting endophytes represent a focus for endophytic diversity and ecology.



There are three methods presently in use for detecting and identifying endophytic fungi in plant tissue: (1) histological observation; (2) surface sterilization of the host tissue and isolation of the emerging fungi onto appropriate growth media; and (3) detection by specific chemistry, (e.g. direct amplification of fungal DNA from colonized plant tissue), having first ascertained that there are no fungal residues on the plant surface (Schulz and Boyle, 2005). Light microscopy may be useful for screening purposes (Cabral *et al*., 1993) and SEM and TEM to visualize fungal structure within the plant tissue (Christensen *et al*., 2002). Tissue for light microscopy may be observed directly, following vital staining to ascertain that the fungus is living or after fixation,

clearing and staining. Additionally, if the visualized hyphae infected the host naturally, it is difficult to determine the taxon to which they belong. Isolation of fungi following surface sterilization onto appropriate growth media is usually the initial step for investigation endophytes. The most common procedure first employs a surfactant such as ethanol and followed by sterilizing agent, such as sodium hypochlorite (Bills, 1996). The surface sterilization has to be optimized for the host with regard to tissue sensitivity, age, and the organ being sterilized. It is preferable to check the effectiveness of surface sterilization by imprinting treated tissue on a fungal growth medium, If no colonies develop from the imprint, the sterilization can be assumed to have been effective (Schulz *et al*., 1998). Molecular methods have been used not only for fungal taxonomy but also to identify isolates that do not sporulate in culture. Molecular methods also permit identification of fungi that are viable but not culturable from the hosts. It is an importance consideration that surface sterilization may not have denaturized the DNA of epiphytes, though sodium hypochlorite is relatively effective for this purpose.

The transmission of endophytes has been categorized in two ways; horizontal and vertical transmission. Grass endophytes colonize their hosts systemically and several species are transmitted vertically by seed to the next host generation. Incontrast, all plants studied to date, including liverworts, mosses, ferns, conifers, and angiosperms, are infected by multiple lineges of horizontally transmitted fungal endophytes by spore. The majority of these endophytes are Ascomycota, although some Basidiomycota and a few Zygomycota are known. Diverse associations with host plants have been reported, ranging from mutualistic relationship (Schulz *et al*., 2002) and cryptic commensalism (Deckert *et al*., 2001) to latent and quiescent pathogen (Sinclair and Cerkauskas, 1996). The stability or the variability of the asymptomatic interaction depends on numerous factors such as environmental stress, senescence of the hosts, virulence of the endophytes and the host defense response (Schulz and Boyle, 2005).

Two major groups of endophytic fungi have been recognized, reflecting differences in evolutionary relatedness, taxonomy, plant hosts, and ecological functions:

1. Clavicipitaceous endophytes (C-endophytes), which infect some grasses
2. Nonclavicipitaceous endophytes (NC-endophytes), which can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies, conifers, and angiosperms.

They differ in important ways and should not be regarded as biologically or ecologically homologous (Table 2.1).

Table 2.1 Comparison of characteristics of endophytes occurring in grass and nongrass hosts

| Endophytes of grass hosts | Endophyte of nongrass hosts |
|---|---|
| -Few species, Clavicipitaceae | -Many species, taxonomically diverse |
| -Extensive internal colonization | -Restricted internal colonization |
| -Occurring in several host species | -Most species with limited host species |
| -Systemic, seed transmitted | -Nonsystemic, spore transmitted |
| -Host colonized by only one species | -Host infected by several species concurrently |

## 2.1 Clavicipitaceous endophytes (C-endophytes)

The first records of endophytic fungi in grasses date back to the late 19$^{th}$ century. The grass endophytes that cause such effects belong to the family Clavicipitaceae (Ascomycota), tribe Balansiae. There were five genera (*Atkinsonella, Balansia, Balansiopsis, Epichloe,* and *Myriogenospora*) total ≈ 30 species. *Balansia* is the largest genus, with 15-20 species. Unlike *Claviceps*, a related and well-known genus of transient ovarian parasites of grasses and sedges, the Balansiae fungi are systemic and perennial (Clay, 1986). Most of the species are endophytic, with hyphae that occur intercellularly in leaf and stem tissues. Clavicipitaceous fungal endophytes of the asexual from, *Neotyphodium* (previously *Acremonium*) systemically infect the

intercellular spaces of plants belonging to several grass genera. The sexual form, *Epichloe* has a parasitic nature and form external stromata and thus have the potential to be transmitted horizontally. *Epichloe* species require fly pollinators for fertilization. The flies are in the genus *Botanophila* (Diptera). Female flies transfer spermatia in their gut a they feed and oviposit. After oviposition, the fly fertilizes the stroma by dragging her abdomen while excreting faeces on the stromal surface; larva feed on the fungal stroma. Flies are the third mutualist in this association. *Neotyphodium* can be observed as hyphal accumulations in infected grass seeds or as single, long and often convoluted hyphae that grow between the cells of the host's leaf tissue. During the vegetative life cycle of the plant, these fungi do not induce any symptom, however, when the plant reproductive cycle starts, grass endophyte interactions can be of three types, depending on the species involved in the host-fungus association (Figure 2.1 and 2.2). In type I interactions, during the plant reproductive cycle an external fungal stroma prevents the emergence of the inflorescence, thus sterilizing the plant. This symptom is known as "choke disease" of grasses. Type II interactions are characterized because a few infected plants in a population are sterilized by stromata, but most infected plants remain asymptomatic, and produce seeds which are infected by the fungus. When those infected seeds germinate, they give rise to infected plants. The type III interactions, infected plants do not show any symptoms, and the fungi use seed transmitted to the next generation (White, 1988). In these interactions in which infected plants remain asymptomatic and the fungus is seed transmitted (type II and III), endophyte infection may be beneficial for the plant host. Relative mutualism or antagonism of an *Epichloe*-grass symbiosis is largely related to the path or paths of symbiont transmission.

It has been reported that *Festuca* and *Lolium* species infected by *Neotyphodium* and *Epichloe* endophytes are more resistant to several species of insects than uninfected plants. Also, seed production and germination is greater in infected *F. arundinacea* plants and *L.* perenne plants are resistant to drought stress than uninfected plants (Siegel et *al.,* 1987; Clay, 1987). The interest in grass-endophyte interactions started

when it was demonstrated that a series of health problems occurring in grazing livestock, known as fescue toxicosis, were caused by the consumption of *F. arundinacea* infected by the endophyte *N. coenophialum* (Bacon et *al.,* 1977). Similar problems also occur in sheep fed with *L.* perenne infected by *N. lolii.* These problems are caused by the production of toxic alkaloids by *Neotyphodium* and *Epichloe* endophytes in infected plants. Endophyte-related livestock toxicoses are considered a serious problem in New Zealand and the United States, where ryegrass and tall fescue pastures are the main component of the livestock diet.

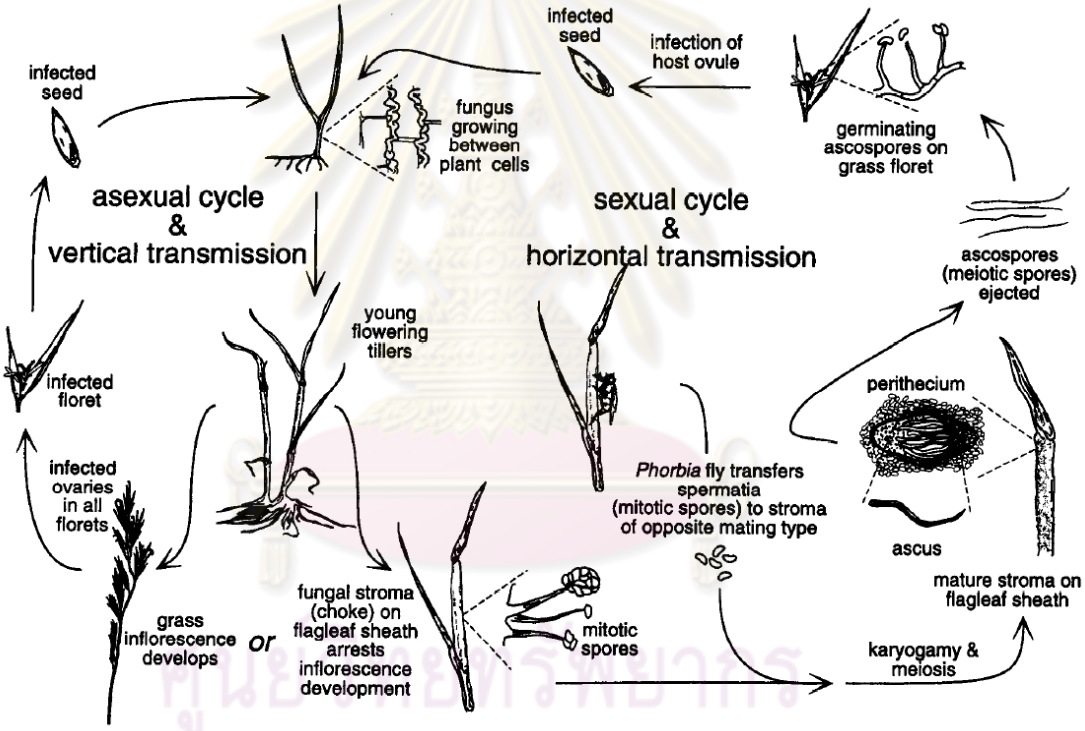


Figure 2.1 An example of pleiotropic symbiosis involving coordinated life cycles of *Epichloe festucae* and its host grass, *Festuca rubra*. The alternative life cycles (clockwise and counterclockwise loops) occur on different tillers of a symbiont-infected plant. In the symbiont's asexual life cycle (clockwise loop) it systemically infects the seeds produced on the mother plant and it thus transmitted vertically through successive host generations. The synbiont's sexual cycle (counterclockwise loop) is initiated by a fungal growth (stroma) that envelops a young flowering tiller and prevents the inflorescence from maturing. Following fertilization and maturation, meiosis spores

are ejected and mediate horizontal (contagious) transmission. Antagonistic *Epichloe* species sterilize most or all flowering tillers of their host plants and are never (or perhaps rarely) transmitted vertically.

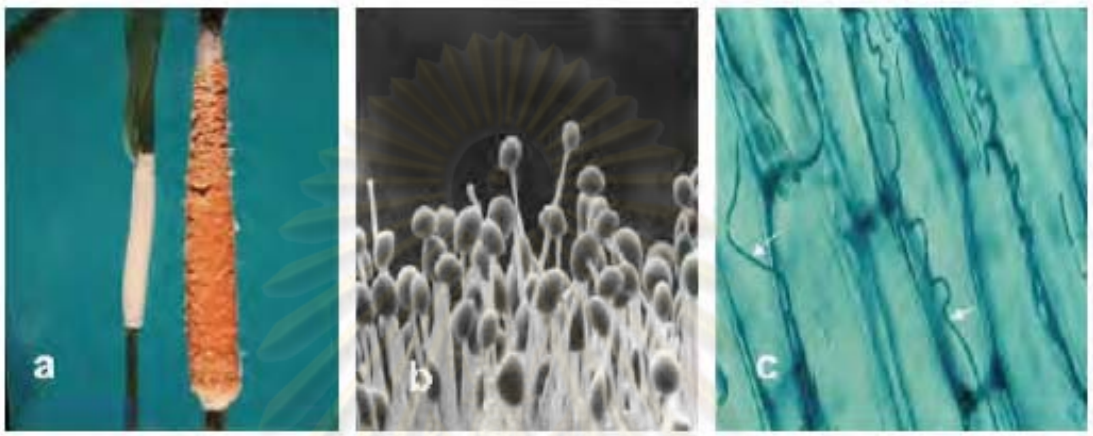


Figure 2.2 Choke disease of *Epichloe amarillans* on autumn benigrass, *Sphenopholis abtusata.* (a) Early phase of disease expression showing white-to-ivory conidiomata (left) completely covering the inflorescence of bentgrass, and at right a mature yellow-to-orange stromata bearing perithecia, around the culm, enclosing the inflorescence primodia, leaf primodia and sheath. (b) A scanning electron micrograph of the conidioma in (a) showing details of the conidiogenous cells with reniform-to-hemispherical conidia (spermatia) born at the tips of conidiophores, typical of the anamorphic state *Neotyphodium*. (c) Light microscopy (40x) of aniline blue stained intercellular hyphae running vertically along the intercellular spaces of the host cells.

Endophytic fungi in the genus *Neotyphodium* confer many beneficial effects to their host plants, including resistance to pests, diseases, grazing and environmental stresses (Belesky and Fedders, 1995; Clay, 1989; Clay *et al*., 1989; Gwinn and Gavin, 1992; Malinowski and Belesky, 2000; Read and Camp, 1986). Although endophytic fungi are beneficial to their host grasses, they also produce alkaloid toxins that are harmful to livestock (Aldrich *et al*., 1993; Porter, 1995). Ergovaline is one of the ergot toxins produced by several *Neotyphodium* spp., especially those infecting tall fescue

(*Festuca arundinacea*). Ergovaline consumption in livestock has been associated with poor weight gain, hormonal imbalances leading to reduced fertility, lactation and gangrene of the animal's limbs (Porter, 1995). However, a direct cause and effect relationship between ergovaline and these symptoms has not yet been demonstrated. Nutrients are cycled between the host and fungus. The loss of plant resources to the fungus, and the potential of some fungi to grow rapidly, indicates that the host regulates development of colonies. Each plant host has a range of physical, chemical, constitutive and induced controls over the spread of fungi within tissues. An enormous diversity of phenolic and other deterrent plant compounds are associated with the presence of endophytic fungi, in fact more than are associated with potent pathogens in the same host. In addition, presence of endophytes upregulates plant responses to pathogens. In the absence of plant controls, proliferation of endophytes through tissues would be expected.

Defence chemicals are produced by the fungus, or the host when infected by the fungus. The chemicals disrupt several metabolic processes in herbivores that eat the grasses. The active metabolites are found in at least four broad categories (Figure 2.3):

1. The ergopeptides ergovaline and ergovalinine are based on lysergic acid amide. Generally known as ergot alkaloids, they induce various metabolic disorders that reduce reproductive rates and growth of grazing animals. The signs include elevated temperature, vasoconstriction, and reduced prolactin in blood. Serum prolactin is a growth hormone that regulates feed intake in mammals. The ergot alkaloids also influence animal gait.

2. Tremogenic neurotoxins Lolitrem A and B are indole diterpenoids. Along with paxilline alkaloids they induce various stiff legged gaits in sheep grazing on infected grass. In addition, they are toxic to or deter feeding by various insects.

3. The unusual Pyrrolopyrazine Peramine is thought to influence insect activity. It is widely found to deter insect feeding. While peramine is the only compound so far chemically characterised, related lipophilic compounds have been indicated in some other endophyte associations.

4. Pyrollizidine-based loline alkaloids reduce feeding by some insects, and acts in concert with the other compounds to reduce feeding and reproductive rates of many more insects. They also appear to be a factor in the allelopathic properties of infected fescue.

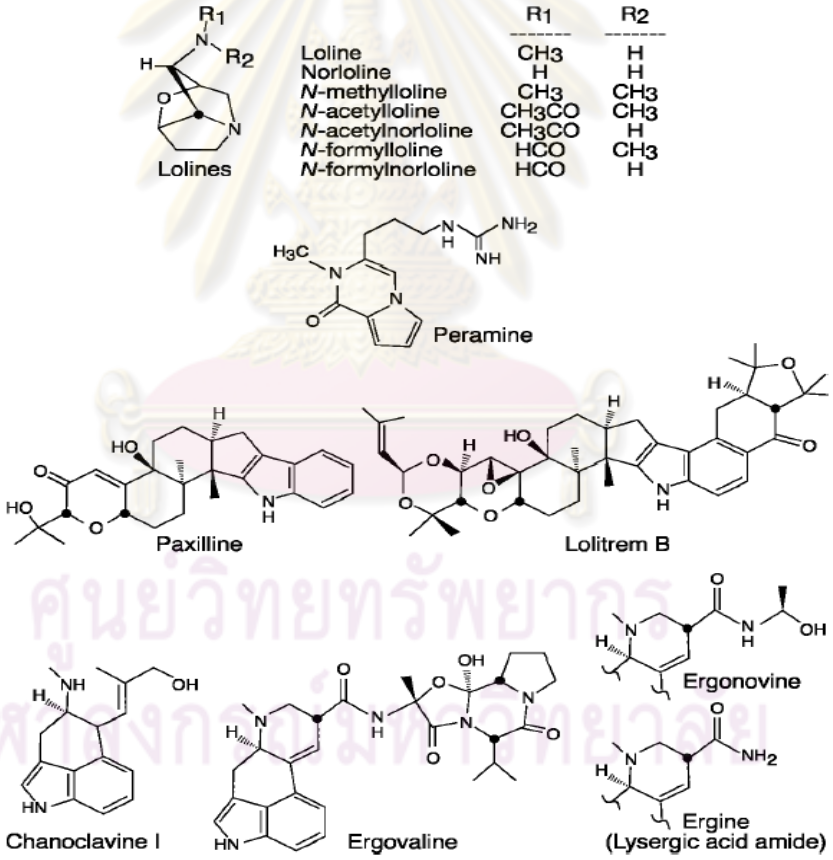


Figure 2.3 Representative of the four class of alkaloids produced by *epichloe* endophytes of grass, namely, l-aminopyrrolizidine alkaloids (lolines), peramine (the only known pyrrolopyrazine), indolediterpenes (here represented by paxilline and loliterm B),

and ergot alkaloids (ergovaline, ergonovine, and ergine). Also shown in chanoclavine I, a clavine alkaloid precursor to the ergot alkaloids.

## 2.2 Non-clavicipitaceous endophytes (NC-endophytes)

The non-clavicipitaceous fungal endophytes are diverse, both phylogenetically and with respect to life-history strategy. Several studies have shown that the majority of endophytic fungi are Ascomycota, including many mitosporic fungi classified as Deuteromycota (Petrini, 1986), and they have been isolated from every organ of almost all sampled plants (e.g. Petrini 1991, Stone *et al*., 2000). Colonisation can be inter- or intracellular, localized or systemic. Some endophytes have been found to be non-aggressive, not causing disease, some to be latent pathogens, others to play mutualistic roles within their hosts.

Non-clavicipitaceous endophytes, horizontally transmitted from plant to plant by spores in all type of plants including woody gymnosperms and angiosperms. In woody plants, newly flushed leaves are generally endophyte-free but soon become inhabited with fungal spores which are dispersed via air, rain splashes and animal vectors from senescent and abscissed previous season's leaves. Germination spores invade the plant by cuticular penetration, entry through stomata or wounds. Leaves and twigs appear to host different endophyte assemblages (Barengo *et al*., 2000) suggesting that foliar endophytes are unable to lurk in and disperse from twigs to leaves. Thus, the lifespan of the foliage limits the lifespan and generation time of foliar fungi (Saikkonen, 2007). Saikkonen *et al*. (2004) suggested that the size, complex architecture, and long age of sexual maturity of the woody plant probably constrain the window for (a) systemic growth, (b) vertical transmission and (c) the length of the latency period of foliar fungi. These are the life history traits which determine the spatial and temperal distribution of endophytic fungi in plant population, as well as the nature of fungus-plant interaction along the antagonism-mutualism continuum (Saikkonen *et al*., 2004). Their reasoning was that endophytic fungi are unable to grow systemically through highly differentiated

and hierarchically organized woody tissues to all above-ground parts of the tree, and that long age of maturity decreases opportunities for vertical transmission of the fungi via host seeds (Saikkonen *et al*., 2004).

One of factor determinants for endophytic fungi seems to be the leaf age because infection frequencies accumulate over time, the age of leaves and needles is strongly positively correlated with the fungal colonization (Arnold and Herre, 2003; Bernstein and Carroll, 1977; Hata *et al*., 1998; Petrini and Carroll, 1981; Stone, 1987). The detected trends in endophyte distribution pattern correlate with the age structure of leaves in the crown, liquid precipitation such as rain, dew and fog, as well as growth form of the tree affecting the microclimate within the crown (Bahnweg *et al*., 2005). For example, rain splashes bring propagules from the fallen previous years leaves in soil and vegetation to the lower part of the tree and colonization success in higher in shade and humid closed canopy compared to sun leaves of the outermost branches (Saikkonen, 2007).

The global study about fungal endophytes abundances and diversity oppear to be (a) highly abundant and hyperdiverse in tropics (Arnold *et al*., 2000, 2001; Fröhlich *et al*., 2000), (b) higher during the wet seasons compared to dry season in semi-arid regions and tropics (Faeth and Hammon, 1997; Rodrigues, 1994; Chareprasert *et al*., 2006), (c) high in rainy slopes of mountains (Carroll and Carroll, 1978) and (d) to increase with increasing annual precipitation (Helander *et al*., 1998). Most mycologists agree that fungal diversity on a global scale is greatest in tropical forests, however, the true scale of tropical fungal biodiversity is not known. Asymptomatic leaves of tropical woody angiosperms consistently contain multiple species of fungal endophytes in moist forest ( Arnold *et al*., 2001).

The species composition of endophytes typically comprises of a few dominant species and numerous only sporadically detected species. The dominant fungal species are presumed to be specific to the host tree species or closely related species

because each host species usually harbours a characteristic assemblage of endophytic fungi. An endophyte in one plant could be a pathogen of the other depending on the balance between pathogenicity and endophytism of the microorganism in the different host (Saikkonen *et al*., 2004). The relationship of the endophyte to the host plant may have begun to evolve from the time that higher plants first appear on the earth, hundreds of millions year ago. Evidence of plant-associated microbes has been discovered in fossilized tissue of stems and leaves. It is possible to image that some of these endophytic microorganisms may have developed genetic systems allowing for the transfer of information between themselves and the higher plants (Strobel, 2003). Schulz *et al*. (1999) showed that infections without visible symptoms, colonization led to the synthesis of higher concentrations of potentially antimicrobial compounds. In vitro, endophytic fungi produce more herbicidally active substances than soil fungi. Schulz *et al*. (1999) therefore hypothesise that the host-endophyte interaction is a case of balanced antagonisms: pathogen overcome the host's defences to the extent that they cause visible damage, whereas endophytic virulence is only sufficient to be able to infect and colonise without causing visible damage. If the balance shifts, the endophyte may turn pathogenic.

## 2.3 Study of bioactive secondary metabolites of endophytic fungi in non-clavicipitaceous endophytes (NC-endophytes)

Endophytes are ubiquitous with rich biodiversity, which have been found in every plant species examined to date (Strobel and Daisy, 2003). It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes. It is estimated that there may be as many as 1 million different endophyte species, however only a handful of them have been described, which means the opportunity to find new and targeting natural products from interesting endophytic fungi among myriads of plants in different niches and ecosystems is great.

The search for new products for pharmaceutical and agrochemical industries is an on-going process that requires continual optimization (Dreyfuss and Chapela, 1994). In optimizing the search for new bioactive secondary metabolites, it is relevant to consider that (1) the secondary metabolites a fungus synthesizes may correspond with its respective ecological niche (Gloer, 1997) and (2) that metabolic interactions may enchance the synthesis of secondary metabolites. Thus, the fungi screened should originate from biotopes from biochemical purposes and they should have metabolic interactions with their environment (Schulz *et al.,* 2002). Endophytic fungi are one source for intelligent screening and fulfill both criteria. They grow within their plant hosts without causing apparent disease symptoms (Petrini, 1991) and growth in this habitat involves continual metabolic interactions between fungus and host. Screening is not a random walk through a random forest. The biological activities and the metabolites produced are associated with the respective biotope and/or host.

Strobel and Daisy (2003) described a specific rationale for the plant selection strategy for endophytic isolation as follows (a) plants from unique environmental setting, especially those with an unusual biology and possessing novel strategies for survival are seriously considered for study. (b) Plants that have an ethnobotanical history (use by indigenous people) that are related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. (c) Plants that are endemic, that have an unusual longevity, or that have occupied a certain ancient land mass, such as Gonwanaland, are also more likely to lodge endophytes with active natural products than other plants. (d) Plants growing in area of great biodiversity also have the prospect of housing endophytes with great biodiversity.

Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. Bills *et al*. (2002) described a metabolic between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those

isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other tropical substrata.

One of the most surprising finding of endophyte studies is the isolation of the taxol-producing endophyte *Taxomyces andreanae* (Stierle *et al*., 1993) Taxol, a highly functionalized diterpenoid, is found in each of the world 's yew (*Taxus*) species. This compound is the world's first billion dollar anticancer drug and it is used to treat a number of other human tissue-proliferating diseases as well. Its cost makes it unavailable to many people worldwide. Therefore, alternative sources are needed. The finding of *T. andreanae* provided another alternative approach for taxol production by fermentation. Since then, several research groups successively have reported their findings on taxol-producing endophytes. Huang *et al*. (2001) found antitumor and antifungal activities in endophytic fungi isolated from three kinds of pharmaceutical plants, *Taxus mairei* which one of major source of taxol, *Cephalataxus fortune* and *Torreya grandis* plants, collected from China.

Tan and Zou (2001) reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological role. These secondary metabolites of endophytes are synthesized *via* various metabolic pathways, e.g. polyketide, isoprenoid, or amino acid derivation, and belong to diverse structural groups, i.e. steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasines.

Liu *et al*. (2001) found endophytic culture extracts from *Artemisia annua*, well recognized for its production of antimalarial drug artemisinin, produce bioactive compounds against phytopathogenic fungi. Endophytes usually produce the enzyme necessary for the colonization of plant tissue. Most of the investigated endophytes utilize xylan and pectin, show lipolytic activity and produce non-specific peroxidases and

laccases (Leuchtmann *et al*., 1992), glucanse (Moy *et al*., 2002) and chitinase (Li *et al*., 2004). Production of extracellular cellulose and hemicellulases other than xylanases are widespread but usually limited to organisms derived from selected hosts or even host tissue (Leuchtmann *et al*.,1992). Substances isolated by Schulz *et al*. (2002) from endophytic fungi originate from different biosynthetic pathways and belong to diverse structural groups: terpenoids, phenols, xanthones, steroids, isocoumarins, cytochalasines, tetralones, benzopyranes and enniatines.

In 2004, Weber *et al*. found another novel antibiotic phomol isolated from fermentations of an endophytic fungus, a *Phomopsis* species, from the medicinal plant *Erythrina crista*. Kim *et al*. (2004) found two new fusicoccane diterpencs named periconicins A and B, with antibacterial activities isolated by bioassay guided fractionation from an endophytic fungus *Periconia* sp., collected from small branches of *Taxus cuspidate*. *Muscodor albus*, xylariaceaous endophytic fungus originally isolated from *Cinnamomum zeylanicum*, produces a mixture of volatile organic compounds (VOCs) in culture and its spectrum of antimicrobial various test organisms including plant-pathogenic fungi, including yeasts and bacteria (Strobel *et al*., 2001). Potential applications for *M. albus* and its VOCs are currently being investigated. These include uses for treating various seeds, fruits and cut flowers, to reduce or eliminate harmful or disease-causing micro-organisms (Mercier and Jimenez, 2004).

Stinson *et al*. (2003) found an endophytic isolate of *Gliocladium* sp. was obtained from the Patagonian Eucryphiacean tree-*Eucryphia cordifolia*, produced a mixture of volatile organic compounds (VOCs) lethal to plant pathogenic fungi such as *Pythium ultimum* and *Verticillum dahlia.* Some of volatile bioactive compounds exuded by this fungus are also the same as those produced by *M. albus*. The endophytic fungus *Pestalotiopsis leucothes* isolated from *Trypterygium wilfordii* was found to produce three compounds which have variable effectd on T- and B-cells and monocytes, and represented a new source of immunomodulatory compounds or treatment of human immune mediated disease (Kumar *et al*., 2005).

*Edenia gomezpompae*, a newly discovered endophytic fungus isolated from the leaves of *Callicarpa acuminate* collected from the ecological reserve E1 Eden, Mexico produced four naphthoquinone spiroketal, including three new compounds and palmarumycin, with antifungal activities ( Macias-Rubalcava *et al*., 2008). The current study found two new solanapyrone analogues, solanapyrones N and O, which were isolated from the fermentation culture of *Nigrospora* sp. YB-141, an endophytic fungus isolated from *Azadirachta indica* growing in the tropical region of southwest China as well as the antifungal activities towards seven phytopathogenic fungi (Wu *et al*., 2009).

You *et al*. (2009) found antifungal secondary metabolites from endophytic *Verticillium* sp., were isolated from roots of wild *Rehmannia glutinosa* which is an important herb in China. The strong antifungal activity against the phytopathogenic fungi suggests that the endophytic fungus *Verticillium* sp. could protect the host by producing secondary metabolites; some metabolites are potentially natural product-derived agrochemicals.

## 2.4 Mangrove forest and their importances

The term “mangrove” refers to a tidally influenced wetland ecosystem within the intertidal zone of tropical and subtropical latitudes. Mangrove also designates the marine tidal forest that includes trees, shrubs, palms, epiphytes and ferns (Tomlinson, 1986). Mangroves are distributed circumtropically, occurring in 112 countries and territories. Among the marine ecosystem, mangroves constitute the second most important ecosystem in productivity and sustained tertiary yield after coral reefs (Sridhar, 2004). There are 9 orders, 20 families, 27 genera and roughly 70 species of mangroves occupying a total estimated area of 181 000 $km^2$ (Spalding *et al.,* 1997). The most diverse biogeographical regions are in the Indo-West Pacific. Indonesia, Australia, Brazil and Nigeria have roughly 43% of the world’s mangrove forests (Alongi, 2002). In Thailand, the estimated mangrove area in 2000 was 2,062 km2 (RFD 2004) or approximately 1.2% of the world mangroves with the dominant species belonging to the

genera *Rhizophora*, *Ceriops* and *Bruguiera* in the family Rhizophoraceae, *Sonneratia* in the family Sonneratiaceae and *Avicennia* in the family Avicenniaceae (Aksornkoae, 1993). There are approximately 27 species in Thailand (Miles *et al*., 1999).

Mangroves are a valuable ecological and economic resource, being important nursery grounds and breeding sites for birds, fish, crustaceans, shellfish, reptiles and mammals; a renewable source of wood; accumulation sites for sediment, contaminants, carbon and nutrients; and offer protection against coastal erosion (Alongi, 2002). It is now important that the possible utilization for other purposes including as a source for chemical constituents with potential medicinal and agricultural value (Table 2.2). The mangrove leaves have been extracted a variety of compounds including taraxerol careaborin and taraxeryl cis-p-hydroxycinnamate from leaves of *Rhizophora apiculata* (Kokpol *et al*., 1990). The some Substances in mangroves have long been used in folk medicine to treat disease (Bandaranayake, 1998).

Table 2.2 indicates the utilization of several of mangrove plants (Miles *et al*., 1999)

| Mangrove species | Utility |
|---|---|
| *Acanthus illicifolius* | Leaf juice used as hair preserver, fruit pulp as blood purifier, dressing for boils and snake bite, leaf preparation used for rheumatism. |
| *Rhizophora* sp. | *R. mucronata* bark used to treat diarrhea, dysentery, and leprosy; fruit sap used as a mosquito repellent. |
| *Xylocarpus* sp. | bark extract is used to treat cholera. |
| *Excoecaria agallocha* | fish and arrowhead poison in Thailand. It is known to cause blindness and skin eruptions in the Philippines. It is used as medication for toothache in Malaysia. |
| *Sonneratia ovata* | Fruit is eatable and used to treat sprains, fermented juice used as anticoagulant. |

Fungi collected on mangrove plants can be divided into two groups: those inhabiting parts of the hosts which are submerged for some time of the day and those that grow above the high tide line. The first group includes the marine species, while the second group embraces the terrestrial species. Both groups can overlap at the high water mark (Kohlmeyer, 1969). Mangrove are home to a group of fungi called “manglicolous fungi.” These organisms are vitally important to nutrient cycling in these habitats (Hyde and Lee, 1995; Kohlmeyer *et al*., 1995). Kohlmeyer and Kohlmeyer (1979) were the first to review this group. They recognized 43 species of higher fungi, including 23 Ascomycetes, 17 Deuteromycetes, and 3 Basidiomycetes. Hyde (1990) listed 120 species from 29 mangrove forests around the world. These included 87 Ascomycetes, 31 Deuteromycetes, and 2 Basidiomycetes.

Researchers found a variety of secondary metabolites compounds from mangrove fungus including spiromassaritone and massariphenone (Wahab *et al*., 2007), enalin A and B with hydroxymethyl furfural and three cyclodipeptides were extracted from the mangrove fungus *Verruculina enaria* (*Lin et al*., 2002), diaporthelactone which showed cytotoxic activity against KB (oral human epidermoid carcinoma) and Raji human tumor cell lines (Lin *et al*., 2005), Aspergiolide A, an anthraquinone derivative with naphtho[1,2,3-de]chromene-2,7-dione skeleton, has been isolated from cultures of a marine-derived fungus *Aspergillus glaucus*, showed cytotoxicities against A-549, HL-60, BEL-7402, and P388 cell lines (Du *et al*., 2007). Mayer and Hamann (2005) reviewed research on the pharmacology of marine chemicals continued to be global in nature involving investigators from Argentina, Australia, Brazil, Canada, China, Denmark, France, Germany, India, Indonesia, Israel, Italy, Japan, Mexico, Netherlands, New Zealand, Pakistan, the Philippines, Russia, Singapore, Slovenia, South Africa, South Korea, Spain, Sweden, Switzerland, Thailand, United Kingdom, and the United States, classified 106 marine chemicals derived from a diverse group of marine animals, algae, fungi and bacteria.

## 2.5 Study of endophytic fungi from mangrove plants and theirs secondary metabolites

The first report on fungal endophyte in leaves of mangrove plant was from India in 1998, Suryanarayanan *et al*. isolated endophytic fungi from leaves of *Rhizophora apiculata* Bl. and *Rhizophora mucronata* Lamk., more endophytes could be isolated during the rainy months than during the dry period. Hyphomycetes and sterile forms were more prevalent than ascomycetes or coelomycetes. *Sporormiella minima, Acremonium* and sterile fungi were isolated from both plants irrespective of the season. In 2000, Suryanarayanan and Kumaresan studied four halophytes belonging to three dicotyledon families, *Acanthaceae*, *Aizoaceae* and *Chenopodiaceae* occur in Pichavaram mangrove forest, India. All the halophyte species (*Acanthus ilicifolius*,

*Arthrocnemum indicum*, *Suaeda maritima* and *Sesuvium portulacastrum*) studied harboured fungal endophytes.

Kumaresan and Suryanarayanan (2001) studied the fungal endophytes in seven dominant mangrove species of Pichavaram mangrove forest in south India. Mitosporic fungi, ascomycetes and sterile mycelia were isolated from the leaves of the mangrove host studied and the endophyte assemblage of each mangrove species was dominanted by different endophyte species. In 2002, Kumaresan and Suryanarayanan studied the endophyte assemblages in young, mature and senescent leaves of *R. apiculata* and they found the number of species as well as the number of isolates of endophytes that could be recovered from the leaves increased with leaf age. More than 200 species of endophytic fungi were isolated and identified from mangrove plant in China, which are mainly *Alternaria*, *Aspergillus*, *Cladosporium*, *Clolletotrichum*, *Fusarium*, *Paecilamyces*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phyllosticta* and *Trichodema* (Liu *et al*., 2007). Most endophytic fungi have a wide rang of hosts, and a few only have single host. The composition and dominant species on each mangrove plant as well as with different parts and age of host or with seasons, are different; e.g., among 290 strains of endophytic fungi obtained from Fugong in Fujian Province, China, *Penicillium*, *Alternaria*, *Dothiorell* and nonsporulating groups were the dominant genera from *Kandelia cande*, while the endophytic fungi from *Bruguiera gymnorrhiza* mainly belonged to *Cephalosporium*, *Altermaria,* and *Penicillium* (Yang *et al*., 2006). Twenty-five endophytic fungi comprised three ascomycetes, 20 mitosporic fungi and two sterile fungi were recovered from two halophytes (*Acanthus ilicifolius* and *Acrostichum aureum*) of a west coast mangrove habitat. Species richness and diversity were high in stems of *Acanthus ilicifolius* and roots of *Acrostichum aureum*. The most dominant endophyte was *Colletotrichum* sp. in prop roots of *Acanthus ilicifolius*, and yeast sp. 1 in rhizomes of *Acrostichum aureum*. *Acanthus ilicifolius* showed dominance of a single species (*Colletotrichum* sp.), while in *Acrostichum aureum,* multiple species dominance was seen (*Acremonium* sp., *Penicillium* sp. and Yeast sp. 1).

Mangrove plants were proven to be a rich source of endophytic fungi. Many secondary metabolites with novel structures and biological activities have been characterized from mangrove-derived endophytic fungi. Considering the number of novel bioactive compounds that have been isolated from mangrove endophytic fungi and the fact that the number of the compounds is increasing rapidly. In 2003, Chen *et al*. found the extract of the endophytic fungus exhibited cytotoxicity toward NCI4460 and Bel-7402, and high activities against *Heliothis armigera* and *Sinergasilus* spp. This endophytic fungus strain was collected from the dropper of *Kandelia candel* from an estuarine mangrove on the South China Sea coast.

In 2007, Maria *et al*. reported mangrove endophytic fungi of southwest coast of India were assessed for the production of extracellular enzymes amylase, cellulase, chitinase, laccase, lipase, protease and tyrosinase by culture plate method. Cellulase and lipase activity was present in all fungi. Moreover more than 50% of the fungi tested showed antimicrobial activity. Xu *et al*. (2007) found seven new phenolic and quinone derivatives with anticancer activity from the fungus Penicillium sp. was isolated from the stems of mangrove plants, *Aegiceras corniculatum* (Aegiceratacеae) .

In 2008, Li *et al*. (2008) found a varity of compounds from liquid fermentation cultures such as new benzaldehyde derivatives were characterized from the liquid fermentation cultures of *Eurotium rubrum*, an endophytic fungus that was isolated from the inner tissue of stems of the mangrove plant *Hibiscus tiliaceus* and in the same year Li *et al*. (2008)also found two new pyrrole alkaloids, and a new indole derivative from an endophytic fungus, *Fusarium incarnatum* (HKI00504), isolated from the leaves of mangrove plant *Aegiceras corniculatum* in China. Chen *et al*. (2008) reported two new amide alkaloids from the mangrove endophytic fungus collected from the south China sea. Amide alkaloids are a class of natural products reported to possess a wide range of pharmacological properties such as anticancer, antimicrobial, and antiviral. Most amide alkaloids have been isolated from the leaves, stem bark, root bark, and seeds of a wide variety of plant species throughout the world. Plant endophytic fungi have also

proved to be sources of this class of secondary metabolites. A strain of *Aspergillus* sp. (w-6) isolated from the inner bark of *A. ilicifolius* collected in Dongzhai Gang, China produced Terpeptin A and B, two new members of the indolic enamides. Terpeptin A and B exhibited modest cytotoxicity against A-549 cell line (*Lin et al*., 2008).

Huang *et al*. (2008) found three metabolites named phomopsin A , B and C, together with two known compounds cytosporone B and C, were isolated from the mangrove endophytic fungus, *Phomopsis* sp. ZSU-H76 obtained from the South China Sea. Some compound from this fungus inhibited two fungi *Candida albicans* and *Fusarium oxysporum* (Huang *et al*., 2008). Wang *et al*. (2008) reported two new metabolites, together with four known compounds mannitol, ergosterol, cerevisterol, and 3b-hydroxy-5a,8a-epidioxyergosta-6,22-diene , produced by the endophytic fungus no. 2106 isolated from the seeds of the mangrove *Avicennia marina* in Hong Kong. Tan *et al*. (2008) found a new hTopo I isomerase inhibitor, was isolated from the mangrove endophytic fungus no. 2240 collected from an estuarine mangrove at the south China sea coast. Three metabolites, sporothrins A, B, and C, from the mangrove endophytic fungus, *Sporothrix* sp. was isolated from the bark of the inshore mangrove tree *Kandelia candel* (Wen *et al*., 2009).

## 2.6 Molecular study of endophytic fungi

Fungal taxonomy is traditionally based on comparative morphological features. The study of endophytic fungi isolates can only be identified if they sporulate on the culture media. Despite the development of various methods to promote endophyte sporulation, e.g. by growing them on modifications of artificial media and under various incubation conditions, numerous isolates do not sporulate. Various optimization of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures (Guo *et al*., 2000). Molecular techniques exhibit high

sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al*., 2006). The development of fungal-specific primer for amplification of the internal transcribed spacer (ITS) region of the ribosomal RNA gene (Gardes and Bruns, 1993) opened the way for direct amplification of fungal DNA from complex substrates containing multiple sources of DNA, such as soil or plant tissue. Early studies demonstrated the utility of the molecular approach to fungal community ecology (Gardes and Bruns, 1996). The primer sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA have been widely used for resolving phylogenic relationships at the species or generic levels of endophytic fungi (Baayen *et al*., 2002; Higgins *et al*., 2007; Huang *et al*., 2009; Goa *et al*., 2003; Morakotkarn *et al*., 2007; Okane *et al*., 2003; Pandey *et al*., 2003; Promputtha *et al*., 2007).

Endophytic fungi comprise a large and diverse group of fungi, so no identification methods will apply to endophytic fungi in general. Direct amplification of DNA for detection and quantification of endophytes from infected hosts may be more general interest. Primer that differ in sequence composition, length, restriction sites, presence of intron sequences and similar characters can be exploited for selective PCR amplification of fungal DNA directly from infected plant host tissue. ITS sequences were determined, aligned, subjected to phylogenetic analysis with PAUP (Phylogenetic Analysis Using Parsimony) and compared with ITS sequences for fungal species in the GenBank database. Conserved sequence motifs were consistent enough to enable disposition of the sequences at least at the family level and in some cases at the genus level. Most of the endophytic fungi were detected and identified by comparative analyses of the ribosomal DNA sequences, especially the ITS region. For example, Huang *et al*. (2009) investigated the taxonomic identities and phylogenetic relationships of fungal endophytes isolated from three plant species, *Artemisia capillaris*, *A. indica*, and *A. lactiflora*, using a combination of morphological and molecular approaches and found that morphological differences among the fungal isolates indicate that diverse distinct morphotypes might be present within the hosts. Thomas *et al*. (2008) used the

molecular technique to identify 46 basidiomycete endophytes isolated from healthy stems and pods of *Theobroma* in Ecuador. A molecular approach using internal transcribed spacer restriction fragment length polymorphism (ITS-RFLP) patterns was used to study the different isolates of a foliar endophytic species of *Phyllosticta* were isolated from different tropical tree species in India to examine genetic variation among the isolates (Pandey *et al*., 2003). Culture-independent DNA methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and ITS sequencing, have been developed for the investigation of complex microbial communities (Bougoure *et al*., 2005, 2007). Most studies on endophyte have been conducted by cultivation. However, as known from environmental microbiology in general, it can be expected that the actual diversity of endophyte colonizing plants will be significantly higher than that suggested by cultivation based approaches. The use of molecular techniques for the direct detection and identification of fungi within natural habitats has been reviewed by Liew *et al.* (1998). There have been only a small number of studies that have used molecular techniques to investigate endophytic fungal communities. Until 2001, Guo *et al.* studied 5.8S gene and flanking internal transcribed spacers (ITS1 and ITS2) of the rDNA were amplified from total DNA extracted from frond tissues of *Livistona chinensis* and found some endophytic fungi were obtained with molecular techniques and were not isolated with traditional methods. This result suggests that some endophytic fungi were not isolated from plant tissues presumably due to the limitations of traditional cultural techniques. Gao *et al.* (2005) studied endophytic fungi associated with *H. japonica* using culture-independent method that relies on the rRNA genes (rDNA) obtained from the plant sample and chose to study the diversity of the internal transcribed spacer (ITS) regions of fungal rDNA, which have been identified as discriminative targets for molecular analysis of fungal communities by using the methods of molecular biology such as RFLP analysis and sequencing of rDNA ITS library and the molecular phylogenetic analysis showed that a broad spectrum of fungi. Molecular techniques appear to offer a complementary method for the detection of endophytic fungi, particularly those that cannot be incubated on artificial media and can be directly amplified from plant tissues (Guo *et al*., 2001).

# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 Reagents

- Ammonium hydrogen phosphate $(NH_4)_2HPO_4$ was used Merck KGaA, Germany.
- Potassium dihydrogen phosphate ($KH_2PO_4$) was used Merck KGaA, Germany.
- Trizma base, minimum 99.9% titration was used Sigma-Aldrich Co., Inc., Singapore.
- Ethyl acetate
- Ethylenediaminetetraacetic Acid (EDTA) was used Sigma-Aldrich Co., Inc., Singapore.
- Ethanol absolute, Analytical grade, ACS. was used Scharlau Chemie S.A., Spain.
- Sodium hydroxide (NaOH) was used Merck KGaA, Germany.
- 100 bp Ladder Sharp DNA Marker was used Fermentas International Inc., Canada.
- pT7Blue T-Vector (NV004; Novagen)
- TaKaRa DNA Ligation Kit Ver. 2 (#6022; Takara Shuzo)
- *E.coli* JM109 competent cells (#9052; Takara Shuzo)
- 25 mM $MgCl_2$
- 2.5 mM dNTP mixture
- 10x $NH_4$ buffer



## 3.2 Instruments

- Incubator (Model 800, Memmert GmbH and Co. KG., Western Germany)
- Incubator shaker (Model SK-737, Amerex Instruments, Inc., USA)
- Autoclave (Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan)
- Hot air oven (Model UC 30, Memmert GmbH and Co. KG., Western Germany)
- Spectrophotometer (Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, USA)

- Laminar flow 'clean' (Model V6, Lab Service Ltd., Part)
- Cold room (Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K)
- 4-Digit precision weighting balance (Model AG 204, Mettler Toledo, Switzerland)
- Microwave (Model 000502174, Thai Cityelectric Co. Ltd.,Thailand and National model NE-E72 High power 700 w, Tokyo, Japan)
- Hot plate stirrer (Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- Vortex mixer (Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, USA)
- Nano Spin (Model NS-060, Nippon Genetic Co., Ltd., Japan)
- Water bath (Model WB14, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- pH meter (Mettler-Toledo International Inc., New York, U.S.A.)
- Gel Documentation system (Bio-Rad Laboratories Gel Doc TM XR, California, U.S.A.)
- Electrophoresis chamber set (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland)
- High Speed Refrigerated Centrifuge (Beckman Coulter TM Avanti J-30I, Palo Alto, California, U.S.A.)
- Microscope (Model CH 30RF200, Olympus Optical Co., Ltd., Japan)
- Kubota Refrigerated Microcentrifuge 6500 (Kubota Corporation, Tokyo, Japan)
- UV-VIS spectrometer model V-530 (PC) (PerkinElmer instruments Lambda 25, Massachusetts, U.S.A.)
- Takara Thermal Cycler Dice Standard TP-65 (TaKaRa Bio Inc., Otsu, Shiga, Japan)
- Microhomogenizer Tomy Micro Smash™ (Model MS-100 Serial no. MS031134 Active DNA™, Tomy Digital Biology, Tokyo, Japan)
- Dry Thermo Unit (Model DTC-1B, Bio-Version, Taitec copporation, Japan)

- Digital Image Stocker (Model DS-100 toyobo, URL)
- Himac Compact centrifuge (Model RXII series , Hitachi CF 16RXII, Japan)

## 3.3 Studied site characteristics

Healthy mangrove leaf samples were collected from different regions of Thailand whithin the same period in May of the year 2006 (Table 3.1, Figure 3.1). There were 3 sampling sites:

3.3.1 Khung Kraben bay is in Chanthaburi Province.The bay is fringed in the inner parts by mangrove forest which is about 500 - 800 width from landward to seaward edge and the mangrove area is around 160 ha (Figure 3.2).

3.3.2 Pranburi Forest Park located in Klongkao-Klongkob National Reserve Forest, Pranburi Distrtict, Prachuap Khiri Khan Province located in the lower central region connecting with the south of Thailand and consists of mixed forest and mangrove forest (Figure 3.3). The weather of the forest is influenced by monsoon and land breeze. It consists of three seasons such as rainy Season beginning in the June to middle of November,Winter begining the middle of November to February and Summer which starts in March to May.

3.3.3 Ranong Biosphere Reserve located on the Andaman coastline of southern Thailand, in Ranong Province, 650 km southwest of Bangkok. In the area of Ranong Biosphere Reserve, the estuarine mangrove is relatively undisturbed and represents one of the best-developed mangrove forests and is classified as old growth stands (Figure 3.4). The mangrove forest in Ranong Biosphere Reserve covers about 67,506 rai or 27,002 acres where *Rhizophora apiculata* is the dominant plant species. (Ranong Biosphere Reserve, 2000). Ranong is renowned as the wettest province in Thailand, which is the result of the influencing monsoon from the Andaman Sea that brings an abundant rainfall. The rainy season is from May to December whereas the cold season is in January and the summer season from February to April (Ranong Province, 2002).

Table 3.1 – Characteristics of sampling sites

| Region | Studied site (Province) | Sampling time | Geographical location (lat. N, long. E) |
|---|---|---|---|
| Eastern Thailand | Chanthaburi | 4 May 2006 | 12°34.317'N, 101°53.964'E |
| Central Thailand | Prachuap Khiri Khan | 13 May 2006 | 12°24.816'N, 099°59.114'E |
| Southern Thailand | Ranong | 10 May 2006 | 09°52.542'N, 098°36.249'E |

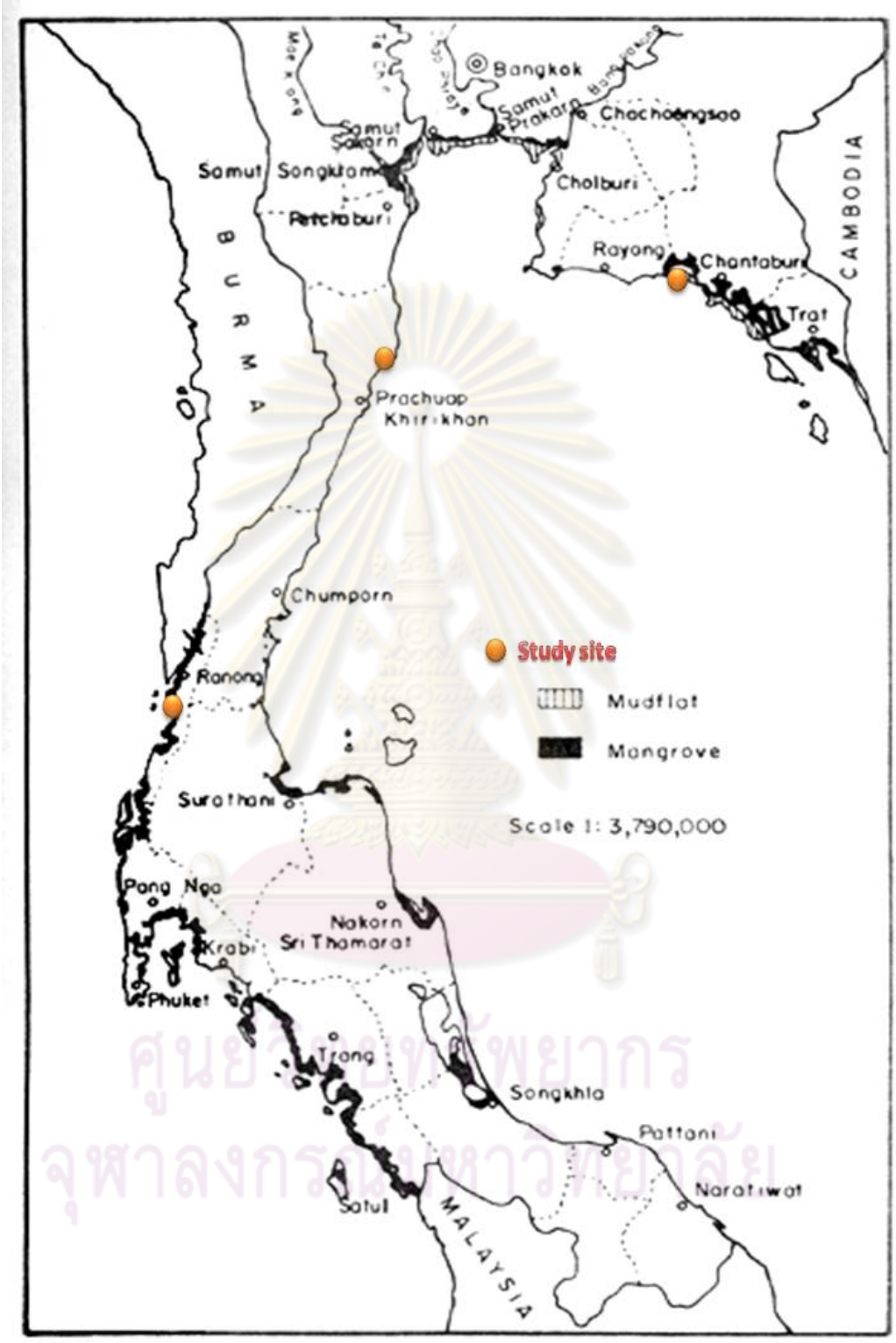


Figure 3.1 Mangrove map of Thailand

Figure 3.2 Location of  Khung Kraben bay in Chanthaburi Province.

Figure 3.3 Location of Pranburi Forest Park in Prachuap Khiri Khan Province

Figure 3.4 Location of Ranong Biosphere Reserve in Ranong Province

## 3.4 Studied mangrove plant details

The dominant mangrove plant species were chosen from each area as shown in Table 3.2.

Table 3.2 The selected mangrove plants for fungal endophyte isolation

| No. | Code | Plant Family | Plant species | Study site (Province) |
|---|---|---|---|---|
| 1 | AAC | Avicenniaceae | *Avicennia alba* Bl. | Chanthaburi |
| 2 | LLC | Combretaceae | *Lumnitzera littorea* Voigt | Chanthaburi |
| 3 | RAC | Rhizophoraceae | *Rhizophora apiculata* Bl. | Chanthaburi |
| 4 | RMC | Rhizophoraceae | *Rhizophora mucronata* Poir. | Chanthaburi |
| 5 | SAC | Sonneratiaceae | *Sonneratia alba* J. Smith | Chanthaburi |
| 6 | CDP | Rhizophoraceae | *Ceriops decandra* Ding Hou | PrachuapKhiri Khan |
| 7 | RAP | Rhizophoraceae | *Rhizophora apiculata* Bl. | PrachuapKhiri Khan |
| 8 | TPP | Malvaceae | *Thespesia populneoides* (Roxb.)Kostel. | PrachuapKhiri Khan |
| 9 | AIR | Acanthaceae | *Acanthus ilicifotius* Linn. | Ranong |
| 10 | RAR | Rhizophoraceae | *Rhizophora apiculata* Bl. | Ranong |
| 11 | RMR | Rhizophoraceae | *Rhizophora mucronata* Poir. | Ranong |
| 12 | XGR | Meliaceae | *Xylocarpus granatum* Koen. | Ranong |
| 13 | XMR | Meliaceae | *Xylocarpus moluccensis* Roem. | Ranong |

### 3.4.1 Family Acanthaceae

*a. Acanthus ilicifotius* Linn. (Figure 3.5)

**Common name**: Ngueak plea monamogoen

**Description**: It is a viny shrub or tall herb, upto 1.5 m high, scarcely woody, bushy, with very dense growth. Shallow tap roots, but occasionally stilt roots are conspicuous. Leaf simple, opposite, decussate, cauline, exstipulate, petiole short, flattened, glabrous, pulvinous to sheathing base. Flower bisexual, typically zygomorphic, complete, erect, sessile, hypogynous. Fruit 1 cm green and 2.5 - 2.0 cm long, kidney shaped 4 seed drupe, Seed 0.5 - 1.0 cm long. These mangrove plants have leaves which look like the

spiny holly leaves. In fact, not all the leaves have the spiny edges that give them their common name. Leaves growing in deep shade can be totally spineless. Unlike some mangrove plants, Sea Holly do not exclude salt at the root level. In fact, their sap is salty and excess salt is secreted through the leaves, to be removed by rain or wind. Sometimes, the salt can be seen as a white crystalline layer on the upper surface. The plant produces a cluster of flowers which appear in neatly organized spikes at branch tips. Flowers have a single large petal large, showy and light violet.

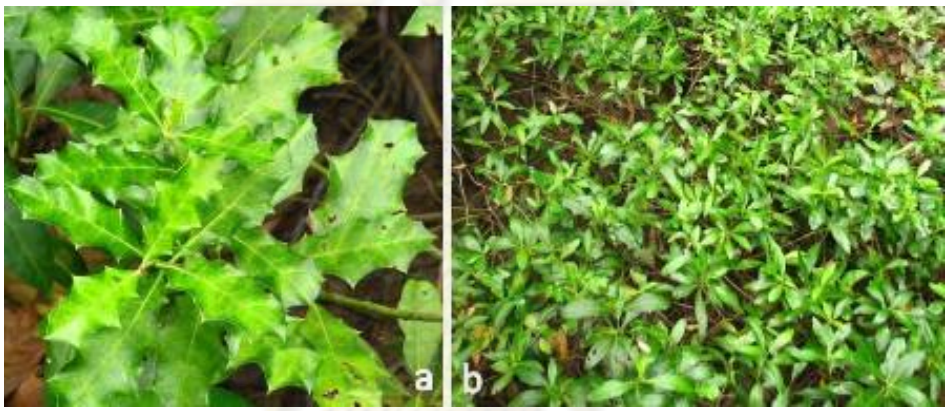


Figure 3.5 *Acanthus ilicifotius* (a) leaves (b) trees

### 3.4.2 Family Avicenniaceae

*a. Avicennia alba* Bl. (Figure 3.6)

**Common name**: Samae khaeo

**Description**: The shrub does not grow more than 20 m. The dark green leaves, 15 cm long by 5 cm wide, have a silvery gray under leaf and grow in opposites. Orange yellow flowers, borne in a racemose inflorescence, have a diameter of 3 to 4 mm when expanded. The bark is smooth, greenish black, finely fissured and does not flake. The fruits are grayish green and conical in shape extended into a beak up to 4 cm long. *Avicennia alba* grows in dry riverbanks to muddy portions of the seashore. Roots: pencil-like pneumatophores emerge above ground from long shallow underground roots.

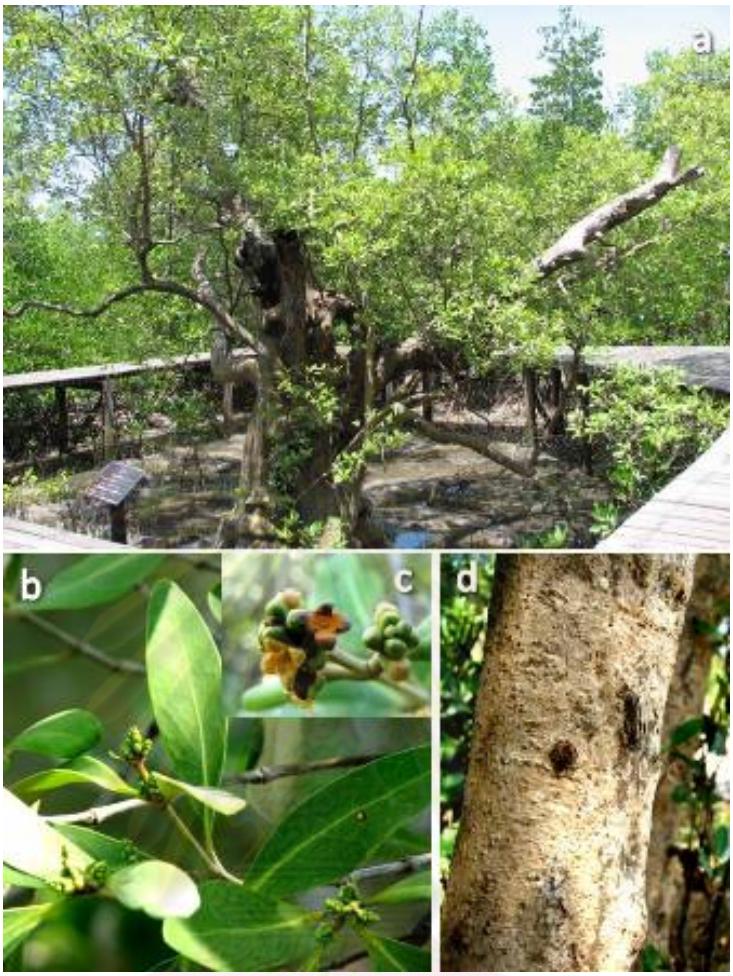


Figure 3.6 *Avicennia alba* (a) tree (b) leaves (c) flowers (d) stem



### 3.4.3 Family Combretaceae

a. *Lumnitzera littorea* Voigt (Figure 3.7)

**Common name** : Churanda

**Description**: Trees, 8-15 m tall with knee-bent aerial roots from the base of the stem. Leaves 1.5-4.5 x 0.8-1.5 cm, ovate or ovate-elliptic, coriaceous, emarginate at apex, cuneate at base. Flowers 10-12 mm long, red, shortly pedicelled, in terminal recemes. Fruits 9-12 mm long, ellipsoid-oblong, longitudinally ribbed. Flowering and fruiting during May-July. This species is easily distinguished from *Lumnitzera racemosa* Willd. by its red-flowering recemes.


Figure 3.7 *Lumnitzera littorea* (a) leaves (b) flowers (c) tree (d) old flowers (e) stem

### 3.4.4 Family Malvaceae

a. *Thespesia populneoides* (Roxb.)Kostel. (Figure 3.8)

**Common name**: pho-thale

**Description**: Small tree, young growth and leaves persistently and closely brownish-lepidote, giving a bronzed or coppery appearance; leaves generally deltoid to subcordate, or cordate with a very shallow broad sinus, tending to be caudate acuminate, usually with prominent domatia in axils of main nerves; stipules subdate to lanceolate, very early caducous; peduncles up to 10-12 cm long, ebracteate, not jointed, usually curved downward so flowers are drooping, involucral bracts 3, reduced, very early caducous, triangularovate, almost verticillate, represented in all but very

young buds by linear scars; calyx 8-10 mm long, truncate or with minute mucrolike teeth; corolla campanulate *5-6* cm long, yellow, center dull reddish to dark maroon; staminal column included, style exserted from staminal column but included in corolla; young fruit and buds exuding yellow gum when cut; mature fruit with two distinct layers, a smooth exocarp separated from a hard tough fluted endocarp by a loose fibrous-spongy mesocarp which partially disintegrates at maturity, the exocarp then dehiscing into 4-6, usually 5, valves, the lines of dehiscence following the median ridges of the cells of the endocarp; seeds several in a cell, broadly obovoid, covered by a dense short pubescence of erect bulbous hairs.


Figure 3.8 *Thespesia populneoides* (a) tree (b) leaves (c) fruit

### 3.4.5 Family Meliaceae

a. *Xylocarpus granatum* Koen. (Figure 3.9)

**Common name**: Taboon-Kao

**Description**: Medium-sized crooked much-branched evergreen tree up to 10 m. tall (taller elsewhere); bark smooth and yellowish, or brown and green and flaking; surface roots laterally compressed and forming a spreading network of ribbon-like pneumatophores with the upper edges protruding above the mud and suggesting a mass of snakes. Leaves paripinnate, drying orange-brown; petiole and rhachis up to 8·5 cm. long, glabrous; leaflets up to 12 × 5 cm., usually much smaller, opposite, 1–2 (3)-jugate, elliptic, oblong-elliptic or obovate-elliptic, apex usually rounded, rarely obtuse or aduceused, base narrowly or broadly cuneate, glabrous, coriaceous, venation prominent on both sides; petiolules 2–5 mm. long. Flowers whitish or pale pink, in lax racemes of (2) 3-flowered cymes; peduncle plus rhachis 4–7 cm. long; bracts minute, usually aduceus. Calyx about 3 mm. long, glabrous, lobed to the middle, lobes rounded. Petals 5–6·5 × 2·5 mm., glabrous. Staminal tube 4–5 mm. long, glabrous. Ovary less than 1 mm. in diam.; style 1·5 mm. long; disk fused to the lower half of the ovary. Fruit large, up to 20 cm. in diam., obscurely 4-sulcate. Seeds 4–8 cm. long.

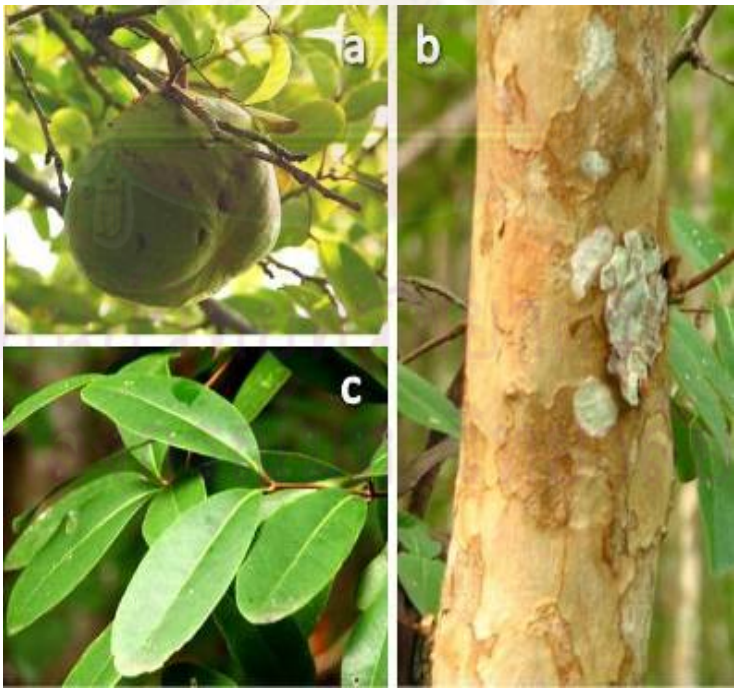


Figure 3.9 *Xylocarpus granatum* (a) fruit (b) stem (c) leaves

b. *Xylocarpus moluccensis* Roem. (Figure 3.10)

**Common name**: Taboon-dum

**Description**: Medium-sized much-branched spreading semi-evergreen tree up to 13 m. tall (much taller elsewhere), sometimes flowering as a shrub; bark rough like an Ulmus; ribbon-like buttresses absent. Leaves paripinnate, drying yellow-green, petiole and rhachis up to 16 cm. long, glabrous; leaflets up to 12 × 6 cm., opposite, 2–3 (4)-jugate, ovate or ovate-lanceolate, tapering from near the base to a subacuminate apex, base variable, asymmetric, glabrous, subcoriaceous, venation closer and less prominent than in *X. granatum* Poir . Flowers white or greenish-white in lax racemes or panicles of 3–7-flowered cymes; peduncle plus rhachis 5–14 cm. long; bracts minute, usually caducous. Calyx c. 2 mm. long, glabrous, lobed to beyond the middle, the lobes of irregular outline, often apiculate. Petals up to 7 × 4 mm., glabrous. Staminal tube 4–5·5 mm. long, glabrous. Ovary less than 1 mm. in diam., style 1 mm. long; disk situated beneath ovary. Fruit up to 8 cm. in diam., obscurely 4-sulcate. Seeds 3·6–7 cm. long.

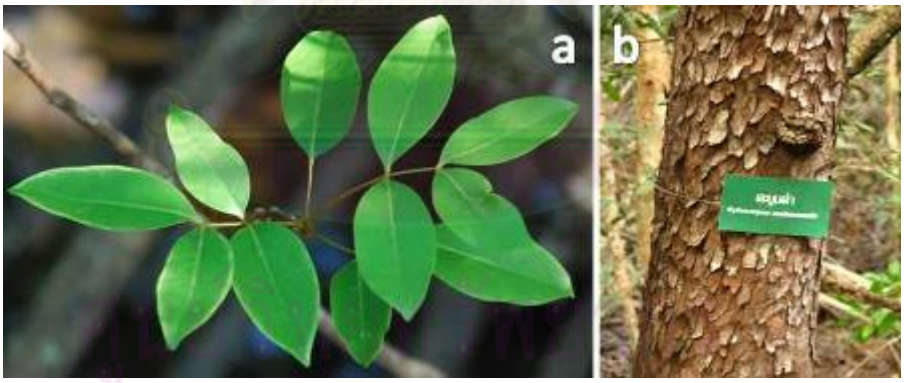


Figure 3.10 *Xylocarpus moluccensis* (a) Leaves (b) stem



### 3.4.6 Family Rhizophoraceae

a. *Ceriops decandra* (Griff.) Ding How (Figure 3.11)

**Other Names**: *Bruguiera decandra* Griff., *Ceriops roxburghiana* Arn.

**Common name**: Prongkae, Prongnu

**Description**: A small to medium-sized straight, columnar, evergreen tree, under favourable conditions reaching up to 35 m in height and the trunk up to 35 cm in diameter with short basal buttresses. The bark and leaves is a source of tannin. The sap of the bark yields a black dye used in the batik industri and a decoction of the bark has medicinal properties. The wood is used for posts, poles, tools, boat ribs and firewood and charcoal.

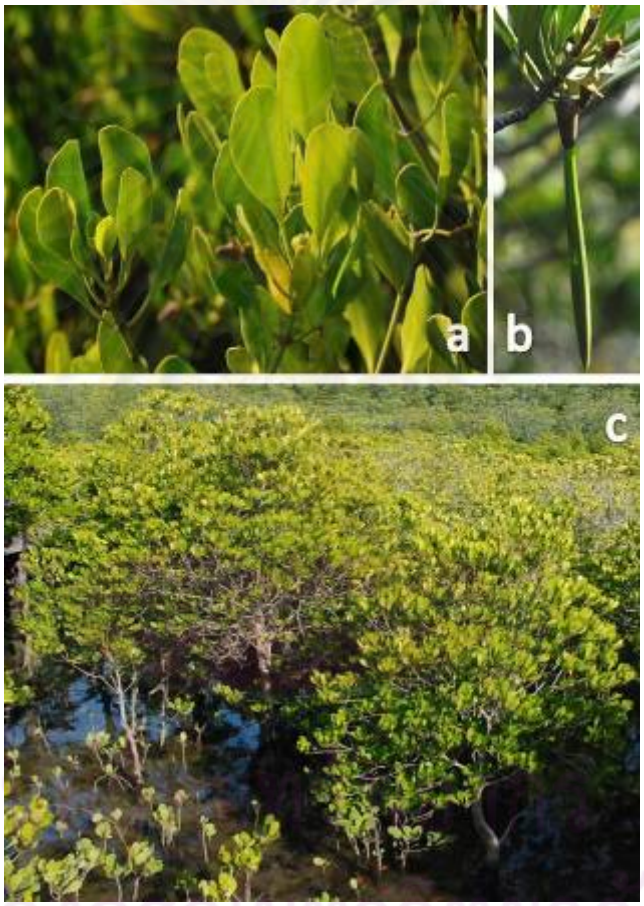


Figure 3.11 *Ceriops decandra* (a) leaves (b) seedlings (c) trees

b. *Rhizophora apiculata* Bl. (Figure 3.12b)

**Synonyms**: *Rhizophora candelaria*, *Rhizophora conjugate*

**Common name**: Kong-kang Bai-Lek, Pangka Bailek

**Description**: An evergreen, medium sized, much-branched tree that is capable of

reaching 20 m height but most of the individuals found in Maldives are only about 6 to 10 m tall. It is characterized by the presence of numerous stilt roots, which are looping from branches and trunk bases and provide support to trees; they also function as air-breathing roots. Bark is grey or dark grey in colour and sometimes longitudinally fissured. Leaves are simple, opposite in arrangement, narrowly elliptic, apiculate (with abrupt slender tip) and smaller than that of other *Rhizophora* species. Inflorescence is a two-flowered cyme on short, stout, dark grey peduncle and axillary in position. Calyx is four lobed, greenish yellow inside and reddish green outside. Petals are four in number and white coloured; not hairy. Viviparous propagules are shorter than that of *R. mucronata,* 25 to 30 cm long, green to brown in colour, warty or relatively smooth, buoyant and dispersed by currents.

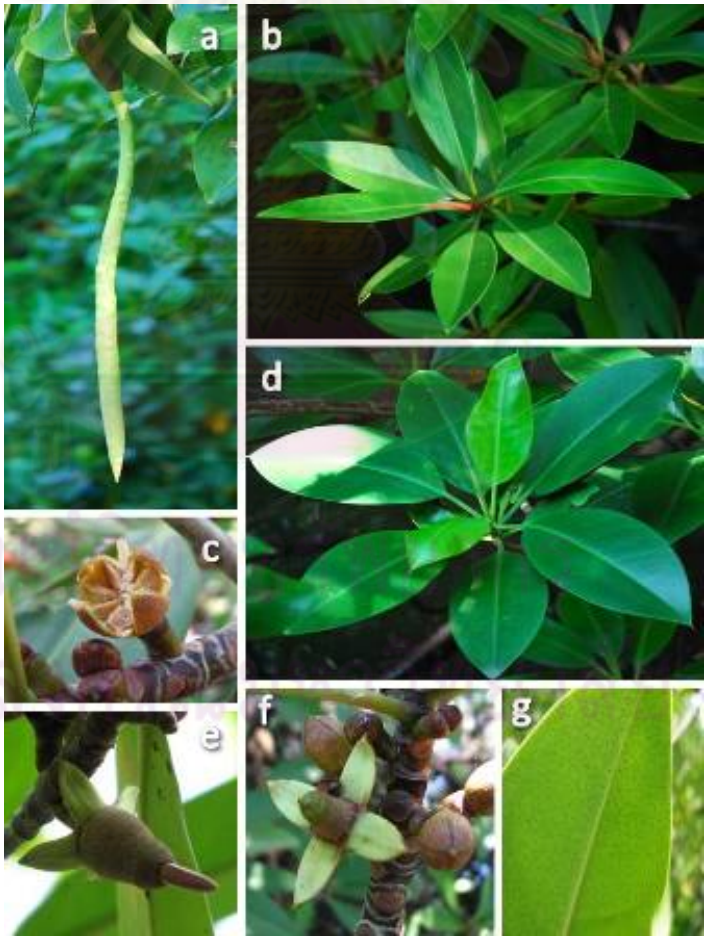


Figure 3.12 *Rhizophora* species (a) seedling (b) *Rhizophora apiculata* leaves (c) flower (d) *Rhizophora mucronata* leaves (e-f) young seedling (g) leaves

c. *Rhizophora mucronata* Poir. (figure 3.12d)

**Synonym**: *Rhizophora macrorrhiza*

**Common name**: Kong-Kang Bai Yai, Pangka Baiyai

**Description**: An evergreen, medium to tall tree that may reach 25 to 30 m height. As in the case of *Rhizophora apiculata* numerous, much-branched, hoop or pile-like stilt roots loop from branches and stems and provide support to trees. These stilt roots also function as above-ground breathing roots. Bark is brown or reddish, smooth and sometimes scaly. Leaves are single, opposite, leathery, broadly elliptic to oblongelliptic in shape with very distinct black dots on the under surface and tipped with fine spine (mucor). Leaf stalk is 3 to 5 cm long. Inflorescence is dichotomously branched cyme, four to eight flowered and axillary in position; peduncle is slender, yellow and 2 to 3 cm long (in *Rhizophora apiculata* peduncle is stout and dark grey in colour). Flowers are creamy white, fleshy and fragrant. Calyx is deeply four lobed and pale yellow. Petals are four in number, light yellowish, densely haired along the margin. Viviparous propagules are longer than that of *Rhizophora apiculata*, 40 to 70 cm long, 2 to 2.5 cm in diameter, cylindrical, warty, green to yellowish green in colour, buoyant and dispersed by currents.

### 3.4.7 Family Sonneratiaceae

a. *Sonneratia alba* J. Smith (Figure 3.13)

**Common name**: Lumpou-thalay

**Description**: Grows up to 15m tall. Bark: Cream, grey to brown bark, slight vertical fissures. Roots: No buttresses or prop roots. It has pneumatophores that are cone-shaped. Leaves: Rounded, leathery, opposite, upper and underside of leaf similar. Flower: White,pom-pom-like, open only for one night. Fruit: Large (4 cm) green, leathery berries with a star-shaped base. Contains 100-150 tiny seeds that are white, flattened and buoyant. *Sonneratia alba* can tolerate wide fluctuations in salinity and often grow on exposed, soft but stable mudbanks low on the tidal mudflats. It is believed that they

store excess salt in old leaves which they later shed. The bark of young *Sonneratia* is covered with a layer of wax, probably to protect it against water loss and attacks by creatures great and small.


Figure 3.13 *Sonneratia alba* (a) stem (b) fruit (c) young flowers (d) flower (e) tree (f) leaves



## 3.5 Leaf sampling

Healthy leaves are defined as leaves undamaged by herbivores and free of overt symptoms of disease. Three hundred healthy leaves were randomly collected from ten individual plants (30 leaves per plant) for each species of mangrove plant from the lower part of the crown and placed into opened plastic bags and processed within 24 h of collection (Figure 3.14). In the case of trees which were growing along the border of the water channels, the leaves were collected from at least 1.0 m above the high tide

level. All studied mangrove plants were woody plants except *Acanthus ilicifolius* Linn. which is a viny shrub or tall herb, upto 1.5 m high.


Figure 3.14 Leaf sampling

## 3.6 Media

Malt extract agar (MEA) was selected for the endophyte isolation as being a medium proven to isolate a diverse taxonomic population. Potato dextrose agar (PDA) and Malt extract agar (MEA) were selected for morphology observations. For the isolation of endophytic fungi, the antibiotic, streptomycin was added at a final concentration of 50 μg/ml to prevent bacterial contamination.

## 3.7 Endophyte isolations

Prior to surface sterilization, all apparently healthy leaves were harvested, washed in running tap water to remove dust particles on leaf surfaces. To kill fungal propagules adhering to the cuticle, three hundred healthy lamina segments (about 0.5 $cm^2$), cut from the middle portion (including mid-rib) of leaves were then surface-sterilized, following the protocol described in Kumaresan and Suryanarayanan (2001), by soaked in 70% ethanol for 5 s, then in sodium hypochlorite (4% available Chlorine) for 90 s. Finally, the leaves were rinsed in sterile distilled water for 10 s and dried with sterile filter paper. The surface sterilized leaves segments were plated out in 9 cm diam. Petri dishes containing 2% malt-extract agar supplemented with streptomycin sulphate

150 $mg^{-1}$), a medium that yields large numbers of diverse endophytic isolates (Fröhlich *et al*., 2000). In order to test the effectiveness of surface sterilization, sterilized leaves were imprinted onto MEA before incubation. The absence of growth of any fungi ascertained that the surface sterilization procedure was effective (Schulz *et al*., 1993). About 300 leaf segments were placed in 30 Petri dishes containing MEA, with 10 segments per dish. The plates were sealed with parafilm and incubated at room temperature. Every 2 days for 4 weeks, each leaf segment for fungal growth. Hyphal tips from distinct colonies emerging from leaf segments was examined subcultured on to new 2% MEA, without antibiotics, plates to obtain pure cultures. All endophytic isolates were stored on MEA slants and kept in distilled water for a living collection.

## 3.8 Fungal endophytes identification

All isolations were identified using classical morphological characteristics in conjunction with molecular data.

### *3.8.1 Morphological study*

All isolates were then conservatively assigned to morphospecies based on morphological characters, including spore production, spore characteristics, aerial mycelium form, colony and medium colour, colony surface texture, margin characters, exudates and growth rates on MEA. Xylariaceous taxa produced stromata like structures and were sorted into different taxa based on stromatal and colony morphology. Cultures which did not sporulate after two months or more after subculture onto different media (oat meal agar and potato dextrose agar) were considered as sterile mycelia. The microscopical analyses were based on observations by light microscopy on an Olympus CH2 research microscope using a 40x dry objective. Specimens for light microscopy were mounted in water, lactophenol-cotton blue for observation of spores and other characteristics, and then identified. Nomenclature of the fungi follows Sutton(1980), Ellis (1971) and Barnett and Hunter(1987).

*3.8.2 Molecular study*

All isolates that were categorized as a member of each genus were studied further using molecular techniques; DNA extraction, Polymerase Chain Reaction and DNA sequencing.

3.8.2-1 DNA preparation and extraction protocol from endophytic fungal isolates

Endophytic fungal cultures were inoculated into 100 ml malt extract broth and incubated at room temperature for 3-4 days. Mycelium was harvested and put into 1.5 ml extraction tubes to fill about one third of each tube with put beads size 2 mm about 3-5 beads per tube Add 700 µl 2 × CTAB lysis buffer was added to homogenize the rinsed pellet and the solution then at 65 °C for about one hour. Then 700 µl choroform/ isoamyl alcohol (24:1 v/v) was added to the solution and completely mixed them by a vortex mixer and then centrifuged at 15000 rpm for 8 mins at room temperature. The supernatant 650 µl was removed to a new micro-tube, being careful not to transfer the debris at the interface and then steps 3 and 4 again. Add 700 µl isopropanol was added to the supernatant, mixed and kept at -20°C for 10 min (or on the ice for 30 min). Centrifuged at 8000 rpm at 4 °C for 10 min to precipitate DNA. The DNA pellet was washed with 500 µl of 70% ethanol and centrifuged at 8000 rpm at 4 °C for 5 mins. The pellet was air-dried and dissolved in 100 µl of sterile distilled water and stored at -20°C until use for PCR. RNAase treatment: samples were treated with 1 µl RNase (10 mg/ml) and the DNA solution incubated at room temperature for 30 mins. 60 ul 20% PEG was added and keep the tube standing in the ice for about 30 mins and then centrifuged at 1500 rpm at 4 °C for 10 mins. The DNA pellet was washed using 70% ethanol 500 µl centrifuged 5 min 15000 rpm 4 °C then the pellet was air-dried and dissolved in 100 µl sterile distilled water and stored at -20°C until used.

3.8.2-2 ITS amplification and ITS fragment analysis

The ITS region of each endophytic isolate was amplified with two primers, ITS3 (5'-GCATCGATGAAGAACGCGCAGC-3') (White *et al*., 1990) and ITS4 (5'-TCCTCCGCTTATTGATAGC-3') (Gardes and Bruns, 1993). The primer ITS4 was labeled with Texas Red (Greiner Bio-one). The PCR reaction mixture 100 μl contained compositions as shown in Table 3.3. The amplification reactions were performed in a thermal cycler TP65 (Takara Shuzo Co., Tokyo). The PCR cycle was as follows; initial denaturation at 94°C for 2 mins, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 1 minute, and finished by a final extension at 72°C for 7 mins. PCR products were run on a 1% agarose gel at 140 V. The DNA samples were amplified by using ITS 3 and 4 based on the highly conserved regions of the 5.8S, ITS2 and 28S rRNA genes (Figure 3.15)

Table 3.3 PCR reaction mixture for primer ITS3-4

| Reagent | Volume (ul) |
|---|---|
| Distilled water | 55 |
| 10x $NH_4$ buffer | 10 |
| 25 mM dNTP | 16 |
| 50 $MgCl_2$ | 5 |
| 20 uM ITS-3 | 1.5 |
| 20 uM ITS-4 | 1.5 |
| Biotaq™ DNA polymerase | 1 |
| DNA template | 10 |

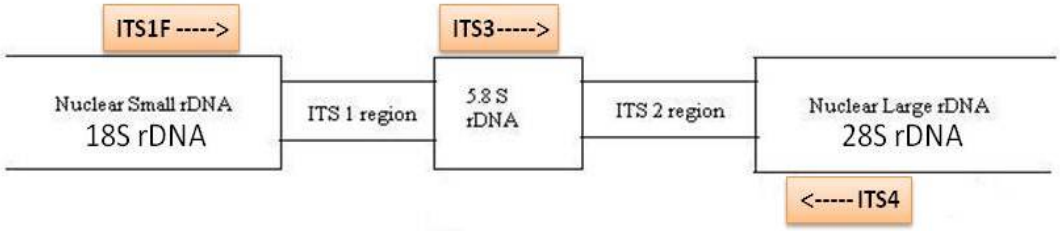


Figure 3.15 Locations of PCR primers for internal transcribed spacers on the nuclear rDNA map

The ITS3-4 fragments were denatured at 94°C for 5 minitues and electophoresed on 6% Long Ranger acrylamide gels (FMC Bioproducts Co., ME), with 6.1 M urea, and 1.2x TBE (0.1 M Tris [hydroxymethyl] aminomethane, 3 mM EDTA and 0.1 M boric acid), in sequencer (SQ-5500E, Hitachi Electronics Engineering Co., Tokyo). DNA size standards (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK) were loaded every ten lanes on the gels. Band patterns were analyzed using FRAGLYS 3.0 software (Hitachi Electronics Engineering Co., Tokyo). Samples showing the identical sizes of ITS3-4 were considered to belong to the same genotype. From each group one sample was arbitrarily chosen and used for sequence analysis.

3.8.2-3 Sequencing

The ITS regions were amplified from the representative sample of each genotype using primer ITS1F 5'-CTTGGTCATTTAGAGGAGTAA-3') and ITS4. The PCR mixture and condition were performed as previously described. The ITS1F-4 fragments were cloned using pT7 Blue vector (Novagen W1) and transformed into *Escherichia coli* strain JM109 (Takara Shuzo Co., Tokyo). Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (GE Healthcare, UK) using T7 and M13 forward primers labeled with Texas Red (Hitachi Instruments Service Co.) in a SQ-5500E sequencer. Some sample were sent to Macrogen company (Tokyo, Japan) for sequencing.

3.8.2-4 Phylogenetic analysis

The ITS1F -4 sequences were used as query sequence to search for identity sequences for GenBank DNA database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS sequences were automatically aligned with chosen ITS sequences obtained from GenBank DNA database from the Table 3.4 . The aligment was carried out using Clustal X (http://innprotweiznann.ac.li/software/ClustalX.html), and manually improved in MacClade 4 (Maddison and Maddison 2001). Phylogenetic tree was constructed in Neighbour-joining (NJ) modes of PAUP*4.08b (PPC) (Swofford, 1999). Neighbour-joining analysis were performed after Kimura's two-parameter model. Bootstrap values were calculated by 1,000 replications.

Table 3.4 Fungal strains used in the study and their accession numbers

| GenBank Accession No. | Taxon |
|---|---|
| AF39372Q2 | *Cladosporium oxysporum* |
| EU497957 | *Cladosporium cladosporioides* |
| EU552111 | *Colletotrichum gleosporioides* |
| AY266386 | *Colletotrichum truncatum* |
| EU056740 | *Colletotrichum capsici* |
| AB1583161 | *Coriolopsis caperata* |
| FJ624265 | *Daldinia eschscholizii* |
| AM749921 | *Daldinia placentiformis* |
| AF001025 | *Diaporthe phaseolorum* |
| AF577815 | *Diaporthe phaseolorum* |
| FJ612855 | Fungal sp. ARIZ |
| DQ062671 | *Glomerella cingulata* |
| FJ459944 | *Glomerella cingulata* |
| EU273524 | *Guignardia mangiferae* |
| EU167584 | *Guignardia vaccinii* |
| DQ201126 | *Hypoxylon anthochroum* |
| AY216475 | *Marasmius cladophyllus* |
| EF026121 | *Nemania primolutea* |
| AF201757 | *Nodulisporium* sp. |
| AB251918 | *Pestalotiopsis disseminata* |
| AF377296 | *Pestalotiopsis microspora* |
| FJ481027 | *Pestalotiopsis vismiae* |
| EU002931 | *Phomopsis* sp. |
| AB505409 | *Phomopsis* sp. |
| AY601920 | *Phomopsis bougainvilleicola* |
| AF079777 | *Phomopsis oryzae* |
| EU571098 | *Phomopsis vaccinii* |
| EF155505 | *Schizophyllum commune* |
| DQ322141 | *Xylaria* sp. |
| FJ175173 | *Xylaria mellissi* |
| AB440118 | *Xylariaceae* sp. |

## 3.9 Data analysis

The Colonization frequency (CF%) of a single endophyte species was calculated as the number of segments colonized by an endophyte species divided by total number of segments × 100. The percentage contribution to an endophyte assemblage by the dominant endophyte (DE%) was calculated as the CF% of the dominant endophyte divided by the sum of CF% of all endophytes in an assemblage×100.

## 3.10 Fungal cultivation and metabolite extraction

### 3.10.1 Fungal cultivation

Each fungal endophyte isolate was grown on MEA at room temperature (25-30 °C) for 1-2 weeks depending on the individual fungal growth rate. The agar cultures were then cut into 7 mm diameter disks by a flamed cork hole borer. One disk was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth per flask. All cultures were incubated for 8 weeks at room temperature (25-30 °C) under static conditions.

### 3.10.2 Metabolite extraction

The culture broths were filtered through a filter paper and were extracted with ethyl acetate three times. The organic phases were pooled and evaporated under reduced pressure to dryness using a rotary evaporator. Crude extractes were dissolved in 10% (v/v) dimethylsulphoxide (DMSO) and used for antimicrobial and cytotoxic screening.

## 3.11 Evaluation of the antimicrobial activity

### 3.11.1. Test microorganisms

The *in vitro* antimicrobial susceptibility tests were performed using two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923, two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922), the yeasts (*Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 5169).

### 3.11.2 Procedures

a. *Preparation of medium*

Nutrient agar plates were poured into dishes of 90 mm internal diameter. If the plates were not required for immediate use they were stored in a refrigerator and protected from desiccation. They were normally used within seven days, but if kept for a longer period provided, they were subjected to quality control procedures before use. Immediately before inoculation the plates were allowed to reach room temperature and the surface of the plate was dry. If not, the plates were dried (with lids ajar) prior to inoculation. There should be no visible droplets of moisture on the surface of the agar or on the lids of the plates when they are inoculated. Nutrient agar was used for bacteria and Potato dextrose agar was used for yeasts.



b. *Preparation of inoculum*

From a pure culture of the test organism, four or five colonies were taken with a wire loop and transferred onto 5 ml of nutrient broth and potato dextrose broth. Incubation of the broth was at 35-37°C for 2-8 hours (bacteria) and room temperature for the yeasts. The turbidity of the cell suspension was adjusted by adding additional nutrient broth or sterile saline, as required, until the turbidity of the cell suspension was

equivalent to the turbidity of a McFarland 0.5 barium sulphate standard. The cell suspension was discarded if it was not used within 15 to 20 mins after preparation.

c. *Inoculation of the test plate*

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess inoculum. The agar was inoculated by streaking the swab across the entire surface. The entire surface of each plate was inoculated by inoculating the surface completely in three different directions to ensure uniform growth (Figure 3.16). The surface of the medium was allowed to dry for 3-5 minutes.

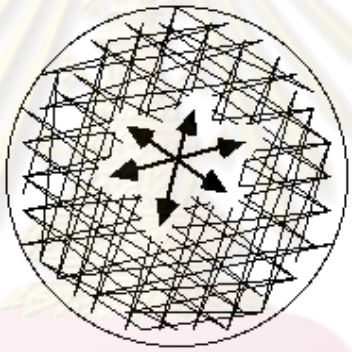

Figure 3.16 The entire surface of the plate is inoculated in three directions as indicated to ensure uniform growth.



d. *Application of culture filtrate*

Wells were made on the agar by removing disks cut with a flamed cork hole borer. 50 μl of culture fitrate was pipetted into the agar wells. This was absorbed by the medium surrounding the wells. All bacterial plates were incubated at 35- 37°C overnight and at room temperature for the yeast plates (approximately 18 hours). Inhibition zones around the wells were measured in cm using a ruler.

### 3.11.3 Preparation of McFarland turbidity standard

McFarland standards were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria was within a given range. Original McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. An opacity standard was prepared by mixing barium chloride ($BaCl_2$) and sulfuric acid ($H_2SO_4$). The resultant precipitate, barium sulfate ($BaSO_4$), is very insoluble. A new standard was prepared every six months.

1. The turbidity standard was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.36 N $H_2SO_4$ (1% v/v).
2. 4 to 6 ml were distributed in screw-cap tubes of the same size as those used in preparing the culture suspension.
3. Tubes were tightly sealed and stored in the dark at room temperature.
4. The turbidity standard was vigorously shaken on a mechanical vortex mixer just before use.

## 3.12 Detection of anticancer activity

The cytotoxic effect of fermentation broths was tested by the MTT (3-3,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide) assay. The cell lines used were five human cancer cell lines. The test cell lines are listed in Table 3.5. The MTT assay protocol follows that described by Palaga *et. al*. (1996).

Table 3.5 Human cancer cell lines for assay.

| Type of human cancer cell line | Reference strains |
|---|---|
| A375 (Human malignant melanoma) | ATCCno.CRL-1619 |
| SW620 (Human colorectal adenocarcinoma) | ATCCno.CCL-227 |
| Kato III (Human gastric carcinoma) | ATCCno.HTB-103 |
| HepG2 (Human liver hepatoblastoma) | ATCCno.HB-806 |
| Jurkat (Human acute T cell leukemia) | ATCCno.CRL-2063 |

**3.12.1 Seeding in 96-well plate**

Seed cells were cultured in 25 t-flask in RPMI-1640 medium containing serum 10 ml, sodium piruvate 1 ml, Hepe 1 ml, streptomycin 50 μl and 10 μl penicillin G at 37°C with 5% $CO_2$, 95% air and complete humidity. Once they reached ~90% confluency, they were detached using 2-2.5 ml of 0.025%trypsin-EDTA and then incubated at 37°C about 2 mins and 5 ml of RPMI-1640 media was added to trypsinized cells. Centrifuged in a sterile 15 ml falcon tube at 1000 rpm in the swinging bucke rotor for 5 min. Media was removed and cells resuspended to 1.0 ml with RPMI-1640 media and then counted by means of trypan blue and a hemocytometer. Media was used to dilute the cells to 2-5×$10^5$ cells/ml and 100 μl/well added onto 96-well plates and incubated overnight. For background absorption, some wells were left cell-free as blanks.

**3.12.2 Treating cells with crude extracts**

cells were treated with all crude extracts and If removing media, this had to be done very carefully as. This is where most variation in data may occur. A final volume of 100 μl per well was obtained. Three wells were left untreated as controls. Each condition was done in triplicate (n=3). The plates were incubated overnight at 37°C in a humidified incubator plus 5% $CO_2$.

### 3.12.3 MTT assay for evaluating cell viability

MTT reagent (10 μl/100 μl per well of the 96 well plate) was added and Incubated at 37°C for 4 hours with 5% $CO_2$, 95% air and complete humidity. After 4 hours, the MTT solution was removed and added 100 μl of isopropanol solution added, covered with tinfoil and agitated on an orbital shaker for 15 min. A purple colour becomes visible at this stage and optical density (OD) of the wells was determined using a plate reader at a test wavelength of 540 nm. Cell viability was calculated as $(A_{sample}-A_{blank})/(A_{control}-A_{blank})$x100% while absorbance obtained from cells incubated with 10%(v/v) DMSO in sterile distilled water to give a final concentration of 0.1% (v/v) in the medium was used as the control and cell free medium as the blank.

# CHAPTER 4
# RESULTS

## 4.1 Diversity of endophytic fungal communities in mangrove plants from Kung Krabaen bay

Endophytic fungi from five mangrove plant species *(Avicennia alba, Lumnitzera littorea, Rhizophora apiculata, Rhizophora mucronata, Sonneratia alba)* were isolated from leaves collected at Kung Krabaen bay at the Chanthaburi site in May 2006. The total range of species identified together with their percentage colonization frequencies are presented in Table 4.1 and Figure 4.1. A total of 8 genera and 8 species of fungi were identified by classical morphology characteristics but there were some cultures which remained sterile and some isolates were unknown species. This raised the total of isolated fungi to 10 species. Species of *Phyllosticta* were the dominant fungi isolated in all mangrove plants. There were differences in the species isolated and, in particular, in the colonization frequency in each host plant. *Avicennia alba* had the maximum taxa of endophytic fungi. The highest colonization frequency was found in *L. littorea* (79.9%) and the lowest in *A. alba* (19.3%).

Table 4.1 Percentage colonization frequency (CF%) of endophytic fungi isolated from five mangrove plant species at the Chanthaburi site

| Endophyte species | Plant host species | | | | |
|---|---|---|---|---|---|
| | *A. alba* | *L. littorea* | *R. apiculata* | *R. mucronata* | *S. alba* |
| *Aspergillus* sp. C1 | 1.0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. C1 | 1.3 | 2.3 | 1.0 | 3.0 | 2.0 |
| *Colletotrichum* sp.C1 | 2.3 | 7.3 | 2.7 | 6.7 | 13.3 |
| *Curvularia cf. branchyspora* | 0 | 0 | 1.7 | 0 | 0 |
| *Daldinia eschscholzii* | 1.7 | 0 | 3.3 | 2.0 | 0 |
| *Pestalotiopsis* sp. C1 | 1.7 | 0 | 4.7 | 4.3 | 0 |
| *Phomopsis* sp. C1 | 3.3 | 11.0 | 3.0 | 0 | 0 |
| *Phyllosticta* sp. C1 | 5.0 | 59.3 | 25.0 | 14.3 | 35.3 |
| *Xylaria* sp. C1 | 1.0 | 0 | 4.0 | 4.7 | 3.3 |
| Sterile mycelium C1 | 2.0 | 0 | 0 | 0 | 0 |
| Unknown species C1 | 0 | 0 | 0 | 3.0 | 0 |
| **Total percentage colonization frequency** | **19.3** | **79.9** | **45.4** | **38.0** | **53.9** |

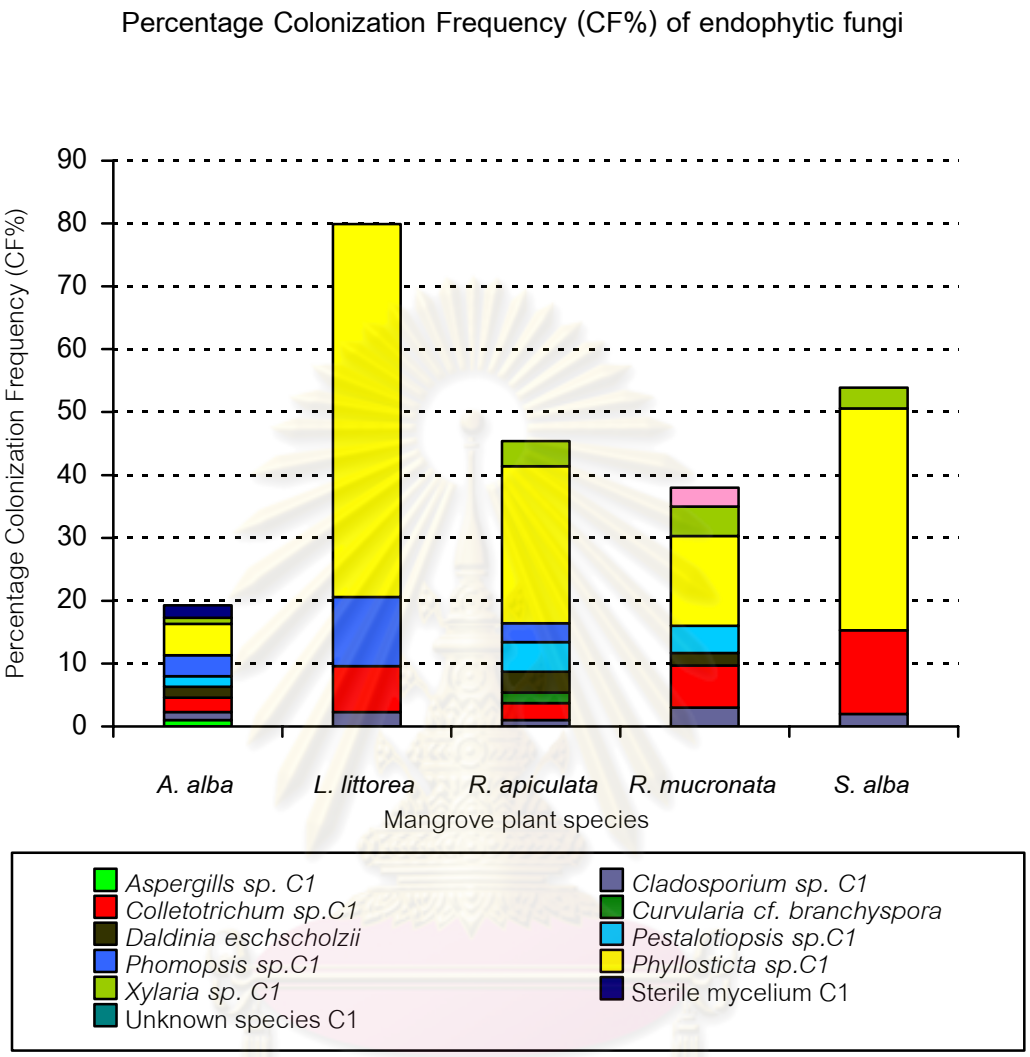


Figure 4.1 Percentage colonization frequency (CF%) of endophytic fungi isolated from five mangrove plant species at the Chanthaburi site

### 4.1.1 Diversity of endophytic fungi from *Avicennia alba* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.2. Fifty-five fungal isolates were obtained and these were assigned to 8 genera and 8 species. Species of *Phyllosticta* (5.0%) followed by *Phomopsis* sp. C1 (3.3%) were the most frequently isolated fungi.

Table 4.2 Endophytic fungi of *A. alba* leaves from Chanthaburi site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Aspergillus* sp. C1 | 3 | 1.0 |
| *Cladosporium* sp. C1 | 4 | 1.3 |
| *Colletotrichum* sp. C1 | 7 | 2.3 |
| *Daldinia eschscholzii* | 5 | 1.7 |
| *Pestalotiopsis* sp. C1 | 5 | 1.7 |
| *Phomopsis* sp. C1 | 10 | 3.3 |
| *Phyllosticta* sp. C1 | 15 | 5.0 |
| *Xylaria* sp. C1 | 3 | 1.0 |
| Sterile mycelium C1 | 6 | 2 |
| **Total isolates** | ***58*** | |
| **Total colonization frequency** | | **19.3** |

### 4.1.2 Diversity of endophytic fungi from *Lumnitzera littorea* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.3. Two-hundred and forty fungal endophytes belonging to 4 genera and representing 4 species were isolated. *Phyllosticta* sp. C1, *Phomopsis* sp. C1, *Colletotrichum* sp. C1 and *Cladosporium* sp. C1 represented 59.3%, 11.0%, 7.3% and 2.3% of the colonization frequency, respectively

Table 4.3 Endophytic fungi of *L. littorea* leaves from the Chanthaburi site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. C1 | 7 | 2.3 |
| *Colletotrichum* sp. C1 | 22 | 7.3 |
| *Phomopsis* sp. C1 | 33 | 11.0 |
| *Phyllosticta* sp. C1 | 178 | 59.3 |
| **Total isolates** | **240** | |
| **Total colonization frequency** | | **79.9** |

### 4.1.3 Diversity of endophytic fungi from *Rhizophora apiculata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.4. From the 136 fungal isolates obtained, 8 genera and 8 species were recorded. The most frequently recovered endophytic genus was *Phyllosticta* sp. C1 (25.0%). Others found were *Pestalotiopsis* sp. C1 (4.7%), *Xylaria* sp. C1 (4.0%), *Daldinia eschscholzii* (3.3%), *Phomopsis* sp. C1 (3.0%), *Colletotrichum* sp. C1 (2.7%), *Curvularia cf. branchyspora* (1.7%) and *Cladosporium* sp. C1 (1.0%).

Table 4.4 Endophytic fungi of *R. apiculata* leaves from the Chanthaburi site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. C1 | 3.0 | 1.0 |
| *Colletotrichum* sp. C1 | 8.0 | 2.7 |
| *Curvularia cf. branchyspora* | 5.0 | 1.7 |
| *Daldinia eschscholzii* | 10.0 | 3.3 |
| *Pestalotiopsis* sp. C1 | 14.0 | 4.7 |
| *Phomopsis* sp. C1 | 9.0 | 3.0 |
| *Phyllosticta* sp. C1 | 75.0 | 25.0 |
| *Xylaria* sp. C1 | 12.0 | 4.0 |
| **Total isolates** | **136** | |
| **Total colonization frequency** | | **45.4** |

### 4.1.4 Diversity of endophytic fungi from *Rhizophora mucronata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.5. One-hundred and fifteen fungal isolates obtained 6 genera and 6 species were recognised. *Phyllosticta* sp. C1 at 14.3 % was the dominant genus followed by species of *Colletotrichum* sp.C1 (6.7%), *Xylaria* sp. C1 (4.7%), *Pestalotiopsis* sp. C1 (4.3%), *Cladosporium* sp. C1 (3.0%), Unknown species C1 (3.0%) and then *Daldinia eschscholzii* (2.0 %).

Table 4.5 Endophytic fungi of *R. mucronata* leaves from Chanthaburi site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. C1 | 9.0 | 3.0 |
| *Colletotrichum* sp. C1 | 20.0 | 6.7 |
| *Daldinia eschscholzii* | 6.0 | 2.0 |
| *Pestalotiopsis* sp. C1 | 13.0 | 4.3 |
| *Phyllosticta* sp. C1 | 43.0 | 14.3 |
| *Xylaria* sp. C1 | 14.0 | 4.7 |
| Unknown species C1 | 9.0 | 3.0 |
| **Total isolates** | **114.0** | |
| **Total colonization frequency** | | **38.0** |

**4.1.5 Diversity of endophytic fungi from *Sonneratia alba* leaves**

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.6. One-hundred and sixty-two fungal endophytes belonging to 4 genera and representing 4 species were isolated. *Phyllosticta* sp. C1 was still dominant (35.3%). *Colletotrichum* sp. C1 (13.3%) was the next most frequently isolated taxon followed by *Xylaria* sp. C1 (3.3%) and *Cladosporium* sp. C1 (2.0%).

Table 4.6 Endophytic fungi of *S. alba* leaves from Chanthaburi site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. C1 | 6.0 | 2.0 |
| *Colletotrichum* sp. C1 | 40.0 | 13.3 |
| *Phyllosticta* sp. C1 | 106.0 | 35.3 |
| *Xylaria* sp. C1 | 10.0 | 3.3 |
| **Total isolates** | **162.0** | |
| **Total colonization frequency** | | **53.9** |

**4.2 Diversity of endophytic fungal communities in mangrove plants from Pranburi forest Park**

Endophytic fungi from three mangrove plant species *(Ceriops decandra, Rhizophora apiculata, Thespesia populneoides)* were isolated from leaves collected at Pranburi Forest Park at the Prachuap Khiri Khan site in May 2006. The total range of species identified together with their percentage colonization frequencies are presented in Table 4.7 and Figure 4.2. A total of 6 genera and 7 species of fungi were identified by morphological characteristics. Species of *Phyllosticta* were the dominant fungi isolated in *C. decandra and R. apiculata.* While a *Phoma* sp. was the most frequently recovered fungi us from *T. populneoides.* There were differences in the species isolated and, in particular, in the colonization frequency in each host plant. *Thespesia populneoides* had maximum taxa of endophytic fungi. The highest of colonization frequency were found in *T. populneoides* (58.7%) and lowest in *C. decandra* (9%).

Table 4.7 Percentage colonization frequency (CF%) of endophytic fungi were isolated from three mangrove plant species at the Prachuap Khiri Khan site

| Endophyte species | Plant host species | | |
|---|---|---|---|
| | *C. decandra* | *R. apiculata* | *T. populneoides* |
| *Cladosporium* sp.P1 | 1.0 | 2.0 | 16.7 |
| *Cladosporium* sp.P2 | 0 | 0 | 8.7 |
| *Colletotrichum* sp. P1 | 1.3 | 0 | 1.0 |
| *Pestalotiopsis* sp. P1 | 1.7 | 3.7 | 0 |
| *Phoma* sp. P1 | 0 | 0 | 30.0 |
| *Phyllosticta* sp. P1 | 5.0 | 16.0 | 2.3 |
| *Xylaria* sp. P1 | 0 | 1.3 | 0 |
| **Total percentage colonization frequency** | **9.0** | **23** | **58.7** |

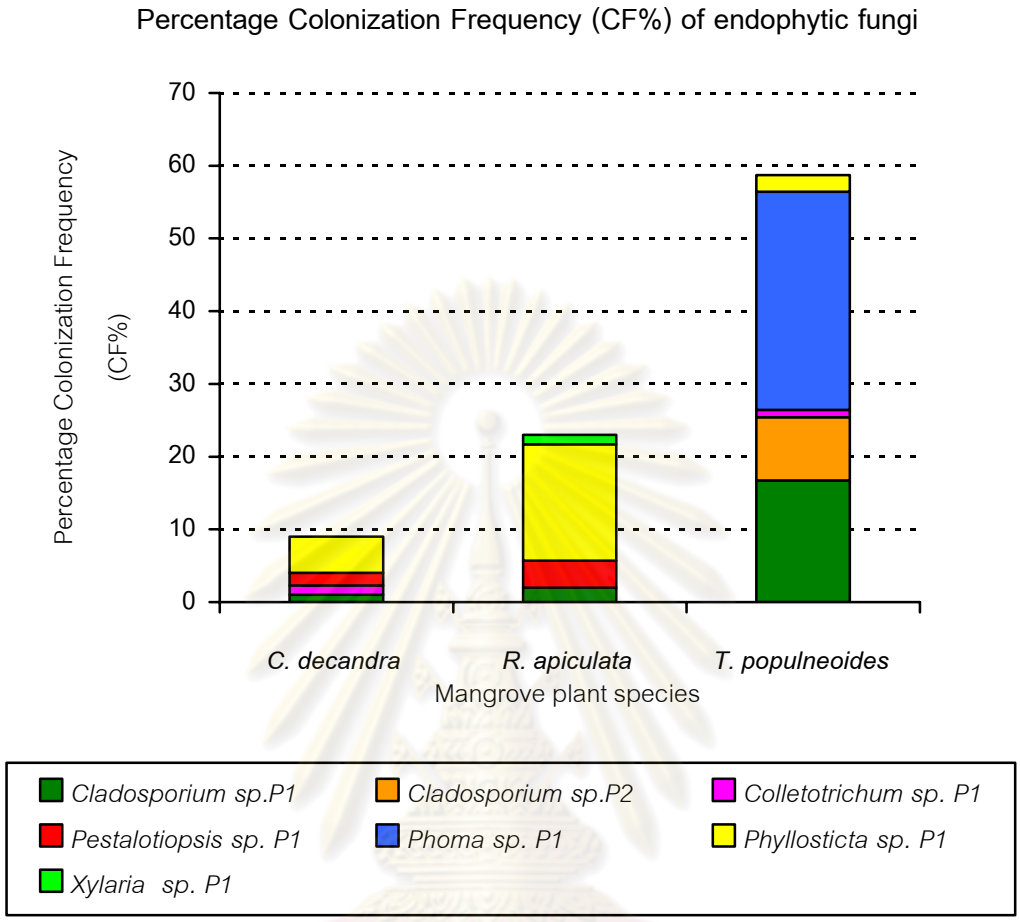


Figure 4.2 Percentage colonization frequency (CF%) of endophytic fungi were isolated from three mangrove plant species at the Prachuap Khiri Khan site

### 4.2.1 Diversity of endophytic fungi from *C. decandra* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.8. Only 27 fungal isolates belonging to *Phyllosticta* sp. P1 (5%), *Pestalotiopsis* sp. P1 (1.7%), *Colletotrichum* sp. P1 (1.3%) and *Cladosporium* sp. P1 (1.0%) were obtained.

Table 4.8 Endophytic fungi of *C. decandra* leaves from the Prachuap Khiri Khan site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. P1 | 3.0 | 1.0 |
| *Colletotrichum* sp. P1 | 4.0 | 1.3 |
| *Pestalotiopsis* sp. P1 | 5.0 | 1.7 |
| *Phyllosticta* sp. P1 | 15.0 | 5.0 |
| **Total isolates** | **27.0** | |
| **Total colonization frequency** | | **9** |

### 4.2.2 Diversity of endophytic fungi from *R. apiculata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.9. Sixty-nine fungal isolates were obtained which represented 4 genera and 4 species. *Phyllosticta* sp. P1 (16%) was the dominant isolate followed by *Pestalotiopsis* sp. P1 (3.7%), *Cladosporium* sp. P1 (2.0%) and *Xylaria* sp. P1 (1.3%).

Table 4.9 **Endophytic fungi of *R. apiculata* leaves from Prachuap Khiri Khan site**

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp.P1 | 6.0 | 2.0 |
| *Pestalotiopsis* sp. P1 | 11.0 | 3.7 |
| *Phyllosticta* sp. P1 | 48.0 | 16.0 |
| *Xylaria* sp. P1 | 4.0 | 1.3 |
| **Total isolates** | **69.0** | |
| **Total colonization frequency** | | **23** |

### 4.2.3 Diversity of endophytic fungi from *T. populneoides* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.10. A total of 183 fungal isolates were obtained. The most frequently isolated taxon was *Phoma* sp. P1 (30%), followed by two species of *Cladosporium* which represented 16.7% and 8.7% of the colonization frequency respectively, with *Phyllosticta* sp. P1 (2.3%) and *Colletotrichum* sp. P1 (1.0%).

Table 4.10 Endophytic fungi of *T. populneoides* leaves from Prachuap Khiri Khan site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp.P1 | 50.0 | 16.7 |
| *Cladosporium* sp.P2 | 26.0 | 8.7 |
| *Colletotrichum* sp. P1 | 3.0 | 1.0 |
| *Phoma* sp. P1 | 90.0 | 30.0 |
| *Phyllosticta* sp. P1 | 7.0 | 2.3 |
| **Total isolates** | **176.0** | |
| **Total colonization frequency** | | **58.7** |

## 4.3 Diversity of endophytic fungal community in mangrove plants from the Ranong site

Endophytic fungi from five mangrove plant species *(Acanthus ilicifotius , Rhizophora apiculata, Rhizophora mucronata, Xylocarpus granatum, Xylocarpus moluccensis)* were isolated from leaves collected at Ranong Biosphere Reserve at Ranong site in May 2006. The total range of species identified together with their percentage colonization frequencies are presented in Table 4.11 and Figure 4.3. A total of 9 genera and 11 species of fungi were identified by morphological characteristics. *Phyllosticta* was the dominant fungi us isolated in *A. ilicifotius and X. moluccensis.* While *Cladosporium* sp. R1 was the most frequently recovered fungi us from *R.*

*apiculata*. *Pestalotiopsis* sp. R1 was the dominant fungi us in *R. mucronata* and *Glomerella* sp. R1 was the most frequency endophytic isolate in *X. granatum*. There were differences in the species isolated and, in particular, in the colonization frequency in each host plant. *Xylocarpus granatum* had the maximum taxa of endophytic fungi. The highest of colonization frequency were found in *A. ilicifotius* (84%) and the lowest in *R. mucronata* (54%).

Table 4.11 Percentage colonization frequency (CF%) of endophytic fungi were isolated from three mangrove plant species at the Ranong site

| Endophyte species | Plant host species | | | | |
|---|---|---|---|---|---|
| | *A. ilicifotius* | *R. apiculata* | *R. mucronata* | *X. granatum* | *X. moluccensis* |
| *Cladosporium* sp. R1 | 3.3 | 21.3 | 5.0 | 2.7 | 0 |
| *Colletotrichum* sp. R1 | 10.0 | 5.0 | 0 | 0 | 6.7 |
| *Daldinia eschscholzii* | 0 | 6.7 | 6.0 | 7.7 | 3.7 |
| *Glomerella* sp. R1 | 18.0 | 2.7 | 2.3 | 19.0 | 7.0 |
| *Pestalotiopsis* sp. R1 | 0 | 17.3 | 20.7 | 3.3 | 6.3 |
| *Phomopsis* sp. R1 | 9.0 | 2.0 | 6.7 | 2.7 | 2.0 |
| *Phomopsis* sp. R2 | 2.7 | 1.7 | 0 | 2.3 | 0 |
| *Phomopsis* sp. R3 | 0 | 0 | 0 | 1.0 | 0 |
| *Phyllosticta* sp. R1 | 35.7 | 5.7 | 3.3 | 4.0 | 22.3 |
| *Xylaria* sp. R1 | 5.3 | 7.0 | 10.0 | 13.3 | 13.3 |
| Sterile mycelium R1 | 0 | 0 | 0 | 3.0 | 3.0 |
| **Total percentage colonization frequency** | **84** | **69.4** | **54.0** | **59.0** | **64.3** |

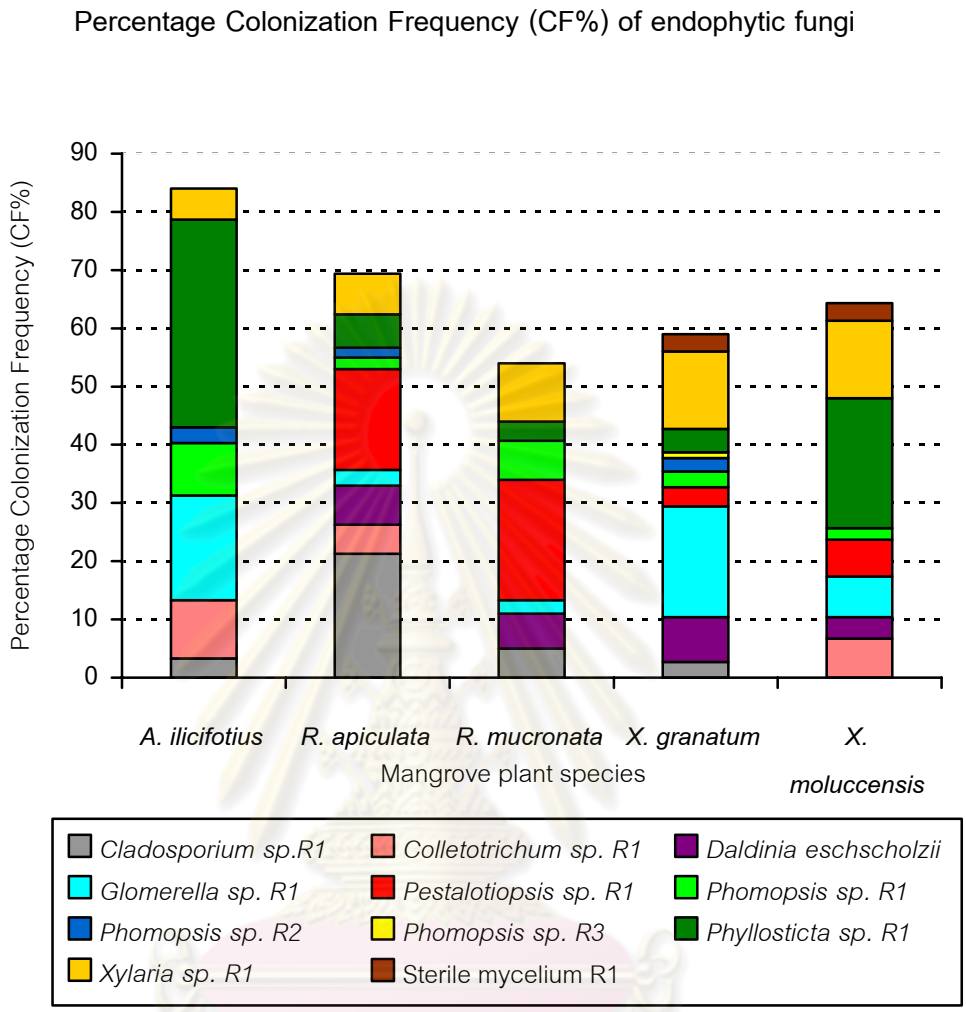


Figure 4.3 Percentage colonization frequency (CF%) of endophytic fungi were isolated from three mangrove plant species at the Ranong site

### 4.3.1 Diversity of endophytic fungi from *A. ilicifotius* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.12. Six genera and seven species were recorded out of the total of 252 fungal isolates. *Phyllosticta* sp. R1 (35.7%) was the dominant isolate followed by *Glomerella* sp. R1 (18.0%), *Colletotrichum* sp. R1 (10.0%), *Phomopsis* sp. R1 (9.0%), *Xylaria* sp. R1 (5.3%), *Cladosporium* sp. R1 (3.3%) and then *Phomopsis* sp. R2 (2.7%).

Table 4.12 Endophytic fungi of *A. ilicifotius* leaves from the Ranong site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp.R1 | 10 | 3.3 |
| *Colletotrichum* sp. R1 | 30 | 10.0 |
| *Glomerella* sp. R1 | 54 | 18.0 |
| *Phomopsis* sp. R1 | 27 | 9.0 |
| *Phomopsis* sp. R2 | 8 | 2.7 |
| *Phyllosticta* sp. R1 | 107 | 35.7 |
| *Xylaria* sp. R1 | 16 | 5.3 |
| **Total isolates** | **252** | |
| **Total colonization frequency** | | **84.0** |

### 4.3.2 Diversity of endophytic fungi from *R. apiculata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.13. A total of 208 endophytic isolates belonging to 8 genera and representing 9 species were obtained. *Cladosporium* sp. R1 at 21.3% was found to be the dominant isolate followed by *Pestalotiopsis* sp. R1 (17.3%), *Xylaria* sp. R1 (7.0%), *Daldinia eschscholzii* (6.7%), *Phyllosticta* sp. R1 (5.7%), *Colletotrichum* sp. R1 (5.0%), *Glomerella* sp. R1 (2.7%) and then *Phomopsis* sp. R1 (2.0%).

Table 4.13 Endophytic fungi of *R. apiculata* leaves from the Ranong site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp.R1 | 64 | 21.3 |
| *Colletotrichum* sp. R1 | 15 | 5.0 |
| *Daldinia eschscholzii* | 20 | 6.7 |
| *Glomerella* sp. R1 | 8 | 2.7 |
| *Pestalotiopsis* sp. R1 | 52 | 17.3 |
| *Phomopsis* sp. R1 | 6 | 2.0 |
| *Phomopsis* sp. R2 | 5 | 1.7 |
| *Phyllosticta* sp. R1 | 17 | 5.7 |
| *Xylaria* sp. R1 | 21 | 7.0 |
| **Total isolates** | **208** | |
| **Total colonization frequency** | | **69.4** |

### 4.3.3 Diversity of endophytic fungi from *R. mucronata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.14. One-hundred and sixty two fungal isolates were obtained and these represented 7 genera and 7 species. *Pestalotiopsis* sp. R1 (20.7%) was the most frequently isolated taxon. *Xylaria* sp. R1 (10.0%) was the next most frequently isolated fungus. *Phomopsis* sp. R1, *Daldinia eschscholzii* , *Cladosporium* sp. R1, *Phyllosticta* sp. R1 and *Glomerella* sp. R1 represented 6.7%, 6%, 5%, 3.3% and 2.3% of these isolates, respectively.

Table 4.14 Endophytic fungi of *R. mucronata* leaves from the Ranong site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp.R1 | 15 | 5.0 |
| *Daldinia eschscholzii* | 18 | 6.0 |
| *Glomerella* sp. R1 | 7 | 2.3 |
| *Pestalotiopsis* sp. R1 | 62 | 20.7 |
| *Phomopsis* sp. R1 | 20 | 6.7 |
| *Phyllosticta* sp. R1 | 10 | 3.3 |
| *Xylaria* sp. R1 | 30 | 10.0 |
| **Total isolates** | **162** | |
| **Total colonization frequency** | | **54.0** |

#### 4.3.4 Diversity of endophytic fungi from *X. granatum* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.15. A total of 168 fungal isolates were recorded and these represented 8 genera and 9 species. *Glomerella* sp. R1 at 19 % isolation frequency was the dominant genus followed by a *Xylaria* species (13.3%), *Daldinia eschscholzii* (7.7%), *Phyllosticta* sp. R1 (4.0%), *Pestalotiopsis* sp. R1 (3.3%), sterile mycelium R1 (3.0%), *Cladosporium* sp. R1 (2.7%), *Phomopsis* sp. R1 (2.7%), *Phomopsis* sp. R2 (2.3%), and *Phomopsis* sp. R3 (1.0%).

Table 4.15 Endophytic fungi of *X. granatum* leaves from the Ranong site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Cladosporium* sp. R1 | 8 | 2.7 |
| *Daldinia eschscholzii* | 23 | 7.7 |
| *Glomerella* sp. R1 | 57 | 19.0 |
| *Pestalotiopsis* sp. R1 | 10 | 3.3 |
| *Phomopsis* sp. R1 | 11 | 2.7 |
| *Phomopsis* sp. R2 | 7 | 2.3 |
| *Phomopsis* sp. R3 | 3 | 1.0 |
| *Phyllosticta* sp. R1 | 12 | 4.0 |
| *Xylaria* sp. R1 | 40 | 13.3 |
| Sterile mycelium R1 | 9 | 3.0 |
| **Total isolates** | 168 | |
| **Total colonization frequency** | | 59.0 |

### 4.3.5 Diversity of endophytic fungi from *X. mucronata* leaves

The total number of isolates obtained and the total range of species identified, together with their percentage colonization frequency (CF%), are given in Table 4.16. One-hundred and ninety-three fungal isolates were obtained and these represented 8 genera and 8 species. *Phyllosticta* sp. R1 (22.3%) was the most frequently isolated taxon. *Xylaria* sp. R1 (13.3%) was the next most frequently isolated taxa and followed by *Glomerella* sp. R1 (7.0%), *Colletotrichum* sp. R1 (6.7%), *Pestalotiopsis* sp. R1 (6.3%), *Daldinia eschscholzii* (3.7%), Sterile mycelium R1 (3.0%) and *Phomopsis* sp. R1 (2.0%).

Table 4.16 Endophytic fungi of *X. mucronata* leaves from Ranong site

| Taxa | Number of isolates | Colonization Frequency (CF%) |
|---|---|---|
| *Colletotrichum* sp. R1 | 20 | 6.7 |
| *Daldinia eschscholzii* | 11 | 3.7 |
| *Glomerella* sp. R1 | 21 | 7.0 |
| *Pestalotiopsis* sp. R1 | 19 | 6.3 |
| *Phomopsis* sp. R1 | 6 | 2.0 |
| *Phyllosticta* sp. R1 | 67 | 22.3 |
| *Xylaria* sp. R1 | 40 | 13.3 |
| Sterile mycelium R1 | 9 | 3.0 |
| **Total isolates** | **193.0** | |
| **Total colonization frequency** | | **64.3** |

**4.4 Diversity of endophytic fungal communities in mangrove plants from three locations**

From 3900 leaf segments (300 from each host species) of 10 different hosts belonging to 7 families, 1,965 isolates of fungal endophytes were isolated. The leaves of all of the woody tree hosts and a shrub host harboured fungal endophytes. The overall infection frequencies, as revealed by the total CF%, varied with the host species; the highest CF% was found in *A. ilicifolius* leaves (84%) from Ranong Province and the lowest in *C. Decandra* (9%) from the Prachuap Khiri Khan site (Table 4.17, Figure 4.4). It was found that the colonization frequency of fungal endophytes from most mangrove plants at Ranong Province was more than 50%. The colonization frequency of endophytic fungi from *A. ilicifotius* leaves was the highest at 84% and the lowest in *R. mucronata* (54%). The highest CF% of mangrove plants from Chanthaburi province occurred in *L. littorea* (79.9%) and the lowest in *A. alba* (19.3%). In the Prachuap Khiri Khan Province site *T. populneoides* exhibited the highest CF% (58.7%) and *C.*

*decandra* was the lowest CF% (9%). *Rhizophora apiculata* was selected as the single species for comparison of colonization frequency in the three experimental sites. It was found that the percentage of fungal endophytic assemblages from Ranong Province was the highest (69.4%) followed by Chanthaburi Province (45.4%) and Prachuap Khiri Khan Province (23%) respectively. The studies of the mangrove plants at the three chosen locations demonstrated differences in their endophytic fungal composition. The endophytic assemblages of the mangrove trees were composed of a number of cosmopolitan species such as *Cladosporium* spp., and a large number of coelomycetous taxa such as *Colletotrichum* spp., *Phomopsis* spp., *Pestalotiopsis* spp. and *Phyllosticta* spp. Species of *Phyllosticta* were the most frequency isolated endophytes from two locations studied (Chanthaburi and Prachuap Khiri Khan Province). The endophyte assemblages of the different mangrove hosts showed that several endophytic species were common to more than one host. Some of these, such as *Cladosporium, Colletotrichum*, *Daldinia eschscholizii, Phyllosticta, Pestalotiopsis, Xylaria* are not host specific.

Table 4.17 Percentage colonization Frequency (CF%) of endophytic fungi isolated from mangrove plants in three locations

| Endophyte species | Plant host species | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AAC | LLC | RAC | RMC | SAC | CDP | RAP | TPP | AIR | RAR | RMR | XGR | XMR |
| *Aspergillus* sp. C1 | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. C1 | 1.3 | 2.3 | 1.0 | 3.0 | 2.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. P1 | 0 | 0 | 0 | 0 | 0 | 1.0 | 2.0 | 16.7 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. P2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.7 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.3 | 21.3 | 5.0 | 2.7 | 0 |
| *Colletotrichum* sp. C1 | 2.3 | 7.3 | 2.7 | 6.7 | 13.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Colletotrichum* sp. P1 | 0 | 0 | 0 | 0 | 0 | 1.3 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 |
| *Colletotrichum* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10.0 | 5.0 | 0 | 0 | 6.7 |
| *Curvularia cf. branchyspora* | 0 | 0 | 1.7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Daldinia eschscholzii* | 1.7 | 0 | 3.3 | 2.0 | 0 | 0 | 0 | 0 | 0 | 6.7 | 6.0 | 7.7 | 3.7 |
| *Glomerella* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 18.0 | 2.7 | 2.3 | 19.0 | 7.0 |
| *Pestalotiopsis* sp. C1 | 1.7 | 0 | 4.7 | 4.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pestalotiopsis* sp. P1 | 0 | 0 | 0 | 0 | 0 | 1.7 | 3.7 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Pestalotiopsis* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17.3 | 20.7 | 3.3 | 6.3 |
| *Phomopsis* sp. C1 | 3.3 | 11.0 | 3.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 4.17 (Continued)Percentage colonization Frequency (CF%) of endophytic fungi isolated from mangrove plants in three locations

| Endophyte species | Plant host species | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AAC | LLC | RAC | RMC | SAC | CDP | RAP | TPP | AIR | RAR | RMR | XGR | XMR |
| *Phomopsis* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9.0 | 2.0 | 6.7 | 2.7 | 2.0 |
| *Phomopsis* sp. R2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.7 | 1.7 | 0 | 2.3 | 0 |
| *Phomopsis* sp. R3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.0 | 0 |
| *Phoma sp.* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30.0 | 0 | 0 | 0 | 0 | 0 |
| *Phyllosticta* sp. C1 | 5.0 | 59.3 | 25.0 | 14.3 | 35.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Phyllosticta* sp. P1 | 0 | 0 | 0 | 0 | 0 | 5.0 | 16.0 | 2.3 | 0 | 0 | 0 | 0 | 0 |
| *Phyllosticta* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 35.7 | 5.7 | 3.3 | 4.0 | 22.3 |
| *Xylaria* sp. C1 | 1.0 | 0 | 4.0 | 4.7 | 3.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. P1 | 0 | 0 | 0 | 0 | 0 | 0 | 1.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.3 | 7.0 | 10.0 | 13.3 | 13.3 |
| Sterile mycelium C1 | 2.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sterile mycelium R1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3.0 | 3.0 |
| Unknown species C1 | 0 | 0 | 0 | 3.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total CF% | 19.3 | 79.9 | 45.4 | 38.0 | 53.9 | 9.0 | 23.0 | 58.7 | 84.0 | 69.4 | 54.0 | 59.0 | 64.3 |

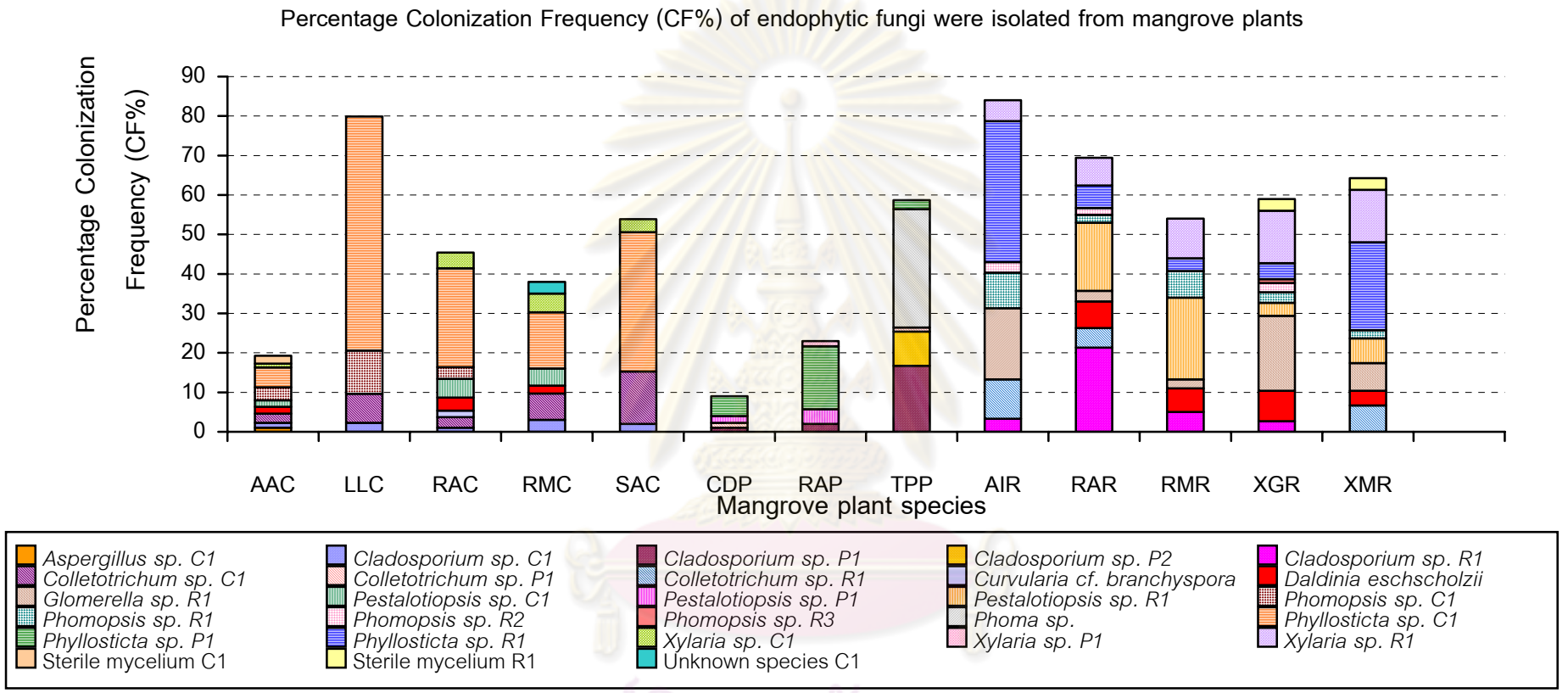


Figure 4.4 Percentage Colonization Frequency (CF%) of endophytic fungi isolated from mangrove plants in three locations (sampling site: R, Ranong Province; C, Chanthaburi Province; P, Prachuap Khiri Khan Province

All of the dominant species from Chanthaburi were of the genus *Phyllosticta* and this was also the dominant genus in *R. apicula* and *C. decandra* occurring in the Prachuap Khiri Khan site. The dominant endophytic species in mangrove plants from Ranong Province were different for each host plant in the mangrove community (Table 4.18)

Table 4.18 Percentage contribution by the percentage Dominant Endophyte (DE%) to endophyte assemblages of mangrove plants.

| Plant host species | Study site (Province) | Dominant endophyte species | DE% |
|---|---|---|---|
| *Avicennia alba* | Chanthaburi | *Phyllosticta* sp. C1 | 25.90 |
| *Lumnitzera littorea* | Chanthaburi | *Phyllosticta* sp. C1 | 74.21 |
| *Rhizophora apiculata* | Chanthaburi | *Phyllosticta* sp. C1 | 55.07 |
| *Rhizophora mucronata* | Chanthaburi | *Phyllosticta* sp. C1 | 37.63 |
| *Sonneratia alba* | Chanthaburi | *Phyllosticta* sp. C1 | 65.49 |
| *Ceriops decandra* | Prachuap Khiri Khan | *Phyllosticta* sp. P1 | 55.56 |
| *Rhizophora apiculata* | Prachuap Khiri Khan | *Phyllosticta* sp. P1 | 69.56 |
| *Thespesia populneoides* | Prachuap Khiri Khan | *Phoma* sp. | 51.10 |
| *Acanthus ilicifotius* | Ranong | *Phyllosticta* sp. R1 | 42.50 |
| *Rhizophora apiculata* | Ranong | *Cladosporium* sp. R1 | 30.69 |
| *Rhizophora mucronata* | Ranong | *Pestalotiopsis* sp. R1 | 38.33 |
| *Xylocarpus granatum* | Ranong | *Glomerella* sp. R1 | 32.20 |
| *Xylocarpus moluccensis* | Ranong | *Phyllosticta* sp. R1 | 34.68 |

## 4.5 Identification of endophytic fungi by morphological characteristics

*Descriptions of the genus*

***Curvularia* sp.** (Figure 4.5)

Colonies effuse, brown, grey or black, hairy, cottony or velvety. Mycelium immersed in natural substrata. Stromata often large, erect, black, cylindrical, sometimes branched, formed by many species in culture. Conidiophores macronematous, mononematous, straight or flexous, often geniculate, sometimes nodose, brown, usually smooth. Conidiogenous cells polytretic, integrated, terminal, sometimes later becoming intercalary, sympodial, cylindrical or occasionally swollen, cicatrized. Conidia solitary, acropleurogenous, simple, often curved, clavate, ellipsoidal, broadly fusiform, obovoid or pyriform with 3 or more transverse septa  or dark brown, often with some cells, usually the end ones, paler than others, sometimes with dark bands at the septa, smooth or verrucose; hilum in some species protuberant.

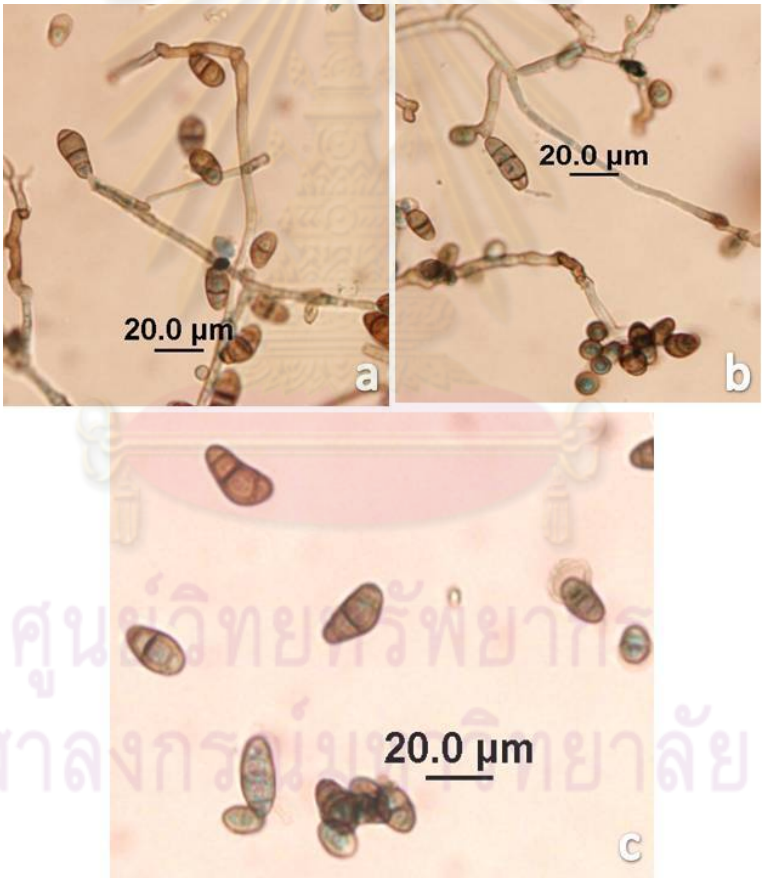


Figure 4.5 *Curvularia* (a-b) conidia on conidiophores (c) spore

***Cladosporium* sp.** (Figure 4.6)

Colonies effuse or occasionally punctiform, often olivaceous but also sometime grey, buff, brown or dark blackish brown, velvety, floccose or hairy.  Mycelium immersed and

often also superficial. Setae and hyphopodia absent. Conidiophores mavronematous or semimacronematous and sometimes also micronematous; macronematous conidiophores straight or flexuous, mostly unbranched or with branches restricted to the apical region forming a stipe and head, olivaceous brown or brown, smooth or verrucose. Ramo-conidia often present. Conidiogenous cells polyblastic, usually integrated, terminal and intercalary but sometime discrete, sympodial, more or less cylindrical, cicatrized. Conidia catenate as a rule but sometimes solitary especially in species with large conidia, often in branched chains, acropleurogenous, simple, cylindrical, doliiform, ellipsoidal, fusiform, ovoid, spherical or subspherical, often with a distinctly protuberant scar at each end or just at the base, pale to dark olivaceous brown or brown, smooth, verruculose or echinulate, with 0-3 or occasionally more septa.

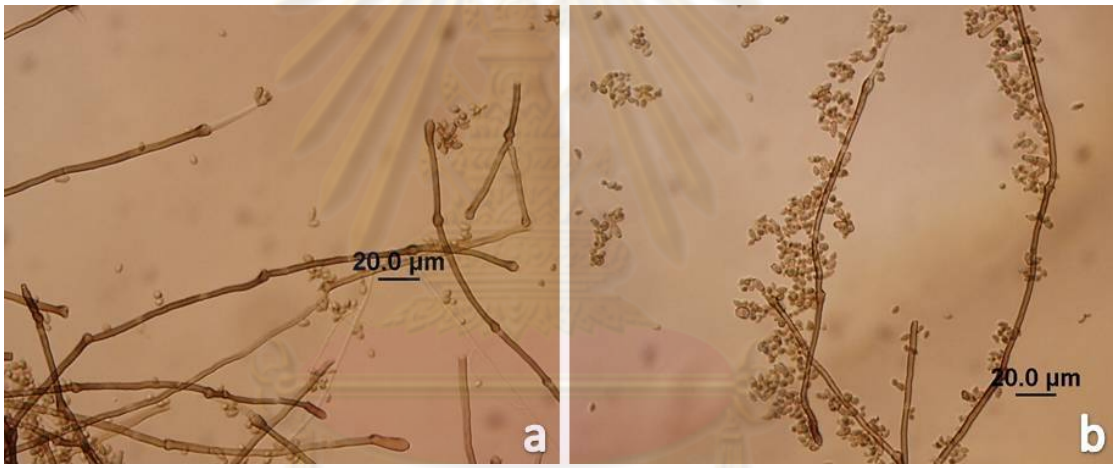


Figure 4.6 *Cladosporium* (a-b) spore

***Colletotrichum*** Cda in Sturm, Deutschlands Flora 3: 41(1831-1832) (Figure 4.7)

Mycelium immersed,branched,septate,hyaline,pale brown or dark brown. Conidiomata acervular, subcuticular, epidermal, subepidermal or peridermal, separate or confluent, composed of hyaline to dark brown, thin- or thick-walled textura angularis; dehiscence irregular. Sclerotia sometimes present in culture, dark brown to black, often confluent, occasionally setose. Setae in conidiomata or sclerotia, brown, smooth, septate, tapered to the apices. Conidiophores hyaline to brown, septate , branched only at the base, smooth , formed from the upper cells of the conidiomata. Conidiogenous cells enteroblastic,phialidic, hyaline , smooth , determinate, cylindrical ,integrated or discrete,

channel minute but occasionally collarette and periclinal thickening quite prominent. Conidia hyaline , aseptate ( except prior to germination ), straight or falcate, smooth, thin-walled, sometimes guttulate , muticate or with the apex prolonged into a simple cellular appendage. Appressoria brown, entire or with crenate to irregular margins, simple or repeatedly germinating to produce complex columns of several closely connected appressoria.

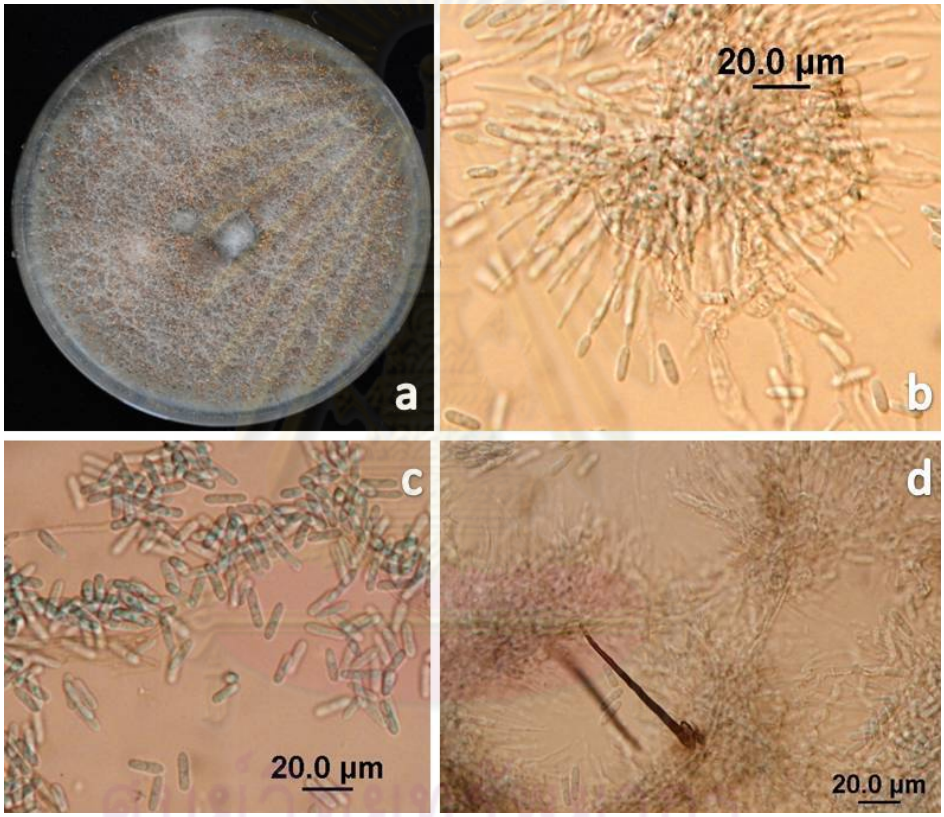


Figure 4.7 *Colletotrichum* sp. (a) culture plate on MEA 7 days (b) conidia produced on conidiophore (c) conidia staining with lacto phenol cotton blue (d) setae

***Daldinia eschscholzii*** (Ehrenb.: Fr.) Rehm., Ann. Mycol. 2: 175. 1904. (Figure 4.8)

Cultural characteristics

Colonies on malt extract agar, under 12 h light and 12 h darkness, reaching edge of a 9 cm diam. Petri dish in 2 weeks; at first white to orange-white in the centre, becoming pale to dull yellow, floccose, finally in patches orange-grey to brownish

grey in the centre, greyish yellow and olive-brown towards the margins, and in between, brown woolly with coarse texture. Reverse at first pale yellow to greyish yellow and finally fairly uniform dark greyish brown. Exudate orange to reddish brown. Agar staining yellowish brown to brown. Odour ether-like with a sweet component. Hyphae sparingly branched, septate, at first hyaline, later light brown, smooth or slightly verrucose, 2-3 μm diam. Conidiogenous structures formed after 3-4 days, at first in the centre, later abundant throughout the whole colony. Directly on the mycelium. Conidiophores mononematous, di-or trichotomously branched, especially towards the apex, regularly septate, at first hyaline and smooth, later light brown and verrucose, up to 280 μm long × 2-3 μm diam. Conidiogenous cells terminal, cylindrical, at first hyaline and smooth, later light brown and verrucose, 10-20 × 2-3 μm, bearing circular refractile to more or less denticulate conidial scars in the somewhat flattened apices. Conidia acrogenous, ellipsoid to obovoid, with a flattened circular abscission scar at the base, hyaline, smooth by L.M. (4)4.5-7(8) × 2-3(3.5) μm.

**Stromata** Turbinate to placentiform, sessile or with short, stout stipe, solitary to infrequently aggregated, smooth, 1.5-4 cm diam × 1-3 cm high; surface brown, vinaceous, dark brick, sepia, greyish sepia or vinaceous grey, blackened and varnished in age; dull reddish brown granules immediately beneath surface, with KOH-extractable pigments livid purple, dark livid or vinaceous purple; the tissue between perithecia brown, pithy to woody; the tissue below the perithecial layer composed of alternating zones. The darker zones dark brown, pithy to woody, 1-0.2 mm thick, the lighter zones white, grey or greyish brown, gelatinous and very hard when dry, becoming pithy to woody, persistent, 0.3-1 mm thick.

**Perithecia** tubular, 0.8-1.6 mm high × 0.3-0.4 mm diam

**Ostioles** obsolete or slightly papillate.

**Asci** 95-125 μm long, with apical apparatus, discoid, 0.5 μm high × 2-2.5 μm broad blueing in Melzer's iodine reagent.

**Ascospores** brown to dark brown, unicellular, ellipsoid-inequilateral, with narrowly rounded ends, 11.3-12.5 × 5.0-6.3 μm, with straight germ slit spore-length on convex side; perispore dehiscent in 10% KOH, conspicuous coil ornamentation; epispore smooth.

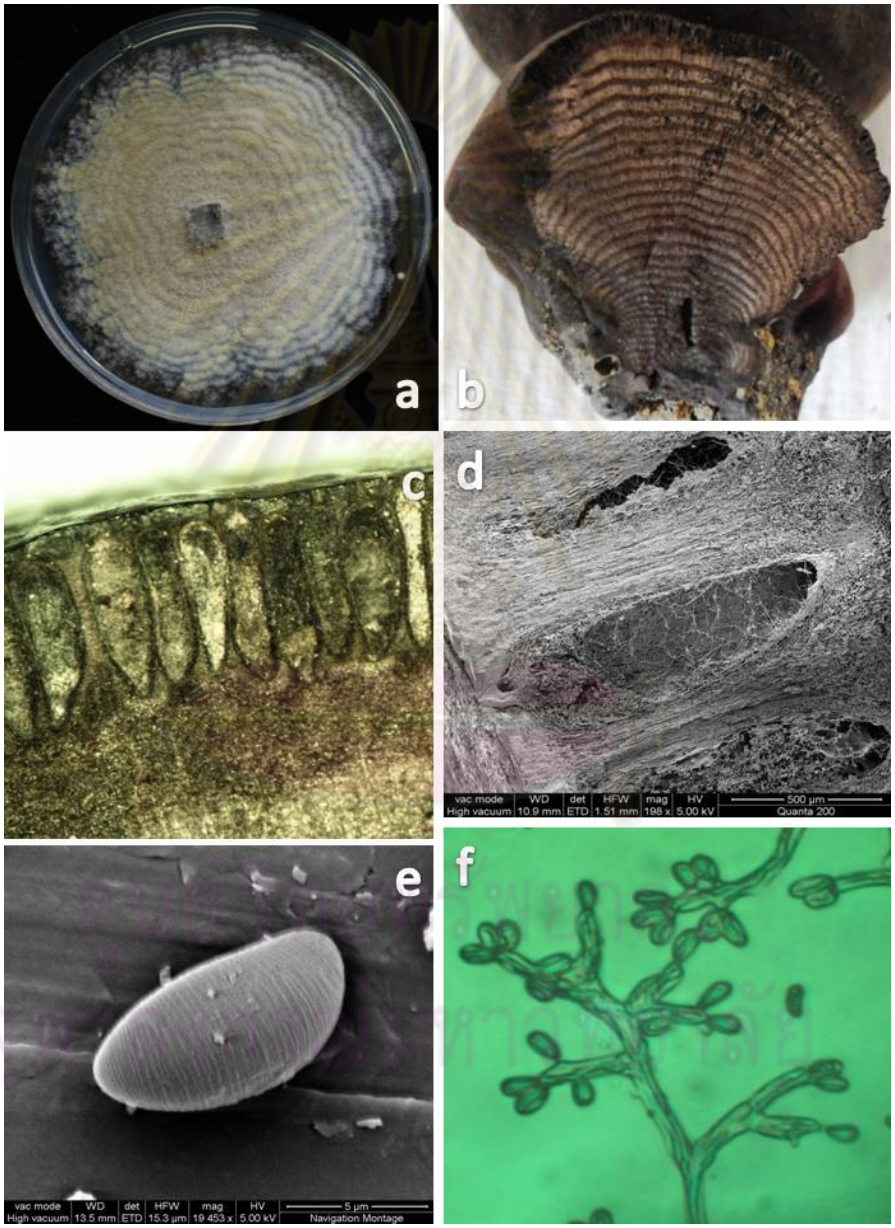


Figure 4.8 *Daldinia eschscholzii* (a) culture plate on MEA 7 days (b) stroma showing the concentric ring (c) perithecia on stereo microscope (d) perithecia on scanning electron microscope (e) ascospore on scanning electron microscope (f) Nodulisporium-like on light microscope

*Glomerella* sp. (Figure 4.9)

The telemorph stage of *Colletotrichum*. Colonies grayish white with sparse aerial mycelium and small dense felty patches, elsewhere reverse white to grey, conidial masses salmon pink: some cultures have abundant grayish white aerial mycelium with poor sporulation and no distinct acervuli (dark race). Perithecia were brown–black, superficial, solitary or in small groups, obpyriform to ovate or ampulliform, 200–520 × 110–320 μm. Asci were cylindrical, narrowing slightly at the apex, unitunicate, evanescent, 53–142 × 5–14 μm, and contained eight ascospores. Ascospores were hyaline, aseptate, oblong, 12–20 × 5–8 μm. Asci 8-spored and ascospores: multi-seriate, very pale grey, narrowly ellipsoid to slightly waisted, 17-23/5-7μm.

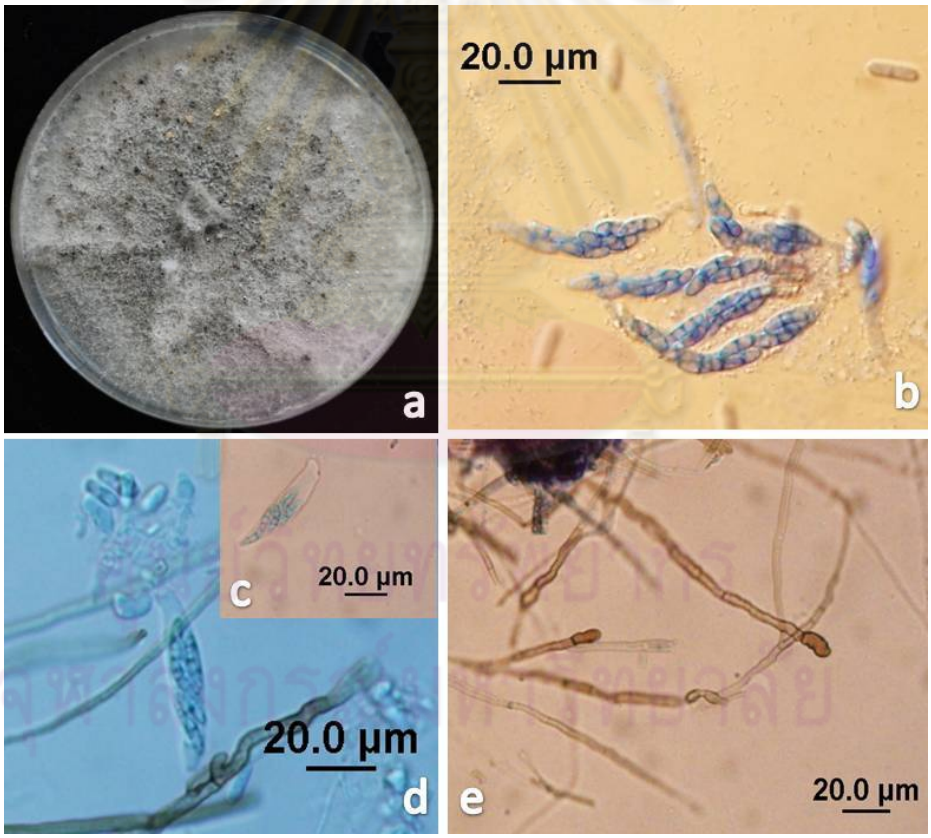


Figure 4.9 *Glomerella* (a) culture plate on MEA (5-7 days) (b) ascus showing spore arrangement (c-d) ascus contained ascospore (e) appressoria

***Pestalotiopsis* sp.** (Figure 4.10)

Mycelium immersed, branched, septate, hyaline, to pale brown. Conidiomata acervular, epidermal to subepidermal, separate or confluent, formed of brown, thin-walled textura angularis. Dehiscence irregular. Conidiophores hyaline, branched and septate at the base and above, cylindrical or lageniform, formed from upper cells of the pseudoparenchyma. Conidiogenous cells holoblastic, annellidic, indeterminate, integrated, cylindrical, hyaline, smooth, with several percurrent proliferations. Conidia fusiform, straight or slightly curved, 4 euseptate; basal cell hyaline, truncate, with an endogenous, cellular, simple or rarely branched appendage; apical cell conic, hyaline, with 2 or more apical, simple or branched, spathulate or espathulate appendages; median cells brown, sometimes versicoloured, thicker –walled, smooth or verruculose.

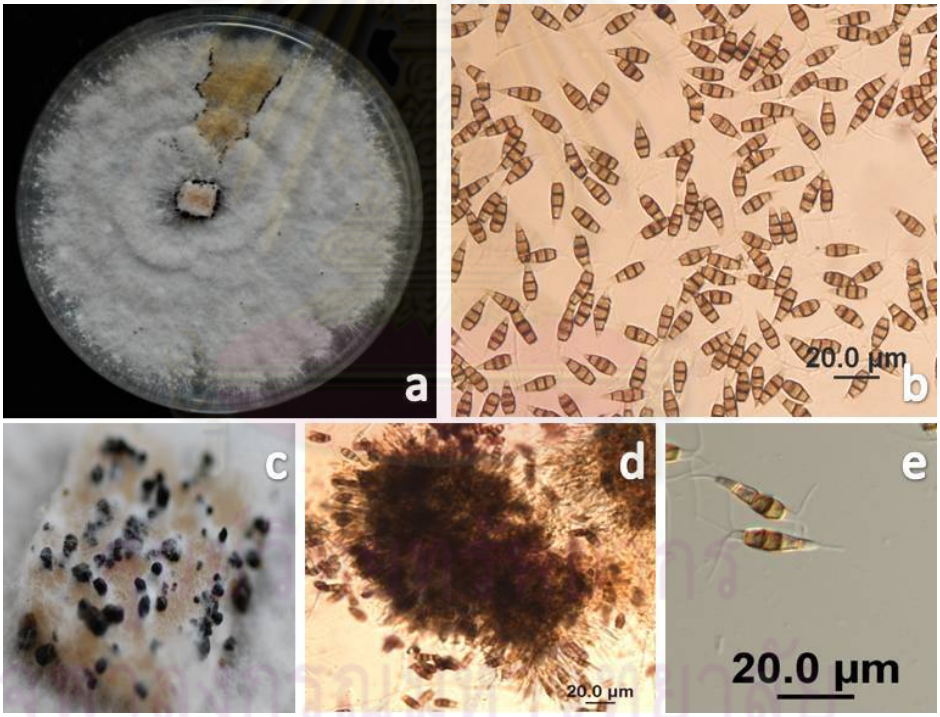


Figure 4.10 *Pestalotiopsis* sp. (a) culture plate on MEA (7-10 days) (b,e) *Pestalotiopsis* conidia showing apical, median, basal cells and appendages (c) black color of spore mass produced on mycelium (d) conidia produced on acervulus conidiomata

***Phomopsis* sp.** (Figure 4.11)

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic, immersed, brown to dark brown, separate or aggregated and confluent, globose , ampulliform or applanate , unilocular , multilocular or convoluted, thick-walled; walls of brown, thin-or thick-walled textura angularis, often somewhat darker in the upper region, lined by a layer of smaller-celled tissue. Ostiole single, or several in complex conidiomata, circular, often papillate. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequently multiseptate and filiform, hyaline,formed from the inner cells of the locular walls. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophores , collarette, channel and periclinal thickening minute. Conidia of two basic types, but in some species with intermediates between the two: $\alpha$-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end ) but sometimes with more guttules, aseptate; $\beta$-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate.

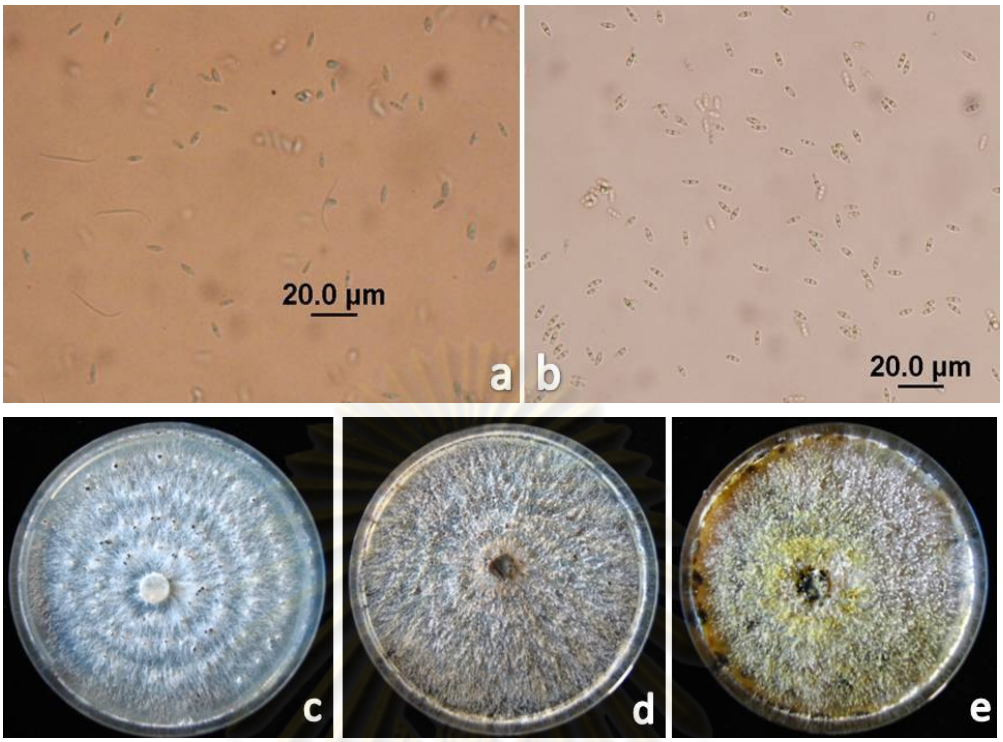


Figure 4.11 *Phomopsis* (a) ∝-conidia fusiform and β-conidia filiform (b) ∝-conidia fusiform (c) culture plate on MEA (10 days) of *Phomopsis* from *R. mucronata* (d) culture plate on MEA (10 days) of *Phomopsis* from *A. ilicifotius* (e) culture plate on MEA (10 days) of *Phomopsis* from *X. granatum*

***Phoma* sp.** (Figure 4.12)

Mycelium immersed, branched, septate, hyaline or pale brown. Conidiomata pycnidial, immersed, or semi-immersed, sometimes becoming erumpent, unilocular, brown, globose, separate or aggregated, occasionally confluent, thin-walled; walls of thin-walled, pale to medium brown textura angularis. Ostioles single or several to each pycnidium, central, not papillate. Conidiogenous cells enteroblastic, phialidic, integrated or discrete, ampulliform to doliiform, hyaline, smooth, collarette and aperture minute, periclinal wall markedly thickened. Conidia hyaline, aseptate or occasionally 1 septate, thin-walled, often guttulate,

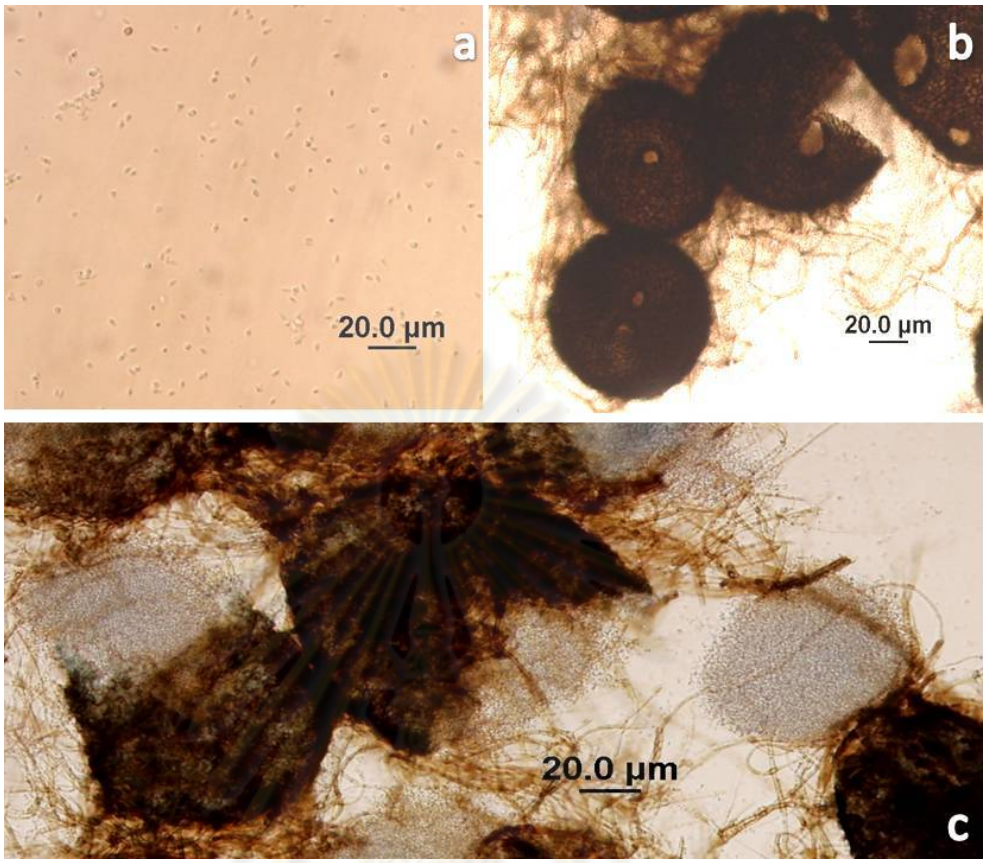


Figure 4.12 *Phoma* (a) conidia (b) pycnidia (c) pycnidia and conidia

***Phyllosticta* sp.** (Figure 4.13)

Colonies on MEA flat, regular, with entire edge; surface leaden-grey in centre, lavender-grey at margin, and leaden-black underneath. Pycnidia immersed to erumpent, globose, subglobose to ellipsoidal. Exuding spore-masses varied per culture me-dium, being colourless and glossy. *Pycnidia* 120–240 × 125–225 µm; pycnidial wall consisting of several layers, 25–70 µm thick; outer wall of pale brown to brown, thickened cells of *textura angularis* to *globularis*; inner wall consisting of one to two pale brown cell layers, that become hyaline toward interior, *textura angularis*. *Ostiole* single, central, 7–8 µm wide, 30–32 µm deep, appearing cylindrical in sec-tion, consisting of thickened, dark-brown cells. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells or branched from a supporting basal cell, 7–25 × 3–6 µm. *Conidiogenous cells* terminal, subcylindrical

to ampulliform or somewhat doliiform, hyaline, smooth, coated in a thin mucoid layer, inconspicuously proliferating once or twice percurrently near apex, 7–17 × 3–5 μm. *Conidia* (10–)12–14(–16) × (5–)6–7(–8) μm, solitary, hyaline, aseptate, thin- and smooth-walled, coarsely guttulate, ellipsoidal to obovoid, tapering toward a narrowly truncate base, enclosed in a thin mucilaginous sheath, μ1 m thick, and bearing a hyaline, mucoid apical appendage, 7–10(–14) × 1–2 μm, straight to flexible, unbranched, tapering towards an acutely rounded tip.

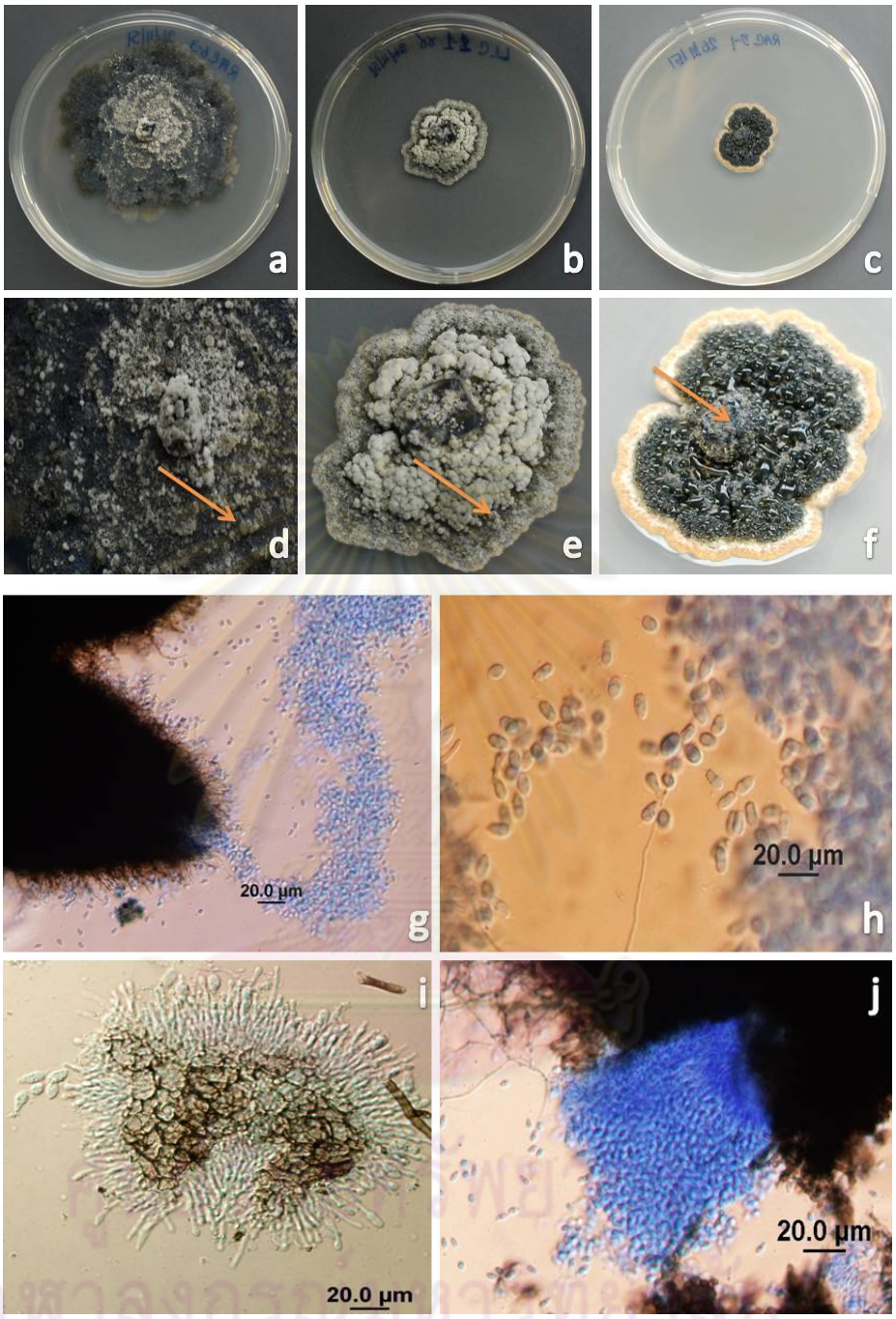


Figure 4.13 *Phyllosticta* (a) culture plate on MEA (10 days) of *Phyllosticta* from *R. mucronata* (b) culture plate on MEA (10 days) of *Phyllosticta* from *L. littorea* (c) culture plate on MEA (10 days) of *Phyllosticta* from *R. apiculata* (d-f) spore produced on mycelium (g) spore (h) spore under light microscope (i) spore produced on conidispore (j) pycnidia contained spore

## 4.6 Identification of endophytic fungi by molecular data and phylogenetic analysis

One hundred and sixty eight endophytic isolates were successfully amplified from the total of 191 endophytic fungal isolates by using primer ITS3 and ITS4 and then were grouped based on ITS region size. From each representative was selected for DNA sequence analysis using the whole ITS region, including ITS1, 5.8S, and ITS2 and the result as shown in the Table 4.19. In addition to the morphological identification, phylogenetic tree were carried out to confirmed with representative sequence. The phylogenetic trees were obtained neighbour joining methods. A NJ tree, containing the representative sequences indicated that the samples contained diverse fungi (Figure 4.14). Of the 30 sequences, 28 sequences related to Ascomycota clade and two sequences belonging to Basidiomycota clade. Further the 28 sequences derived from Ascomycota clade, together with *Guignardia vaccinii*, two species of *Cladosporium*, *Colletotrichum gloeosporioides, Glomerella cingulata, Pestalotiopsis disseminata*, *Diaporthe phaseolorum*, *Hypoxylon anthochroum*, *Phomopsis* sp. and *Xylariaceae* sp.. The 2 sequences derived from the Basidiomycota clade, represented *Schizophyllum commune* and *Marasmius cladophyllus.* *Cladosporium* sp. P2 isolated from *T. populneoides* was placed in the clade of *Cladosporium cladosporioides* wheras *Cladosporium* sp. P1 isolated from *T. populneoides* and *Cladosporium* sp. P1, a fungal endophyte of *R. apiculata* were placed in the clade of *Cladosporium oxysporum.* While *Xylaria* sp. C1 and *Xylaria* sp. R1 were 97% identical to the region of Fungal sp., but the result of NJ analyses, they were found to be most closely related to *Xylariceae* sp. (Figure 4.14). Sterile mycelium R1 were grouped with *Schizophyllum commune* accessions in order Agaricales with 100% bootstrap support. Sterile mycelium C1 was found to be most closely related to *Maramius cladophyllus*. From the phylogenetic tree, *Daldinia eschscholzii* separates from *Pestalotiopsis* sp. with 100% bootstrap support even though they were in the same Order. Species of *Phomopsis* and *Colletotrichum* were divided with each clade by 100% bootstrap support. For Unknown species C1

was 99% identical to the region of *Hypoxylon anthochroum*. The phylogenetic tree indicated that it should be grouped in the *Hypoxylon* and *Nodulisporium* clade.

Table 4.19 Sequence affinity of endophytic fungi mycelium and corresponding terminal fragment lengths for the ITS-2 region

| Group | ITS3-4 size (bp) | Number of sample | Suquence affinity and accession number (BLAST) | | | | Plant species | Sampling site | Endophytic taxa |
|---|---|---|---|---|---|---|---|---|---|
| | | | Accession no. | GeneBank closest species match | Overlap (bp) | Identity (%) | | | |
| 1 | 336 | 7 | AF39372Q2 | *Cladosporium oxysporum* | 587/589 | 99 | *T. populneoides* | Prachuap Khiri Khan | *Cladosporium* sp. P1 |
| 2 | 337 | 8 | EU497957 | *Cladosporium cladosporioides* | 572/574 | 99 | *T. populneoides* | Prachuap Khiri Khan | *Cladosporium* sp. P2 |
| | | | | | | | *R. apiculata* | Prachuap Khiri Khan | *Cladosporium* sp. P1 |
| 3 | 342 | 10 | GQ120498 | *Colletotrichum gleosporioides* | 397/432 | 91 | *R. apiculata* | Ranong | *Colletotrichum* sp. R1 |
| | | | | | | | *A. ilicifotius* | Ranong | *Colletotrichum* sp. R1 |
| 4 | 343 | 15 | FJ612855 | Fungal sp. | 571/584 | 97 | *A. ilicifotius* | Ranong | *Xylaria* sp. R1 |
| | | | | | | | *S. alba* | Chanthaburi | *Xylaria* sp. C1 |
| 5 | 344 | 15 | DQ062671 | *Glomerella cingulata* | 610/613 | 99 | *A. ilicifotius* | Ranong | *Glomerella* sp. R1 |
| | | | AF001025 | *Phomopsis phaseolorum* | 596/601 | 99 | *R. apiculata* | Ranong | *Phomopsis* sp. R1 |
| | | | AF001025 | *Diaporthe phaseolorum* | 592/601 | 98 | *X. granatum* | Ranong | *Phomopsis* sp. R3 |
| 6 | 345 | 9 | DQ201126 | *Hypoxylon anthochroum* | 491/497 | 98 | *R. mucronata* | Chanthaburi | Unknown species C1 |
| | | | U97658 | *Phomopsis* sp. | 596/605 | 98 | *R. mucronata* | Ranong | *Phomopsis* sp. R2 |

Table 4.19 (Continued) Sequence affinity of endophytic fungi mycelium and corresponding terminal fragment lengths for the ITS-2 region

| Group | ITS3-4 (bp) | Number of sample | Suquence affinity and accession number (BLAST) | | | | Plant species | Sampling site | Endophytic taxa |
|---|---|---|---|---|---|---|---|---|---|
| | | | Accession no. | GeneBank closest species match | Overlap (bp) | Identity (%) | | | |
| 7 | 349 | 53 | FJ624265 | *Daldinia eschscholizii* | 563/564 | 99 | *R. mucronata* | Chanthaburi | *Daldinia eschscholizii* |
| | | | EU167584 | *Guignardia vaccinii* | 672/674 | 99 | *L. littorea* | Chanthaburi | *Phyllosticta* sp. C1 |
| | | | | | | | *S. alba* | Chanthaburi | *Phyllosticta* sp. C1 |
| | | | | | | | *R. apiculata* | Chanthaburi | *Phyllosticta* sp. C1 |
| | | | | | | | *A.alba* | Chanthaburi | *Phyllosticta* sp. C1 |
| | | | | | | | *C. decandra* | Prachuap Khiri Khan | *Phyllosticta* sp. P1 |
| | | | | | | | *T. populneoides* | Prachuap Khiri Khan | *Phyllosticta* sp. P1 |
| | | | | | | | *A. ilicifotius* | Ranong | *Phyllosticta* sp. R1 |
| | | | | | | | *R. mucronata* | Ranong | *Phyllosticta* sp. R1 |
| | | | | | | | *R. apiculata* | Ranong | *Phyllosticta* sp. R1 |
| | | | | | | | *X. granatum* | Ranong | *Phyllosticta* sp. R1 |
| | | | | | | | *X. mucronata* | Ranong | *Phyllosticta* sp. R1 |
| 8 | 351 | 20 | AB251918 | *Pestalotiopsis disseminata* | 619/622 | 99 | *C. decandra* | Prachuap Khiri Khan | *Pestalotiopsis* sp. P1 |
| | | | | | | | *R. apiculata* | | |
| 9 | 352 | 8 | AF455536 | *Eurotium amstelodami* | 578/578 | 100 | *A.alba* | Chanthaburi | *Aspergillus* sp. C1 |
| 10 | 382 | 1 | AY216475 | *Marasmius cladophyllus* | 581/641 | 90 | *A.alba* | Chanthaburi | Sterile mycelium C1 |
| 11 | 427 | 9 | EF155505 | *Schizophyllum commune* | 672/674 | 99 | *X. granatum* | Ranong | Sterile mycelium R1 |

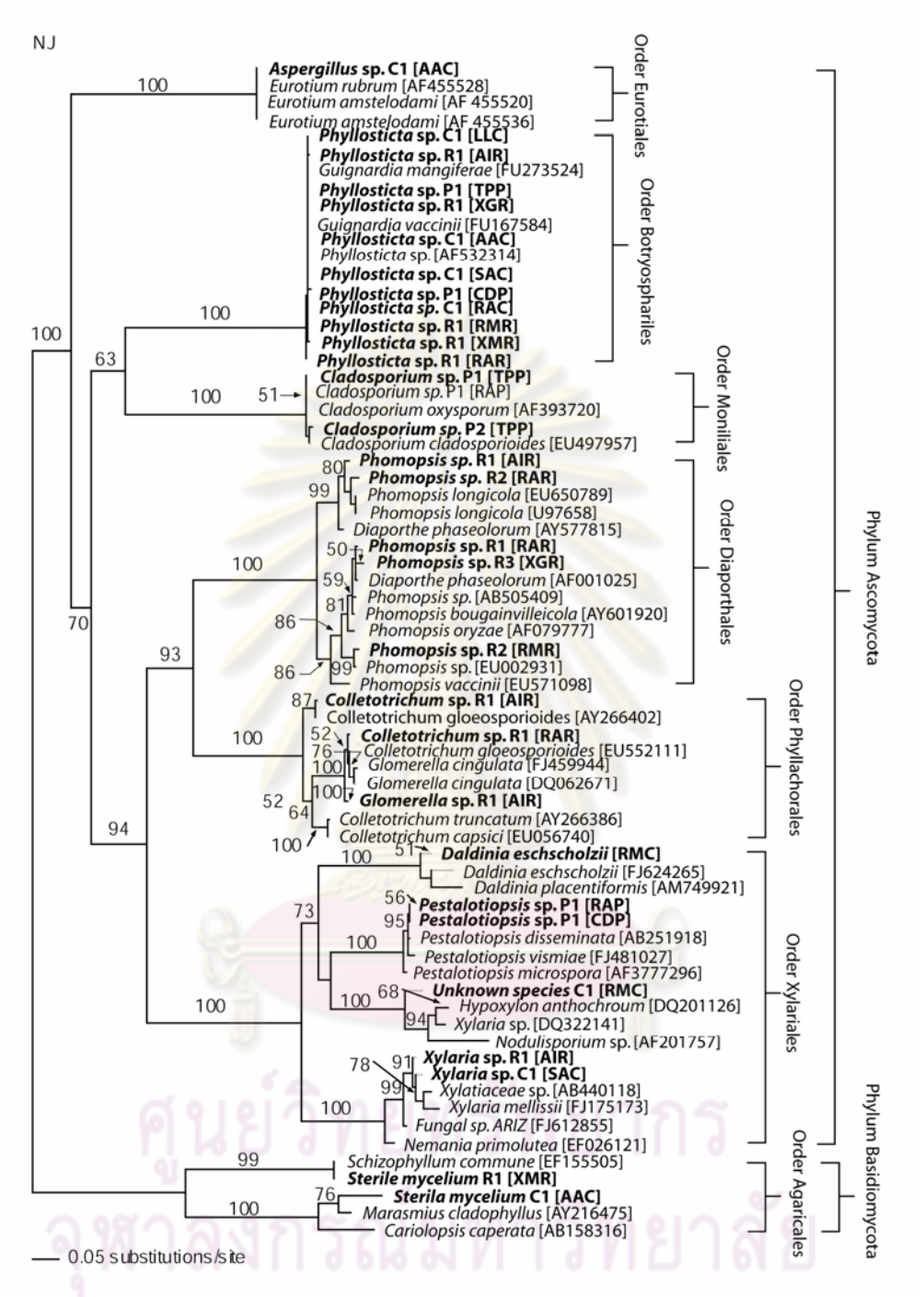


Figure 4.14 Neighbour-joining tree obtained from analysis of the rDNA ITS showing the relationships of the 30 representative sequences (bold) with reference sequences. Bootstrap values (n = 1000 replicates) are reported as percentages. The scale bar represents the number of changes per nucleotide position.

## 4.7 Antimicrobial testing

Evaluation of antimicrobial activity, crude extracts from fermentation brothes of 71 endophytic fungal isolates were tested against a test panel of two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus )* and two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) for antimicrobial activity, as an indication of their capability to produce secondary metabolites of potential therapeutic interest. There were 19 fungal strains which showed antimicrobial activity against at least one of the test strains (Table 4.19). Most of the antimicrobial activities were specific for Gram-positive bacteria. Only 3 fungal strains were found to produce inhibitors against the Gram-negative bacteria selected. Almost all of the fungal strains that exhibited any antimicrobial activity inhibited the growth of *B. subtilis*. There was *Xylaria* sp. R1 isolated from *X. granatum* inhibited only *E. coli*, while *Colletotrichum* sp. P2 isolated from *T. populneoides* was only effective against *S. aureus*. The crude extract of *Cladosporium* sp. P1 isolated from *C. decandra* and unknown species isolated from *R. mucronata* inhibited only Gram-positive bacteria and two species of yeast (*Candida albicans* and *Saccharomyces cerevisiae*). *Xylaria* sp. R1 which was isolated from *A. ilicifotius*$^{R}$ showed the best effect on *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*., with growth inhibition of 43.0, 34.5, 30.6 and 20.6 mm, respectively. All test bacteria and yeasts were also inhibited by a crude extract of *Cladosporium* sp. P1 isolated from *T. populneoides*$^{P}$ and showed the highest growth inhibition zone against *E. coli*, *P. aeruginosa*, *B. subtilis* ,*S. aureus*, *C. albicans* and *S. cerevisiae* with growth inhibition of 36.3, 32.6, 26.3, 14.6, 13.0 and 12.0 mm, respectively.

Table 4.20 Anitmicrobial activity of crude ethyl acetate extracts derived from endophytic fungi (diameter of zone of inhibition in mm; n = 3, mean ±SD). (Test microorganisms: Bacteria - Pa, *Pseudomonas aeruginosa* ATCC 27853; Sa, *Staphylococcus aureus* ATCC 25923 ; Ba, *Bacillus subtilis* ATCC 6633; Ec, *Escherichia coli* ATCC 25922; Sc, *Saccharomyces cerevisiae* ATCC 5169; Ca, *Candida albicans* ATCC 10231) (Sampling site: [R], Ranong Province; [C], Chanthaburi Province; [P], Prachuap Khiri Khan Province).

| Endophytes species | Host plant species | Test microorganisms | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sa | Ba | Ec | Pa | Sc | Ca |
| *Xylaria* sp. R1 | *A. ilicifotius*[R] | 20.6±1.1 | 30.6±1.1 | 43.0±1.4 | 34.5±0.7 | 0 | 0 |
| *Phomopsis* sp. R1 | *A. ilicifotius*[R] | 0 | 10.0±0.0 | 0 | 0 | 0 | 0 |
| *Phomopsis* sp. R2 | *A. ilicifotius*[R] | 0 | 11.3±1.5 | 0 | 0 | 0 | 0 |
| *Colletotrichum* sp.R1 | *A. ilicifotius*[R] | 0 | 10.6±0.6 | 0 | 0 | 0 | 0 |
| *Glomerella* sp.R1 | *A. ilicifotius*[R] | 0 | 11.3±0.7 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. R1 | *R. apiculata*[R] | 0 | 12.7±1.5 | 0 | 0 | 0 | 0 |
| *Pestalotiopsis* sp. R1 | *R. apiculata*[R] | 0 | 10.3±0.7 | 0 | 0 | 0 | 0 |
| *Colletotrichum* sp.R1 | *R. apiculata*[R] | 0 | 12.0±0.0 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. R1 | *R. mucronata*[R] | 0 | 20.0±0.0 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. R1 | *X. moluccensis*[R] | 0 | 20.0±0.0 | 0 | 0 | 0 | 0 |
| *Xylaria* sp. R1 | *X. granatum*[R] | 0 | 0 | 37.5±0.7 | 0 | 0 | 0 |
| *Xylaria* sp. C1 | *A. alba*[C] | 0 | 10.7±0.7 | 0 | 0 | 0 | 0 |
| *Daldinia eschscholzii* | *A. alba*[C] | 0 | 10.3±0.7 | 0 | 0 | 0 | 0 |
| *Colletotrichum* sp.C1 | *A. alba*[C] | 0 | 10.0±0.0 | 0 | 0 | 0 | 0 |
| Unknown species C1 | *R. mucronata*[C] | 0 | 18.0±1.7 | 0 | 0 | 14.0±0.0 | 15.0±0.0 |
| *Daldinia eschscholzii* | *R. apiculata*[C] | 0 | 12.0±0.0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp.P1 | *T. populneoides*[P] | 14.6±0.6 | 26.3±0.6 | 36.3±1.1 | 32.6±0.6 | 12.0±0.0 | 13.0±0.0 |
| *Colletotrichum* sp.P2 | *T. populneoides*[P] | 13.0±1.0 | 0 | 0 | 0 | 0 | 0 |
| *Cladosporium* sp. P1 | *C. decandra*[P] | 15.3±0.6 | 15.0±0.0 | 0 | 0 | 10.0±0.0 | 10.0±0.0 |

## 4.8 Cytotoxicity testing

A total of 84 ethyl acetate extracts from the endophytic fungi were tested for anticancer activities by the MTT assay against A375 (Human malignant melanoma), SW620 (Human colorectal adenocarcinoma), Kato III (Human gastric carcinoma), HepG2 (Human liver hepatoblastoma) and Jurkat (Human acute T cell leukemia). There were 26 endophytic fungal isolates which could produce some compounds which displayed cytotoxicity against some cancer cell lines, which demonstrated that endophytic fungi from mangrove plants are a reliable source for natural anticancer active compounds. (Table 4.20). Most of crude extracts inhibited Jurket, SW 620 and Kato III cancer cell with cell viability below 40%. There were 2 endophytic isolates which showed broad-spectrum inhibition to all cancer cell lines such as *Pestalotiopsis* sp. C1 from *R. apiculata*$^{C}$ and *Cladosporium* sp. P1 from *T. populneoides*$^{P}$. There were seven isolates which acted as specific anticancer activities to Jurkat cells with cell viability below 40%. *Phomopsis* sp. R2 (9.82%) was isolated from *A. ilicifotius*$^{R}$ displayed specific inhibition with Human colorectal adenocarcinoma (SW620). Moreover, It was found that some strains associated with different host plants such as *Xylaria* sp. R1 and *Cladosporium* sp. P1 displayed different anticancer activities. In this study, it was found that the crude extract of *Cladosporium* sp. P1 isolated from both *T. populneoides*$^{P}$ and *C. decandra*$^{P}$ showed the high anticancer potential.

Table 4.21 Anitcancer activity of crude ethyl acetate extracts derived from endophytic fungi (human cancer cell lines including malignant melanoma A375 (ATCC no.: CRL-1619), colorectal adenocarcinoma SW620 (ATCC no.: CCL-227), gastric carcinoma Kato III (ATCC no.: HTB-103), liver hepatoblastoma HepG2 (ATCC no.: HB-8065) and Acute T cell leukemia Jurkat (ATCC no.: CRL-2063). Sampling site: [R], Ranong Province; [C], Chanthaburi Province; [P], Prachuap Khiri Khan Province).

| Endophytes species | Host Plant species | Percentage of cell viability (%) | | | | |
|---|---|---|---|---|---|---|
| | | A375 | HepG2 | SW620 | Jurkat | KatoIII |
| *Xylaria* sp. R1 | *A. ilicifotius*[R] | 81.63 | 72.10 | 71.06 | 20.21 | 71.30 |
| *Phomopsis* sp. R1 | *A. ilicifotius*[R] | 83.61 | 84.61 | 76.49 | 29.09 | 39.12 |
| *Phomopsis* sp. R2 | *A. ilicifotius*[R] | 87.90 | 80.95 | 9.82 | 92.18 | 79.89 |
| *Colletotrichum* sp. R1 | *A. ilicifotius*[R] | 22.04 | 45.37 | 0.57 | 44.82 | 8.24 |
| *Glomerella* sp. R1 | *A. ilicifotius*[R] | 85.41 | 74.23 | 18.27 | 93.10 | 29.00 |
| *Pestalotiopsis* sp.R1 | *A. ilicifotius*[R] | 60.76 | 98.27 | 39.36 | 78.44 | 24.73 |
| *Xylaria* sp.R1 | *R. apiculata*[R] | 20.13 | 41.23 | 11.78 | 44.48 | 28.12 |
| *Cladosporium* sp.R1 | *R. apiculata*[R] | 66.00 | 85.72 | 70.52 | 25.88 | 67.58 |
| *Pestalotiopsis* sp.P1 | *R. apiculata*[R] | 69.33 | 49.08 | 67.52 | 29.20 | 64.06 |
| *Xylaria* sp.R1 | *X. moluccensis*[R] | 50.68 | 80.15 | 97.24 | 24.50 | 24.52 |
| *Phomopsis* sp. R1 | *X. moluccensis*[R] | 74.05 | 95.89 | 95.00 | 32.25 | 55.11 |
| *Pestalotiopsis* sp. R1 | *X. moluccensis*[R] | 98.08 | 96.54 | 76.25 | 36.90 | 81.46 |
| *Xylaria* sp. R1 | *X. granatum*[R] | 97.95 | 72.46 | 60.21 | 28.79 | 27.73 |
| *Xylaria* sp. C1 | *A. alba*[C] | 85.40 | 57.37 | 62.94 | 37.52 | 65.10 |
| *Pestalotiopsis* sp. C1 | *R. apiculata*[C] | 2.00 | 2.75 | 0.28 | 0 | 3.26 |
| *Phyllosticta* sp. C1 | *R. apiculata*[C] | 10.76 | 39.18 | 14.36 | 89.09 | 13.09 |
| *Daldinia eschscholzii* | *R. apiculata*[C] | 53.29 | 83.48 | 2.01 | 38.10 | 59.10 |
| *Phomopsis* sp. C1 | *R. apiculata*[C] | 54.34 | 83.28 | 40.72 | 15.00 | 23.37 |
| *Phomopsis* sp.C1 | *L. littorea*[C] | 64.06 | 75.08 | 36.35 | 76.89 | 35.40 |
| *Cladosporium* sp. P1 | *T. populneoides*[P] | 0 | 9.32 | 6.46 | 0 | 2.90 |
| *Colletotrichum* sp. P1 | *T. populneoides*[P] | 44.96 | 85.59 | 30.31 | 26.55 | 36.56 |
| *Phoma* sp. | *T. populneoides*[P] | 69.79 | 76.98 | 99.09 | 30.00 | 93.84 |
| *Cladosporium* sp. P1 | *C. decandra*[P] | 1.21 | 26.03 | 10.68 | 64.93 | 18.09 |

# CHAPTER 5
# DISCUSSION

## 5.1 Diversity of endophytic fungi in mangrove plants

The leaves of all woody tree hosts and a shrub mangrove host harboured fungal endophytes. The highest colonization frequency was found in *A. ilicifotius* leaves (84%) from the Ranong site. A possible explanation for it could be related to the position of their leaves close to soil because this plant is a shrub and about 1 meter high above ground level allowing maximum capture of any fungal spores in their immediate environment thus increasing the potential for penetration and colonization by endophytes. Moreover this plant is sometimes submerged in mangrove water due to monsoonal and tidal influences and is therefore always exposed to high humidity compatible with high fungal colonization. Saikkonen (2007) found that rain splashes bring propagules from the fallen previous years leaves in soil and vegetation to the lower part of the tree and colonization success is higher in shade and humid closed canopy compared to sun leaves of the outermost branches. *Ceriops decandra* from the Prachuap Khiri Khan site was found to have the lowest colonization frequency (9%) which might be attributed to the occurrence of this plant in open areas with an open canopy usually exposed to sun light all day. Thus the drier growth conditions in which the tissue is characteristically dry and hard, would appear to be significant in explaining the lower colonization frequency in this plant species. Rodrigues (1994) suggested the lower number of isolates recovered could be related to the effects of water stress. It is known that under water deficit some plants may accumulate non-structural carbohydrates. This accumulation generally leads to a build up of carbon-based defenses such as tannins, making the plant less susceptible to fungal endophyte colonization during the dry environment.

The endophyte assemblages from the same plant species growing in different locations may be quite different. A number of factors might cause this phenomenon including vegetation in the vicinity and environmental conditions such as temperature, rainfall and humidity. There can also be effects on the mycoflora resulting from human activities (Mekkamol, 1998). Infection frequency of endophytes are known to vary with altitude, humidity, density of canopy, precipitation and host susceptibility (Petrini & Carroll 1981). Climatic conditions may greatly influence the colonization of plants by endophytic fungi (Petrini, 1991). From observation of the geographical environment of the study areas, it was found that the Ranong forest is the oldest complete natural forest with high density of closed canopy. *Rhizophora apiculata* was selected as the single species for comparison of colonization frequency in the three experimental sites. It was found that the percentage of fungal endophytic assemblages from Ranong Province was the highest (69.4%) followed by Chanthaburi Province (45.4%) and Prachuap Khiri Khan Province (23%) respectively. It is therefore not surprising that endophytic fungi from the Ranong site had a higher colonization frequency because Ranong is the wettest province in Thailand and has heavy rainfall in all months of year where is surrounded by mountain and the sampling site is located on the lowest slope of the mountain. The sampling period was during the rainy season and it was also raining on the day that the samples were collected. Thus the high number of isolates obtained at the Ranong site probably is a reflection on environmental factors such as rainfall and atmospheric humidity. This is in agreement with the findings of other studies where it was found that wet conditions were favourable for fungal sporulation and infection and this caused endophyte infections to increase during the wet season (Wilson, 2000). Global studies a fungal endophyte abundance and diversity appear to be higher during the wet season compared to the dry season (Rodrigues, 1994; Chareprasert *et al*., 2006) and high on the rainy slopes of mountains (Carroll and Carroll, 1978) with increase with increasing annual precipitation (Helander *et al*., 1998). The colonization frequency of endophytic fungi in *R. mucronata* from Ranong Province also occurred at a greater frequency than at the Chanthaburi sites. Furthermore, the size of *Rhizophora apiuclata* leaf was larger than those at the Chanthaburi site and at the Prachuap Khiri Khan site.

This is likely, therefore, to be another contributing factor for the higher rate of colonization of *Rhizophora* in Ranong Province. In explaining the much higher recovery of endophytic fungi, Rodrigues (1994) suggested that the expanded palm leaves represent a better physical trap for spores than folded leaves.

It was found that *Lumnitzera littorea* had the highest CF% in the studied plant communites in Chanthaburi Province even though its leaves were the smallest of all of the mangrove species studied. The results indicated that the CF% of *Lumnitzera* leaves (79.9%) was close to the CF% of other mangrove plants from Ranong although the climatic conditions between these two sites is very different. The leaves of this plant are succulent leaves which is favourable for endophytic colonization. The CF% of *Avicennia alba* growing at the same area of the Chanthaburi site was lowest even though the size of this plants same as *Lumnitzera* leaves but this plant has very thin leaves with low water content which is not conducive to high colonization frequency. This strongly indicated that the characteristics of leaves is one of the important factors influencing colonization by fungal endophytes. It has been reported that salt excretion in leaves of some mangrove species may serve as an important defense against fungal attack. Gillbert *et al*. (2002) found that *Avicennia* leaves supported less superficial fungal growth and endophytic colonization than *Rhizophora* leaves. The effect of high leaf-surface salinity on spore germination could thus explain the restricted fungal colonization, low fungal diversity, and low levels of disease found in *Avicennia* leaves. With the lowest leaf salinity, *Rhizophora* consistently suffered more disease and supported greater fungal infection, fungal diversity, and hyphal growth. Salt concentration may be an important factor determining the outcome of plant-fungus encounters in mangrove forests. Spore germination of most mangrove-associated fungi was strongly inhibited by salt concentrations (Gilbert *et al*., 2002). Amir *et al*. (1996) found that mycelial growth of *Fusarium oxysporum* was not inhibited by salinities less than 2%, but that 3% salt reduced mycelial growth. In contrast, conidial spore germination was inhibited by as little as 0.5% salt. Gillbert *et al*. (2002) suggested that although mangrove species growing in close proximity are likely exposed to the same

density and composition of potential pathogens, disease development may be mediated by foliar salt concentrations. Variation in fungal colonization and disease development across species is not caused by differences in spore availability.

The endophyte assemblages of the different mangrove hosts in Thailand showed that several endophytic species were common to more than one host, indicating that these endophytes have a wide host range. The species diversity of the endophytes varied between the different mangrove hosts suggesting that a selection mechanism was operating in constituting the endophyte assemblages. Some endophytic genera exhibited no host specificity and were recovered from plants belonging to different groups and growing in different geographical locations; these include *Cladosporium* (Azevedo, *et al*, 2000) and Coelomycete genera such as *Pestalotiopsis, Phomopsis* (sexual stage: *Diaporthe*), *Phyllosticta* (sexual stage: *Guignardia*) and *Colletotrichum* (sexual stage : *Glomerella*) were often isolated as foliar endophytes from tropical plants (Aveskamp *et al*., 2008; Chareprasert *et al*. 2006; Fröhlich *et al*., 2000; Ganley *et al*., 2004; Lacap *et al*., 2003; Wipornpan *et al*., 2001;) and were also found in mangrove plants from India (Kumaresan & Suryanarayanan 2001; Suryanarayanan & Kumaresan 2000).

*Phyllosticata* was found to be the dominant genus in all mangrove plants from the Chanthaburi site and also in two plant species from the Prachuap Khiri Khan, *C. decandra* and *R. apiculata*. This is significant as tree endophytes are disseminated horizontally (Miller *et al*., 2009) and that the canopy of all the different host species in a community is exposed to the same fungal inoculum. For *T. populneoides* leaves collected from the Prachuap Khiri Khan site, the dominant fungal endophyte was species of *Phoma*. This may be because these trees are growing in an isolated location far from other mangrove plants that grow in cluster groups thus the inocula of fungal spores was different from the other plants.

The dominant fungal species of almost all mangrove hosts at the Ranong location (*R. apiculata, R. mucronata* and *X. granatum*) were different for each host plant. This phenomenon is similar to a report from Indian findings where it was found that the dominant endophytes were different for each host plant in the mangrove community (Kumaresan and Suryanarayanan, 2001). The dominant foliar endophytes of *C. decandra* and *A. ilicifotius* growing in mangrove forest of India were different from those reported here where the dominant species in *C. decandra was* sterile mycelium and Ascomycetes 2.3 was the dominant isolate in *A. ilicifotius* (Suryanarayan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001). The another report from India found the most dominant endophyte in roots of *A. ilicifotius* was *Colletotrichum* sp. (Maria & Sridhar 2003). Thus, It seem there is a selection mechanism which distributes the different (dominant) endophytes among the different host species in the same mangrove community. This trend is similar to that of coniferous hosts in the Pacifc northwest (Carroll & Carroll 1978). Petrini *et al*. (1982), studying the endophytes of evergreen shrubs in western Oregon, also noticed that one endophyte species of each host invariably comprised the largest portion of the endophyte assemblage and was relatively host specific.

*Phyllosticta* was also found as the dominant endophytic fungus in all mangrove plants in the three study sites and as the morphology of the mycelium and the size of spores were not very different the molecular techniques were used for sequencing and it was found that this genus is similar to *Guignardia vaccinii* (sexual state of the genus *Phyllosticta*), this was very surprising because all of these fungi were of the same species and they did not have genetic variation when considered with the results from the phylogenetic tree for all of mangrove plants. This species therefore had a constant genetic sequencing. From the phylogenetic tree, it were found all of the species of *Phyllosticta* were close to two species of *Guignardia* viz. *Guignardia vaccinii* and *Guignardia mangiferae* which are non pathogenic species, have a broad host range and occur on many woody species (Renato *et al*., 2006; Sieber, 2007).

García-López *et al.* (1989) reported symptoms associated with *Pestalotiopsis disseminata* as the most common causes of leaf lesion in *Rhizophora mangle* in Cuba. Leaves of *Rhizophora* commonly suffered leaf necrosis surrounded by these symptoms were commonly associated with *Pestalotiopsis* spp., and sometimes with species of *Colletotrichum* (Gilbert *et al*., 2002). It was found that the species of *Pestalotiopsis* was identical with the *Pestalotiopsis disseminata* as an endophytic fungus in *R. apiculata and R. mucronata.* The results from this study are certainly supportive of this, although it is not apparent whether all strains isolated as endophytes are capable of producing disease. *Pestalotiopsis* spp. were also common endophytic fungi in many species of tropical plants (Suryanarayanan *et al*., 2005; Tejesvi *et al*., 2006; Hu *et al*., 2007). This fungal genus is ubiquitous in distribution, occurring on a wide range of substrata. Many are saprobes, pathogenic and endophytic on living plant leaves and twigs (Bissett, 1982; Brown *et al*., 1998; Hyde and Fröhlich, 1995; Karaca and Erper, 2001; Rivera and Wright, 2000; Taylor *et al*., 2001; Tuset *et al*., 1999). *Pestalotiopsis* is a complex genus and consists of members difficult to classify at the species level. At present, inter-specific delineation of this genus is based on morphology of the conidia. Some of these *Pestalotiopsis* species have gained much attention in recent years as they have been found to produce many important secondary metabolites (Li *et al*., 2001; Li and Strobel, 2001). Jeewoon *et al*. (2003) found molecular data indicated that the genus contains two distinct lineages based on pigmentation of median cells and four distinct groupings based on morphology of apical appendages.

*Xylaria* species appeared to be the most common endophytic fungi of mangrove trees. They were found in almost all mangrove plants except *L. littorea*, *C. decandra* and *T. populneoides*. Xylaria was not found as endophytic fungi in mangrove plants from India (Kumaresan & Suryanarayanan 2001; Suryanarayanan & Kumaresan 2000). We found endophytic *Hypoxylon* species in *R. mucronata* collected from the Chanthaburi site. Also this fungus, was not reported from Indian studies. There has been a report of *Hypoxylon* as a mangrove fungus of *R. apiculata* and *Avicennia* spp. in India (Venkateswara Sarma *et al*., 2001). The current study found *Daldinia eschscholzii*

as an endophytic fungus in some mangrove plants (*A. alba* and *R. apiculata*) in the Chanthaburi site and in almost all plants in the Ranong site. A large number of specimens of *Daldinia* were formed (Chapter 4 (Figure 4b-f)) on the stem of an *Avicennia* tree following the death of *Avicennia* tree. *Daldinia eschscholzii* which is a wood-decaying fungus is common in tropical forests and is also found an endophytic in tropical plants (Whalley, 1996). So endophytic xylariaceous fungi have been isolated most frequently from plants in tropical regions (Dreyfuss & Petrini 1984, Rodrigues & Samuels 1990, Pereira, Azevedo & Petrini 1993, Rodrigues 1994, Fisher et al. 1995, Lodge, Fisher & Sutton 1996, Rodrigues & Petrini 1997, Frohlich *et al*., 2000, Guo *et al*., 2000, Photita *et al*. 2001).

Saprophytic fungi are fundamental to many aspects of decomposition and energy flow in mangrove forests (Newell 1996). Hyde (1991) observed that the ascomycetes were the most common taxonomic group in the intertidal mangrove region, which probably plays an important role in the turnover of mangrove wood. Sabada *et al.* (1995) reported 44 fungi associated with standing senescent *Acanthus ilicifolius* from Mai Po mangrove, Hong Kong (32 Deuteromycotina, 11 Ascomycotina and 1 Basidiomycotina; very frequent species were *Acremonium* sp.,*Colletotrichum gloeosporioides* cf., *Phoma* sp., *Fusarium* sp., *Tubercularia* sp. and *Phialophora* sp. cf.) (Sadaba *et al*. 1995). Alias et al. (1995) reported 169 species of saprophytic fungi of mangrove from Malaysia (most commonly occurring species: *Halocyphina villosa*, *Kallichroma tethys*, *Lulworthia grandispora*, *Hypoxylon oceanicum*, *Kactylospora haliotrepha*, *Verruculina enalia* and *Savoryella lignicola*). Some researchers (Zhou & Huang 2001) have reported an aspects parts of species and ecological characters of mangrove pathogenic fungi in Shankou, Qinzhou and the estuary of Beilun in Guangxi, China. Twenty-six species of mangrove pathogenic fungi, mainly such as *Colletortichum*, *Pestalotiopsis*, *Alternaria,* and *Phyllosticta* were identified. *Phomopsis mangrovei*, which is probably pathogenic, was described from dying prop roots of *Rhizophora apiculata* in Thailand (Hyde 1996). There were research we found that *Colletotrichum gloeosporioides, Phoma, Phomopsis, Pestalotiopsis , Phyllosticta,* a

species of *Hypoxylon* and two species of Basidiomycota (*Schizophyllum* and *Maramius* ) can live in mangrove plant tissue as an endophytic phase, where the endophyte can inhabit plant organs are at some time in their life colonize internal plant tissues without causing apparent harm to their host, they have a dormant or latent phase within plant tissue prior to causing disease symptoms on the plant host. An endophyte in one plant could be a pathogen of the other depending on the balance between pathogenicity and endophytism of the microorganism in the different hosts (Saikkonen *et al*., 2004). Schulz *et al*. (1999) therefore hypothesise that the host-endophyte interaction is a case of balanced antagonisms: pathogens overcome the host's defences to the extent that they cause visible damage, whereas endophytic virulence is only sufficient to be able to infect and colonise without causing visible damage. If the balance shifts, the endophyte may turn pathogenic.

## 5.2 Identification by using morphological characteristics and molecular technique

Fungal taxonomy is traditionally based on comparative morphological features (e.g., Lodge *et al*., 1996; Sette *et al*., 2006; Crous *et al*., 2007; Zhang *et al*., 2008). However, special caution should be taken when closely related or morphologically similar endophytes are identified, because the morphological characteristics of some fungi are medium dependent and cultural conditions can substantially affect vegetative and sexual compatibility (Zhang *et al*., 2006; Hyde and Soytong, 2007). Furthermore, the conventional methods cannot be applied for identifying fungal isolates that fail to sporulate in culture, which are categorized as mycelia sterilia (Lacap *et al*., 2003). Various optimization of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures (Guo *et al*., 2000). Nevertheless, a large number of fungi still do not sporulate in culture, and these mycelia sterilia are considerably frequent in endophyte studies (Lacap *et al*., 2003). This research also

used different media for identification but some culture still remained sterile mycelium and there were therefore found problems for identification of some fungi. Although it able to produce spores in culture medium for identification, especially the group *of Colletotrichum, Phyllosticta, Phomopsis and Pestalotopsis* these are difficult to distinguish from one another due to their similarity in morphological characteristics (Shenoy *et al*., 2007) because the morphological features changed every time when they were subcultured to a fresh medium.

Therefore molecular analyses were used to identify the fungal endophytes isolated from mangrove plant hosts and to investigated their phylogenetic relationships. Thirty of the representative morphological isolates were further identified with molecular phylogenetic analysis of ITS1-5.8S-ITS2 sequences. There was a good agreement between morphological and ITS-sequence based approaches. This research found some problems when used only ITS2 region size because the different genus of some endophytic fungi had the same size and also some endophytic fungal species had the same ITS2 region size then It should be use the other molecular tool such as RFLP and T-RFLP for solve this problem.

*Colletotrichum, Pestalotiopsis, Phomopsis*, and *Xylaria* were the most frequent endophytes either within or among the hosts,which is consistent with the findings reported in other studies of tropical endophytic fungi (Arnold *et al*., 2000, 2001; Wipornpan *et al*., 2001; Rodrigues *et al*., 2005). *Phomopsis* species are considered to be the asexual phases of *Diaporthe* species, a teleomorphic genus in the family Valsaceae (Girlanda *et al*., 2002). It found that two representative of *Phomopsis* sp. R1 and *Phomopsis* R3 were similar to *Diaporthe phaseolorum* when from examinion of the aphylogenetic tree.

Phylogenetic affinities of isolates based on ITS data are useful for assessing the accuracy and value of BLAST identifications of ITS sequences, which may be limited by misidentified, unidentified, and unrepresented fungi in GenBank. At present,

phylogenetic analysis of DNA sequences remains a more effective method for determining taxonomic placement of sterile endophytes. Phylogenetic analyses also provide complementary information about the evolution of endophytic fungi, which cannot be inferred from BLAST or FASTA searches alone. Molecular techniques have been successfully used for identifying endophytic fungi in recent studies (Promputtha *et al*., 2005; Sette *et al*., 2006; Tedersoo *et al*., 2006; Morakotkarn *et al*., 2007). Our study also shows that molecular identification based on ITS sequences can be used to complement or verify morphological identification of unknown endophytes. It was that the unknown species C1 grouped in the clade *Hypoxylon* and *Nodulisporium* but it also including *Xylaria* sp. (DQ322141) in the group clade. It may be mistake of the data from GeneBank because a group of *Xylariaceae* spp. was separated in another clade with 100% bootstrap support. Based on DNA analysis, problems associated with taxonomic identification of mycelia sterilia could be solved. However, it was found that some of sequences downloaded from GenBank for comparative analysis may not be accurate in their identification. The use of ITS sequences also has limitations in phylogenetic analysis. Further studies using different gene sequences can be conducted to resolve this type of difficulties in the phylogenetic analysis of fungi.

## 5.3 Antimicrobial and anticancer testing

Fungal endophytes can modify plants at genetic, physiologic, and ecologic levels (Weishampel & Bedford, 2006). These modifications induce profound changes in how plants respond to their environments, with potential consequences in terms of spatial variation in vegetation dynamics. Strobel *et al.* (2004) have realized that mangrove fungi were important by adapting to the extreme environment and suggested that they are promising sources for screening for new products, especially the mangrove endophytic fungi. It was believed that the metabolites possibly act as chemical defenses as adaptation of mangrove fungi (Rodriguez *et al*., 2004). Jones *et al.* (2008) reviewed the occurrence of fungal endophytes in marine organisms, especially seaweeds and mangrove plants, and compared them to obligate or marine-

derived fungi. The ability of endophytes to produce a wide range of new or novel bioactive compounds. In the current is already established study, crude extract of endophytic fungi isolates showed broad spectrum of antimicrobial and anticancer activities, emphasizing the potential of endophytic fungi of mangrove plants as producers of novel metabolites. Crude extracts of *Xylaria* sp.R1 and *Cladosporium* sp.p1 showed a wide spectrum of anti-microbial activity and *Pestalotiopsis* sp.C1 show broad spectrum against cancer cell lines. There was recent report from China on the marine-derived fungus *Cladosporium* sp. F14 which had the ability to produce antibiotic and antifouling compounds (Xiong *et al*., 2009). Endophytic *Xylaria* spp. and *Pestalotiopsis* spp. were known to generate antifungal, anti-oxidant and anti-cancer metabolites (Li *et al*., 2008; Liu *et al*., 2007). *Pestalotiopsis* spp. are attracted much attention in recent years for their ability to produce a variety of bioactive secondary metabolites. Those of mangrove endophytic origin therefore ,deserve further study as potential sources of novel metabolites activity. Medicinal properties of different parts of mangrove plants (Bandaranayake, 1998) may be fully or partially dependent on the endophytic fungi. Bioactive product discovery depends on the knowledge of habitats where fungi were abundant and the strength of culture collection (Hyde, 2001). Studies on mangrove endophytic fungi were initiated recently as it has been realised that mangrove plants harbour an extremely diverse endophytic fungal flora (Suryanarayanan and Kumaresan, 2000; Ananda and Sridhar, 2002). Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for biological control (Strobel, 2003, Guo *et al*., 2008). Therefore, the use of endophytic fungi opens up new areas of biotechnological exploitations.

# CHAPTER 6
# CONCLUSION

Mangrove endophytic fungi are of enormous scientific interest because they constitute the second largest part of the earth's marine fungi and endophytic mangrove fungi live in mangrove plants that possess unique structures, metabolic pathways, reproductive systems, and sensory and defense mechanisms because they have adapted to extreme environments. Then the endophytic mangrove fungi represent a source of unique genetic information. Now, DNA of endophytic fungi can be directly amplified from plant tissues (Arnold, 2007; Guo *et al*. 2001). The analysis of environmental DNA samples by molecular approaches may change the perception of microorganisms in a variety of ecosystems. Using the light and electoral microscopy, chemical analysis, and the molecular cultivation independent techniques (such as 18S, 28S, ITS rDNA sequences, and fluorescence in situ hybridization (FISH) together, it possibly detect and qualify the complex diversity of mangrove endophytic fungi inhabiting individual plants and also the natural products from mangrove endophytic fungi, which are known to be a source of bioactive metabolites of biotechnological interest the most of these fungi grow in a unique and extreme habitat are should have high consideration for certainly will play a crucial role in meeting the demand for screening for novel compounds.

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# APPENDICES

# APPENDIX A

## MEDIA

1) MEA : Malt extract agar (according to Blakeslee)

| | | |
|---|---|---|
| Malt extract (powdered) | 20 | g |
| Peptone, bacteriological | 1 | g |
| Glucose | 20 | g |
| Agar | 15 | g |
| Distilled water | 1 | litre |

Final pH 5.0-5.5

2) PDA : Potato-dextrose agar

| | | |
|---|---|---|
| Potatoes, peeled and diced | 200 | g |
| Glucose | 20 | g |
| Agar | 15 | g |
| Distilled water | 1 | litre |

Boil 200 g of peels, diced potatoes for 1 hr in 1 litre of distilled water. Filter, and make up the filtrate to one litre. Add the glucose and agar and dissolve by steaming and sterilise by autoclaving at 121°C for 15 min.

3) Nutrient Agar

| | | |
|---|---|---|
| Peptone | 5 | g |
| Beef extract | 3 | g |
| Agar | 15 | g |
| Distilled water | 1 | litre |

Adjust pH to 7.0±0.1

# BIOGRAPHY

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