DIVERSITY OF SAPROBIC FUNGI ON DEAD LEAVES OF *Magnolia liliifera* AND *Cinnamomum iners* IN NORTHERN THAILAND AND SCREENING FOR THEIR ANTIMICROBIAL ACTIVITY

JUTAMART MONKAI

MASTER OF SCIENCE
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THESIS COMMITTEE

Rampai Kodsueb .....CHAIRPERSON
(Dr. Rampai Kodsueb)

.....ADVISOR
(Assoc. Prof. Dr. Kevin D. Hyde)

.....CO-ADVISOR
(Asst. Prof. Dr. Ekachai Chukeatirote)

.....EXAMINER
(Dr. Itthayakorn Promputtha)

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มหาวิทยาลัย
แม่ฟ้าหลวง

Thesis Title	Diversity of Saprobic Fungi on Dead Leaves of *Magnolia liliifera* and *Cinnamomum iners* in Northern Thailand and Screening for Their Antimicrobial Activity
Author	Jutamart Monkai
Degree	Master of Science (Biological sciences)
Advisor	Assoc. Prof. Dr. Kevin D. Hyde
Co-Advisor	Asst. Prof. Dr. Ekachai Chukeatirote

ABSTRACT

Saprobic fungi play an important role as decomposers in natural ecosystems. These fungi also have the potential to produce bioactive compounds which can be applied for biotechnological uses. The diversity of fungi on decaying leaves of *Magnolia liliifera* and *Cinnamomum iners* collected in dry season at Doi Suthep-Pui forest, Chiang Mai, Thailand was studied. Thirty-five taxa were identified from *M. liliifera* comprising 8 ascomycetes and 27 asexual taxa. The most abundant species found were *Sporidesmium* sp. (80%), *Colletotrichum fructicola* (70%) and *Stachybotrys parvispora* (70%). For *C. iners*, 17 taxa were identified comprising 2 ascomycetes and 15 asexual taxa. Anamorph of *Eutypa* sp. 2 (60%) and *Pleurophragmium* sp. (60%) were the most abundant species on *C. iners*. A few genera and only one species (*Lasiodiplodia theobromae*) overlapped between the two hosts. The fungal diversity of *M. liliifera* is higher than that of *C. iners*. Distinct fungal communities were found between two hosts on each stage of decomposition and incubation periods. Decaying leaves collected in an early stage of decomposition

supported greater fungal diversity than those collected in a later stage of decomposition on both hosts. Saprobic fungi were specific to different tissues types (leaf lamina, midrib, petiole). These results provide evidence for host-specificity in saprobic fungi.

The three taxa obtained from the diversity study of saprobic fungi on leaves of *M. liliifera* and *C. iners* are probably new to science. These taxa were described, illustrated and the nucleotide sequences of ITS and LSU regions were blasted in GenBank to reveal the closest matches. Molecular phylogenetic is needed to help identify these fungal taxa in the future.

Fungi in family *Planistromellaceae* are saprobes and pathogens on various plants and are characterized by multi or uniloculate ascostromata comprising several layers of brown to black thick-walled cells and pseudoparaphyses are not obvious in mature specimens. The type specimens of *Planistromellaceae* were examined and described including *Comminutispora agavacearum, Eruptio acicola, Loratospora aestuarii, Microcyclus angolensis, Mycosphaerellopsis myricariae, Planistroma yuccigenum* and *Planistromella yuccifoliorum.* Phylogenetic analyses based on molecular data from LSU and ITS genes provided three different groups represented by the type species of *Piptarthron, Planistroma* and *Kellermania*. Three genera; *Kellermania, Planistroma* and *Mycosphaerellopsis* were accepted in *Planistromellaceae,* while four other genera are excluded from this family.

Some saprobic fungi were selected for screening of antimicrobial activity. Nine isolates were extracted for secondary metabolites and crude extracts were separated into four fractions (A: petroleum ether, B: acetone, C: ethanol and D: water). The antimicrobial activity of these extracts was assayed *in vitro* by modified disc diffusion methods against nine pathogenic microorganisms. The results showed that *Stachybotrys parvispora* (JM09), *Clonostachys rosea* (JM13), *Beltrania*

rhombica (JM14), *Fusicoccum aesculi* (JM19-1, JM19-2), Ascomycete sp. (JM25), and *Chaetomium* sp. (CI18) were effective against some test microorganisms. *Clonostachys rosea* (JM13) showed the highest antimicrobial activity with a broad spectrum of activity against several bacteria and fungi. Of these four fractions, ethyl acetates represented the maximum antibacterial activity. FTIR spectrums showed that these antimicrobial compounds are composed of various compounds. The results suggest that saprobic fungi could be a promising resource of useful bioactive compounds.

Keywords: Antimicrobial activity/Fungal diversity/*Planistromellaceae*/Saprobic fungi/Taxonomy/Thailand

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CHAPTER 1

INTRODUCTION

1.1 Ascomycetes

Fungi are eukaryotic organisms which are a large group and diverse in morphology, physiology, ecology and genetics (Kendrick, 2000; Kirk, Cannon, Minter & Stalpers, 2008). They have offered various important functions to the ecosystem as decomposers of organic matter, symbionts with algae and many plants, pathogens on plants and animals (Carlile, Watkinson & Gooday, 2001). The utilization of fungi in the agriculture and biotechnology is more recent development and these include such widespread products such as biofertilizers, mycoherbicides, fermented food products, antibiotics, enzymes and organic acids (Hawksworth, 2001a).

1.1.1 Definitions

The term ‘Ascomycetes’ refers to members of the phylum Ascomycota, in those which produce sexual spores called ‘Ascospores’(Carlile et al., 2001; Ingold, 1973). The phylum Ascomycota is the largest group of fungi comprising 3 subphyla: Pezizomycotina, Saccharomycotina and Taphrinomycotina which have been estimated to 15 classes, 68 orders, 327 families, 6355 genera and 64,163 species (Kirk et al., 2008).

1.1.2 Morphological Characters

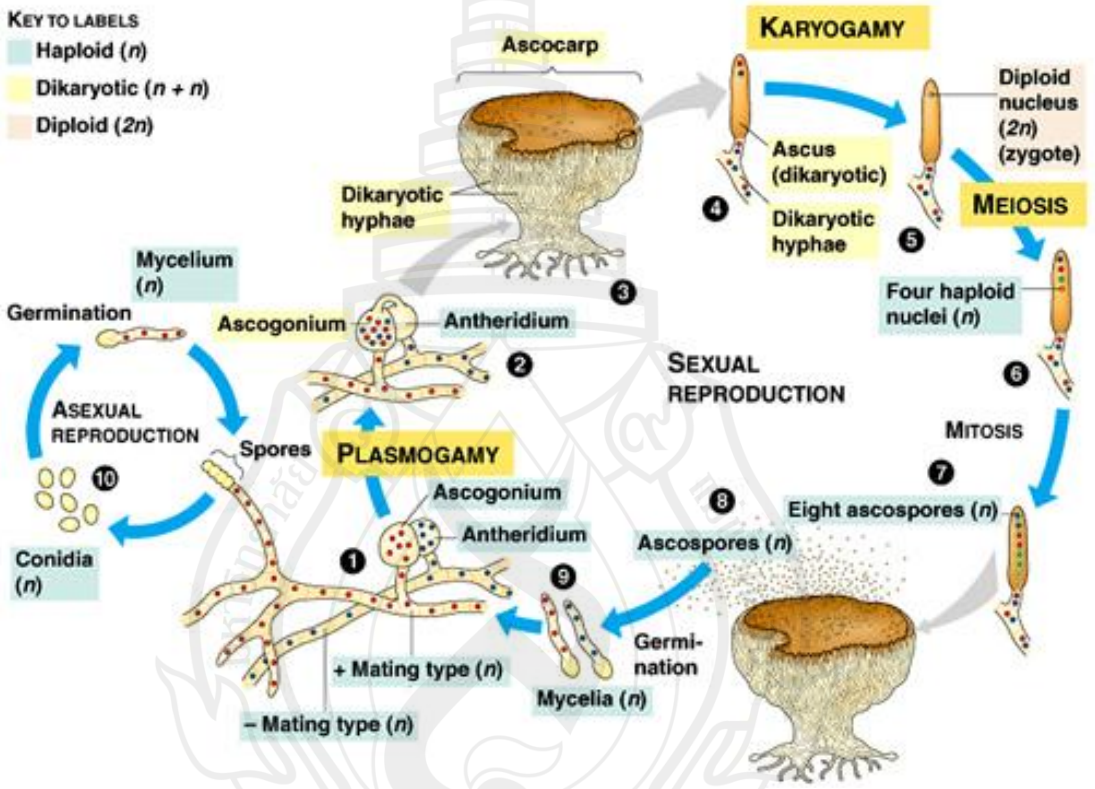
The ascomycetes are characterized by filamentous, branched, septate mycelium and forming fruiting bodies composed of ascomata during sexual reproduction (Carlile et al., 2001; Ingold, 1973). Ascoma (Pl. ascomata) typically

contain a large number of asci. Ascus (Pl. asci) is a spore-producing unit which at maturity usually contains eight ascospores. The ascospores are released into the air through the top of ascus (Ingold, 1973; Kendrick, 2000). Ascomata are formed in various shapes; completely close, openly cupulate, globose, flask-shaped and mostly with apical opening. They can appear solitary or clustered. Their texture can likewise be very variable, including fleshy, like charcoal (carbonaceous), leathery, rubbery, gelatinous, slimy, powdery, or cob-web-like. Also ascomata come in multiple colours. Ascus can be functionally one-layered (unitunicate) or two-layered (bitunicate) which are very varieties of shapes such as globose, clavate, cylindrical, sac-like, sometime consist of the apical pore in the top. All sterile elements between asci are called as 'Hamathecium' which are an important character for fungal taxonomy. Ascospores are also varied in shapes between globose to fusiform or cylindrical. They can be hyaline or variously coloured (often brown or black). The important character is the septation of ascospores which are transversely and/or longitudinally septa ranged from 0 to several septa (Ingold, 1973). However, most ascomycetes also have asexual morph during their life cycle. Asexual spores are called as 'Conidia' which are produced by conidiophores. There are two groups of asexual morph separated by conidium formation. The first group is called as 'Hyphomycetes'; conidiophores that bearing with conidia at their tips arise from simple or aggregate hyphae. Some fungi produce conidiophores inside fruiting bodies called as 'Conidiomata' which are many shapes such as flask-shaped, cushion-shaped and globular shaped. These fungi is called as 'Coelomycetes' (Agrios, 2005).

1.1.3 Life Cycle

The sexual fertilization begins when two compatible haploid mycelia meet each others. Then the female reproduction organ, called as the ascogonium fuse with the antheridium (male reproduction organ). The fertilized ascogonium has now two types of nuclei; one male and one female and forms dikaryotic hyphae (n+n). These dikaryotic hyphae develop the fruiting bodies. At the tip of ascus, there are two nuclei which will then fuse to form a zygote (2n, diploid). This undergoes meiosis to produce four haploid nuclei and a further mitosis undergoes to form eight haploid nuclei each which become ascospores. After that, asci finally disperse ascospores into

the air and ascospores germinate and produce haploid mycelium (n). Continually, the sexual reproduction carries on in the same cycle. Exception in many cases, asexual reproduction also occurs which may produce conidia on aerial hyphae (conidiophores) and/or produce conidia in conidiomata (Carlile et al., 2001; Kendrick, 2000) (see Fig. 1.1).



From **Ascomycota - reproduce sexually via ascospores.** Retrieved March 28, 2013, from http://www.bio.miami.edu/dana/160/160S12_16.htm

Figure 1.1 Life Cycle of Ascomycetes

1.1.4 Significance

Ascomycetes are important in great varieties of aspects. In natural ecosystem, they play an important role to decompose plant litter which is a vital part of global carbon (Cooke & Rayner, 1984). Many ascomycetes associate with mutualistic symbionts to other organisms such as mycorrhizae (Snyder & Allen, 2004), endophytes (Aly, Debbab & Proksch, 2011b) and lichens (Oksanen, 2006). Some ascomycetes are parasites of plants which make an enormous negative effect for crop production (Agrios, 2005). Some cause disease in human and animals (Carlile et al., 2001; Sullivan, Moran & Coleman, 2005). In the biotechnological and pharmaceutical industries, they have been produced potentially fermentation products comprising antibiotics, medicines, commercial enzymes, and other natural products (Bennett, 1998). Some ascomycetes are widely used in production of many foods and drinks such as cheese, beer, wine, bread and soybean products (Khachatourians, 2004). Moreover, many ascomycetes can be used as biological control agents for insect, plant pathogenic fungi and weed management (Butt & Copping, 2000).

1.2 Mode of Life of Ascomycetes

1.2.1 Pathogen

Fungi are able to penetrate into plant tissues if it causes the damage to the host is called as ‘Pathogen’. Some fungi are living within plant tissues and get the nutrition without causing cell death is called ‘biotrophic’. Some fungi are killing cells and absorbing nutrients from dead tissues is called ‘necrotrophic’ (Carlile et al., 2001). A wide range of fungi are important pathogens causing severe diseases of plants. Ascomycetes account for most of plant pathogens which have a broad range of hosts (Agrios, 2005). The diseases of plant pathogenic fungi are associated with many symptoms such as spots, wilt, blight, rots, cankers, die back and damping off. (Doohan, 2005). Most ascomycetes cause the infections by their asexual state during the growing season. The sexual state is always occurred on infected plant organs only in the end of growing season (Agrios, 2005). The mechanism of fungal pathogens is

started from fungi penetrate through the wounds of plants or produce special structures such as appressoria (Doohan, 2005). After that, these fungi also produce degradative enzymes that cause necrotic lesions and low molecular weight metabolites such as phytoxins which damage the plants (Hanson, 2008).

1.2.2 Endophyte

Some fungi live within internal tissues of their host plants without any negative effects refer to 'Endophytic fungi'. Most of these endophytes are ascomycetes which are extremely diverse and can be specific to particular plant species (Aly et al., 2011b; Suryanarayanan et al., 2009). These fungi reside intercellular space of stems, petioles, roots and leaves on living plants with mutualistic symbiosis. Plants provide the proper habitat for fungal growth (Sieber, 2007). The association of endophytes are come from their airborne spores are horizontally transmitted to the host plants. In next plant generations, endophytes may be vertically transmitted through the infection of seeds (Faeth & Fagan, 2002). Endophytic fungi also support plant growth by producing secondary metabolites which are toxic to against invertebrates herbivores, nematodes and pathogen (Aly et al., 2011b; Suryanarayanan et al., 2009). Moreover, these fungi may change the physiology and morphology of host plant to be tolerant in stress environment (Faeth & Fagan, 2002). However, some endophytes can be considered to pathogens in latent period (Arnold, 2007; Sieber, 2007). These pathogenic fungi could potentially be detected as endophytes in healthy tissues long before symptoms develop (Photita, Lumyong, Lumyong, McKenzie & Hyde, 2004).

1.2.3 Saprophyte

Some group of fungi; pathogen and endophytes obtain their nutrients from living organisms. However, other group of fungi called 'Saprophytes' obtain nutrients from dead organisms (Carlile et al., 2001). Plant litter is an important component of natural ecosystems. It is the main source of organic matter and forms a protective layer on the forest floor and is a buffer against microclimatic fluctuations, erosion and soil compaction (Sayer, 2006). Saprobic fungi function on decomposing organic material and recycling nutrients to other organisms (Cooke & Rayner, 1984).

Microfungi growing in decomposing plant debris are mostly vegetative or asexually reproducing states of ascomycetes (Lodge, 1997). Bacteria and fungi also play important roles in decomposing process by production of their extracellular enzymes to utilized organic substances (Calile et al., 2001; Osono, 2007). But fungi are better for decaying some components of plant cells such as cell wall. Because mycelium of fungi can penetrate through plant cells and this help to increase more space for decomposing process (Cooke & Rayner, 1984). Moreover, saprobic fungi can decay the most complex compounds such as cellulose and lignin. (McClaugherty & Berg 1987; Promputtha, Hyde, McKenzie, Peberdy & Lumyong, 2010). There are some evidences that endophytes later become as saprobes because endophytic fungi can decay plant materials after the plant die (Koide, Osono & Takeda, 2005; Osono, Bhatta & Takeda, 2004; Osono et al., 2009; Promputtha et al., 2010; Purahong & Hyde, 2011; Tang, Jeewon & Hyde, 2005).

1.3 Saprobic Ascomycetes: Diversity and Importance

The number of fungi worldwide was estimated at 1.5 million species by Hawksworth (1991, 2001b). However, only 70,000 species are presently described leaving the remaining 1.43 million (or 95%) as being undescribed. It is therefore important to search for these undescribed fungi which occur in unexplored habitats, hosts or poorly studied countries especially in tropical regions (Hawksworth & Rossman, 1997). Fungal diversity in temperate forests have been relatively studied a longer history than in the tropical forests, although the tropics and subtropics are generally thought to be greater fungal diversity than at higher latitudes. The reasons for higher diversity at low latitudes are not always clear, but this might be because of relevant factors such as host diversity, resource abundance and habitat diversity (Lodge & Cantrell, 1995).

The numbers of fungi occurring on a single host and in particular whether they are specific to that host are a significant indicator for assessing global number of fungal species (Hyde et al., 2007). Host-specificity and host-recurrence may be an

appropriate terms for saprobic fungi. Host-specificity is a term where a species is restricted to a particular host or group of related species, but does not occur on other unrelated plants in the same habitat. Host-recurrence is the frequent or predominant occurrence of a saprobic fungus on a particular host or range of hosts, but the fungus also occurs infrequently on other host plants in the same habitat (Zhou & Hyde, 2001). Many fungi which are saprobes on palms appear to be unique (e.g. *Astrosphaeriella*, *Manokwaria*, *Myelosperma*, *Oxydothis*, *Palmicola*, and *Pemphidium*). There are examples of *Oxydothis* species which are known from only a single host, and others from more than one host (Hyde, 1994) Saprobic fungi may be specific at the host genus level (Hyde et al., 2007). The example study was done by McKenzie, Buchanan and Johnston (1999, 2000, 2002) which the fungal species from *Agathis*, *Metrosideros*, *Nothofagus* (southern beech) in Southern Hemiphere in New Zealand were low overlap. Saprobic fungi were more specific at the host family level such as fungal composition in three hosts in *Magnoliaceae* are varied (Kodsueb, McKenzie, Lumyong & Hyde, 2008). Leaf litter fungi also specific to tissue types (Duong, McKenzie, Lumyong & Hyde, 2008; Photita et al., 2001; Photita, Lumyong, McKenzie, Hyde & Lumyong, 2003; Pinnoi, Lumyong, Hyde & Jones, 2006; Promputtha, Lumyong, Lumyong, McKenzie & Hyde, 2002; Yanna & Hyde, 2001). Yanna and Hyde (2001) found that different frond parts of palms supported distinct fungal communities on most samples.

Estimates of global fungal numbers also depend on relevant factors such as the impact of methodology, of geography, and of decomposition stages. The diversity of saprobic fungi on various hosts have been studied in many countries (Hyde, Ho, McKenzie & Dalisay, 2001; Kannangara, Parkinson & Deshappriya, 2007; Lee Mel'nik, Taylor & Crous, 2004; Parungao, Fryar & Hyde, 2002; Paulus, Gadek & Hyde, 2003; Paulus, Kanowski, Gadek & Hyde, 2006; Photita et al., 2001; Polishook, Bills & Lodge, 1996; Przybył, Karolewski, Oleksyn, Łabędzki & Reich, 2007; Santana, Lodge & Lebow, 2005; Wong & Hyde, 2001). Most studies of saprobic fungi have been carried out by using direct observation (moist chamber method), particle filtration or surface washing isolation (Duong, Lumyong & Hyde, 2004; Paulus et al., 2003, 2006; Polishook et al, 1996; Promputtha, Lumyong, Lumyong, McKenzie & Hyde, 2004). Polishook et al. (1996) used particle filtration and moist

chambers incubation to isolate fungal species on leaf litter from two tree species in Puerto Rico. Many more species have been isolated using particle filtration more than by using moist chambers. However, this does not mean that all fungi isolated from particle filtration method are saprobic fungi (Duong et al., 2004).

Geographic factor are also an important criteria in studying microfungi on leaf liiter (Hyde et al., 2007; Parungao et al., 2002; Photita et al., 2001; Somrithipol, Jones & Hywel-Jones, 2002; Yanna & Hyde, 2001). Parungao et al. (2002) studied leaf litter fungi from 13 different tree types on two rainforest sites in North Queensland, Australia. Both of forest sites had similar diversity. There were 36 (63%) of taxa that occurred on only one leaf type, revealing possible host specificity or recurrences. Fungal communities on decaying palm fronds in Australia, Brunei and Hong Kong were studied by Yanna and Hyde (2001). Low numbers of fungi were found common among palms from different sites. This result indicated that fungal communities may be influenced by climate and geographic distribution.

There were some studies focused on saprobic fungi on different stages of decomposition (Seephueak, Petcharat & Phongpaichit, 2010; Seephueak, Phongpaichit, Hyde & Petcharat, 2011; Shanthi & Vittal, 2010; Yanna & Hyde, 2001). Seephueak et al. (2010, 2011) studied fungi on different stages of decaying leaf and branch litter of the rubber tree. They showed that the number of taxa on middle stage decaying branches was higher than new and old decaying fallen branches. Saprobic fungi were examined from two stages of decomposition on *Pavetta indica* leaves by moist chamber technique. Freshly fallen senescent leaves have more fungi than leaves already undergoing active decomposition (Shanthi & Vittal, 2010). These studies were expressed that in different grades of litter shifts in activity of the various species of the mycota occurred indicating peaks and troughs of activity for some of the species.

Lodge (1997) hypothesized that the higher diversity of tree species in less disturbed forest contributed to greater ascomycete diversity. Only few studies on fungal diversity of saprobic fungi in Thailand have been carried out in recent years (Table 1.1). In northern Thailand, forests have greater plant diversity (Gardner, Sidisunthorn & Anusarnsunthorn, 2000). There have been several studies concerning fungal diversity in the forests of northern Thailand in recent years including studies of

Table 1.1 Recent Studies on Diversity of Saprobic Fungi in Thailand

Hosts	Litter types	Sites	Seasons	References
Musa acuminata	Petioles, pseudostems and leaves	Doi Suthep-Pui, Chiang Mai	Wet and dry	Photita et al. (2003)
Dracaena lourieri, *Pandanus* spp.	Leaves, leaf sheaths, prop-roots and seeds	Chiang Dao, Doi Suthep-Pui, Chiang Mai and Phayao	Wet and dry	Thongkantha, Lumyong, McKenzie and Hyde (2008)
Shorea obtusa	Leaves	Naresuan University, Phayao	Wet and dry	Osono et al. (2009)
Ficus species	Leaves	Mae Taeng, Chiang Mai	N/A*	Wang, Hyde, Soytong and Lin (2008a)
Magnolia liliifera	Leaves lamina, vein, petioles	Doi Suthep-Pui, Chiang Mai	Wet	Promputtha et al. (2002, 2004)
Magnolia liliifera, *Manglietia garrettii*, *Michelia baillonii*	Wood	Doi Suthep-Pui, Chiang Mai	Wet and dry	Kodsueb et al. (2008)
Castanopsis diversifolia	Leaves lamina, vein, petioles	Doi Suthep-Pui, Chiang Mai	Wet	Duong et al. (2008)
Thysanolaena latifolia, Saccharum spontaneum	Leaves and stems	Doi Suthep-Pui, Chiang Dao, Mae Rim, Chiang Mai	Wet and dry	Bhilabutra, McKenzie, Hyde and Lumyong (2010)
Eleiodoxa conferta	Leaves, rachides and petioles	Sirindhorn peat swamp forest, Narathiwat	Wet and dry	Pinnoi et al. (2006)
Hevea brasiliensis	Leaves, branches	Para rubber plantations, Nakhon Si Thammarat and Songkhla	Wet and dry	Seephueak et al. (2010, 2011)
Delonix regia	Pods	Boundary areas of Khao Yai National Park	Wet	Somrithipol et al. (2002)

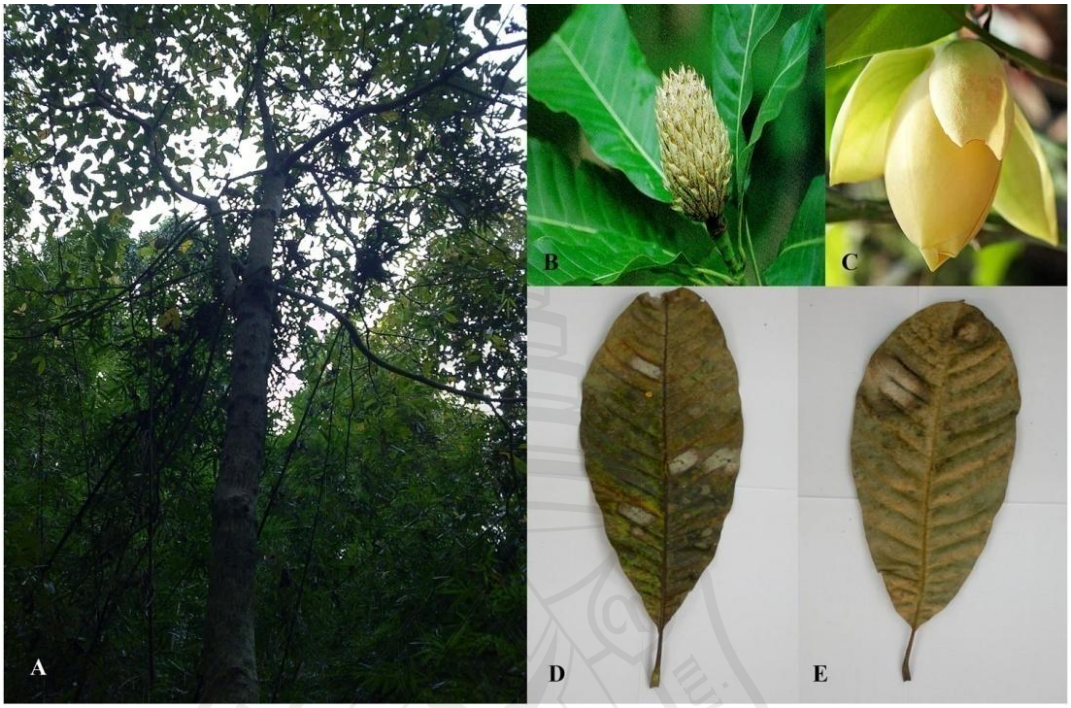
Note. *N/A = Not available

fungi on on monocotyledons (Bhilabutra et al., 2010; Bussaban, Lumyong, McKenzie, Hyde & Lumyong, 2004; Photita et al., 2003; Thongkantha et al., 2008), on *Shorea obtusa* (*Dipterocarpaceae*) (Osono et al., 2009), on *Ficus* species (Wang et al., 2008a), on leaf and woody litter of *Magnoliaceae* (Promputtha et al., 2002, 2004; Kodsueb et al., 2008), on *Castanopsis diversifolia* (Duong et al., 2008). Promputtha et al. (2002) investigated saprobic fungi from *Magnolia liliifera, Meliosma simplicifolia* and *Berchemia floribunda* on the forest of Doi Suthep-Pui National Park, Thailand. Most fungi from all tree species were host-specific. Similarly, fungal diversity on leaf litter of five tree species in Doi Suthep-Pui National Park, Thailand were studied by Duong et al. (2008). Each host species also had different fungal communities. *Castanopsis diversifolia* had the highest diversity of fungi. Studies of saprobic fungi have provided more biodiversity data. Many new taxa have been also described.

1.3.1 *Magnolia liliifera*

Magnolia liliifera (L.) Baill., family *Magnoliaceae*, is in evergreen forest distributed in India, Nepal, Sikkim, Bhutan, Tibet, South China, Thailand, Myanmar, Malaysia, Malay Peninsula and Borneo. In northern Thailand, this tree is commonly located in Doi Suthep-Pui National Park. The tree is up to 15 m high with open irregular crown and smooth, pale brown bark. Leaves are 20-50 × 7-15 cm, narrowly obovate with blunt tip and tapering at the base. Mature leaves are dark green, smooth or with scattered hairs on mid vein below. 9-15 pairs of widely spaced side veins. Stalks are 5-7 cm, swollen at base, stipule scar more than ¾ of total length (Gardner et al., 2000). The characteristic of *M. liliifera* are shown in Fig. 1.2. *M. liliifera* leaves are large and thick, which may provide a large number of fungal species. Promputtha et al. (2002) investigated saprobic fungi from *M. liliifera* leaves on the forest of Doi Suthep-Pui National Park in wet season. Many diverse fungal communities and undescribed species were discovered. Therefore, this host is a good source for study saprobic fungi. Saprobic fungi on decaying woody litter of *M. liliifera* in the forest of Doi Suthep-Pui National Park during the wet and dry seasons were studied by Kodsueb et al. (2008). The results showed that samples collected in the dry season provided greater species richness than samples collected in the wet season. To

establish host-specificity of saprobic fungi, fungi from *M. liliifera* are compared with fungi on leaves of *Cinnamomum iners*, which growing in the same forest area.

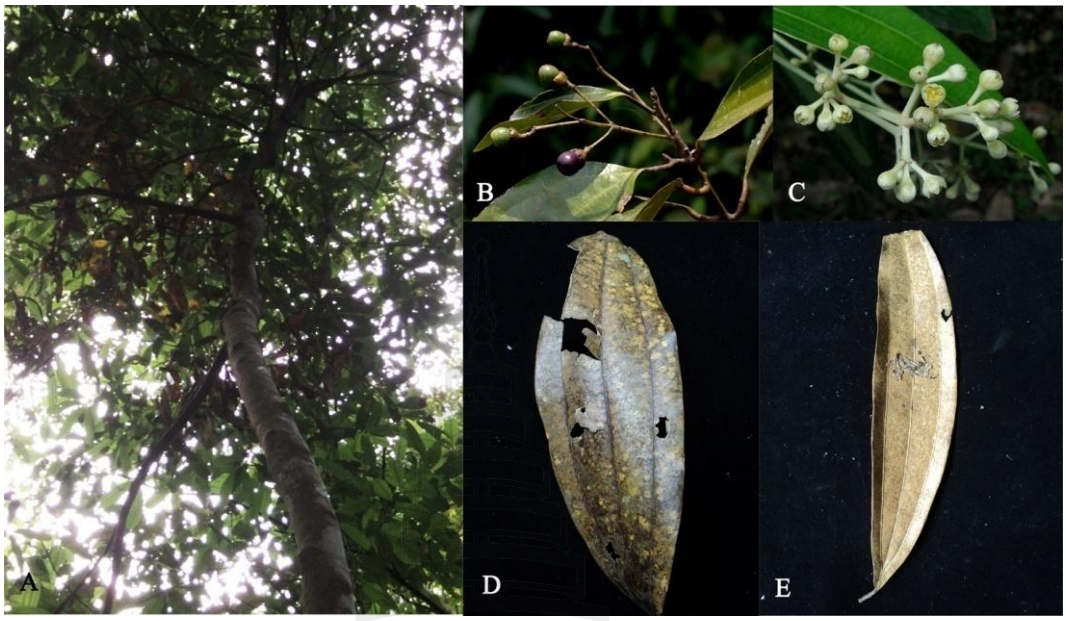


Note. (A) Tree, (B) Fruit (**Endemic and Rare Plants of Thailand.** Retrieved March 28, 2013, from http://www.rspg.or.th/plants_data/rare_plants/rare_plants19.htm (C) Flower (**Tropical Plant catalog.** Retrieved March 28, 2013, from http://toptropicals.com/catalog/uid/Magnolia_liliifera.htm), (D) Recent Fallen Leaf, (E) Dead Leaf

Figure 1.2 *Magnolia liliifera*

1.3.2 *Cinnamomum iners*

Cinnamomum iners Reinw. ex Blume, family *Lauraceae*, is an evergreen tree distributed in India, Myanmar, Thailand, Cambodia, Vietnam, Malay Peninsula and Indonesia. This tree is native in northern Thailand. The tree is high, up to 20 m with dense oval or cylindrical crown. Barks are pale brown or greyish, smooth, thin, inner bark pinkish with a strong smell of cinnamon. Leaves are 8-30 × 3-9 cm, oblong or lanceolate, widest in the middle, blunt or slightly pointed at both ends. Young twigs are pink and silky-hairy. Mature leaves are leathery, completely smooth, dark green above, grey-green (glaucous) below. There are 3 main veins running entire length of leaf, outer pair joined to central one above base of leaf, transverse vein faint, ladder-like. Stalks are 0.8-1.7 cm, twigs slender and shiny, old leaves yellow. The characteristic of *M. liliifera* are shown in Fig. 1.3. The timber is insect resistant used for aromatic joss sticks and the bark yields an inferior grade of cinnamon spice, used for food flavouring and incense (Gardner et al., 2000). The Essential oil from leaves is used for flavorings sweets and confectionery (Jantan, Wiselius, Lim & Sosef, 1995). It has been reported that leaf extract can produced some biological and phamaceutical activities such as insecticidal activity (Jantan, Mat & Hock, 1992; Zaridah, Nor Azah & Rohani, 2006). In Thailand, there have been some studies concerning endophytic fungi from *Cinnamomum* species (Lumyong, Lumyong, McKenzie & Hyde 2002; Suwannarach, Bussaban, Hyde & Lumyong, 2010; Worapong, Inthararaungsom, Strobel & Hess, 2003; Worapong et al., 2001). Worapong et al. (2003) and Suwannarach et al. (2010) discovered new fungal species and strains that can produce volatile compounds. However, there has been no study on the fungal diversity on decaying *C. iners* leaves in Thailand. It is therefore interesting to study these fungi to understand the relationship between fungi and host. In addition to this tree grows in Doi Suthep-Pui National Park, the same forest area as *M. liliifera*. Diversity of saprobic fungi on *C. iners* were studied and compared to those fungi found on *M. liliifera*.



Note (A) Tree, (B) Fruits (***Cinnamomum iners*** **Reinw. ex Blume – LAURACEAE**. Retrieved March 28, 2013, from http://www.biotik.org/laos/species/c/cinin/cinin_08_en.html), (C) Flowers (***Cinnamomum iners*****, flower close-up**. Retrieved March 28, 2013, from http://www.flickr.com/photos/bushblog/5415857076/), (D) Recent Fallen Leaf, (E) Dead Leaf

Figure 1.3 *Cinnamomum iners*

1.4 Secondary Metabolites of Saprobic Ascomycetes

Fungi are well-known as natural resources utilizing for medicinal and biotechnological applications (Hawksworth, 2001a). The economically important products produced by fungi are diverse such as antibiotics, enzymes, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, hormones and proteins. There are two classes of products during fungal life cycle, namely primary and secondary metabolites. Primary metabolites are essential for growth of fungi

includes enzymes, fats, alcohol and organic acids (Murphy & Horgan, 2005). On the other hands, secondary metabolites are not essential for vegetative growth but to resist unfavorable environments, finish cell proliferation, differentiation and entire life cycle to reach the purpose of self-defense and survival (Zhong & Xiao, 2009). In addition, secondary metabolites possess many bioactivities of antimicrobial, anti-inflammatory, antioxidant, anticancer, antiparasitic and immunomodulating etc. (Zhong & Xiao, 2009).

The first fungal metabolite product is mycophenolic acid from *Penicillium glaucoma* discovered in 1896 by Gosio (Bérdy, 2005). In the last two decades, there were many important fungi derived compounds such as penicillins (Fleming, 1929), cephalosporins (Newton & Abraham, 1955), cyclosporin (Dreyfuss et al., 1976), griseofulvin (Rehm, 1980), and lovastatin mevinolin (Albert et al., 1980). Up to now, fungi have been recognized to produce relatively a high number of bioactive products about 38% of all microbial products. Among fungal species, the ascomycetes and basidiomycetes are the most frequent producers of bioactive secondary metabolites with about 6400 compounds (Bérdy, 2005). Most species of which namely to from *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Phoma*, *Alternaria*, *Acremonium* and *Stachybotrys*, *Ganoderma*, *Lactarius* and *Aureobasidium* are good producers to produce several hundreds of compounds (Bérdy, 2005; Zhong & Xiao, 2009). Higher fungi are exhibited diverse bioactive compounds and the drug-like characteristics of chemical structure, which present as a major natural compounds library for new drug discovery (König ,Kehraus, Seibert, Abdel-Lateff & Müller, 2006; Strobel, 2003). Fungal secondary metabolites are in the classes of heterocyclics (e.g. camptothecin, decaturins), polyketides (e.g. aflatoxin, fumonisins), sterols (e.g. ergosterol, phycomysterol), non-ribosomal peptides (e.g. sirodesmin, siderophores), terpenes (e.g. deoxynivalenol (DON), Paclitaxel) (Fox & Howlett, 2008; Zhong & Xiao, 2009). Some secondary metabolites and their bioactivities are listed in Table 1.2.

Table 1.2 Some Secondary Metabolites from Ascomycetes and Their Bioactivities

Species	Metabolites	Activities	References
Acremonium zeae	pyrrocidine A	antibacterial antifungal	Wicklow, Roth, Deyrup and Gloer (2005)
Alternaria brassicicola	alternariol altechromone A herbarin A	antimicrobial antifungal xanthine oxidase inhibitory	Gu (2009)
Aspergillus fumigatus	fumigaclavine C	antifungal mycotoxin	Liu et al. (2004)
Beltrania rhombica	rhombidiol (1) rhombitriol (2)	antibacterial antifungal	Rukachaisirikul, Kaewbumrung, Phongpaichi and Hajiwangoh (2005)
Camposporium quercicola	quercilolin tenellic acid A 2',4'-dihydroxyacetophenone	antibacterial	Wang et al. (2008b)
Cephalosporium sp. IFB-E001	graphislactone A	antioxidant	Song, Huang, Sun, Wang and Tan (2005)
Chaetomium globosum	globosumone A	cytotoxic	Bashyal, Wijeratne, Faeth and Gunatilaka (2005)
Cladosporium herbarum	aspernigrin A	cytotoxic xanthine oxidase inhibitor	Ye, Zhu, Song, Liu and Tan (2005)
Diaporthe sp. CR 146	cytosporone B	antifungal cytotoxic	Brady, Singh, Janso and Clardy (2000)
Fusarium oxysporum strain	vincristine	anticancer	Zhang et al. (2000)

Table 1.2 (Continued)

Species	Metabolites	Activities	References
Nodulisporium sp. MF 5954, ATCC 74245	nodulisporic acid A	insecticidal	Ondeyka et al. (1997)
Ophioceras venezuelense	ophiocerins A-D	antifungal antibacterial	Reategui, Gloer, Campbell and Shearer (2005)
Penicillium janthinellum	shearinine D	anticancer	Smetanina et al. (2007)
Periconia atropurpurea	periconicin B	anticancer	Teles et al. (2006)
Pestalotiopsis microspora	pestacin	antimycotic antioxidant	Harper et al. (2003)
Phoma medicaginis	brefeldine A	antibiotic initiation of apoptosis in cancer cells	Weber, Stenger, Meffert and Hahn (2004)
Phomopsis phaseoli	3-hydroxypropionic acid	nematicidal	Schwarz, Kopcke, Weber, Sterner and Anke (2004)
Stachybotrys cylindrospora	trichodermin trichodermol	antifungal	Ayer and Miao (1993)
Trichoderma viride	viridepyronone	antifungal	Evidente et al. (2003)
Xylaria sp. F0010	griseofulvin dechlorogriseofulvin	antifungal	Park et al. (2005b)

The mostly known secondary metabolites are a class of compounds known as antibiotics (Murphy & Horgan, 2005). The term “antibiotic” is defined as the secondary metabolites isolated from microorganism which inhibits the growth of other microorganisms, in minimal concentration. The antimicrobial activities of secondary metabolites are included mainly antibacterial, antifungal, and/or antiviral

activities (Bérdy, 2005; Zhong & Xiao, 2009). The most famous antibiotics is β-lactams, which regularly known as penicillin discovered by Fleming in 1929. The β-lactams can kill susceptible bacteria by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall (Aly et al., 2011a). The first antifungal natural products from fungi is griseofulvin isolated from *Penicillium griseofulvum* (Rehm, 1980) was commonly used to treat fungal infections of the skin, hair and nails (Aly et al., 2011a). For antiviral compounds, the interesting example is concentricolide isolated from marine ascomycetes, *Daldinia concentrica* which showed effective anti-HIV-1 activity (Qin et al., 2006).

The problems of newly emerging diseases, drug-resistant pathogen and pathogenic microbes, or parasitic protozoans are currently increasing worldwide (Aly et al., 2011a). In last several years ago, the screening of new bioactive secondary metabolites and developing of separation and characterization techniques from higher fungi were intensively attempted. However, only a relatively few number of higher fungi species are chemically investigated (Zhong & Xiao, 2009). Many undescribed fungal species lived in unexplored habitats, which are abundant sources of a wide range of useful natural products with diversified chemical structures and various interesting bioactivities (Hawksworth & Rossman, 1997). It is evident that secondary metabolites from higher fungi will provide more opportunities for finding new lead structures for medicinal chemistry (Zhong & Xiao, 2009). On the basis of drug discovery, there have been various alternative sources including marine fungi (Firáková, Múčková & Šturdíkovál, 2007; Lin et al., 2005), freshwater fungi (Reategui et al., 2005; Rukachaisirikul et al., 2005; Wang et al., 2008b). and endophytic fungi (Aly et al., 2008; Phongpaichit, Rungjindamai, Rukachaisirikul & Sakayaroj, 2006; Wang, Jiao, Cheng, Tan & Song, 2007; Xing, Chen, Cui, Chen & Guo, 2011). Gu (2009) had studied on screening antimicrobial activity of endophytic fungi isolated from *Malus halliana*. It was found that *Alternaria brassicicola* ML-P08 showing strong activity (MICs: 0.31–2.50 mg/ml) was selected for characterization and in vitro antimicrobial assay of its secondary metabolites. *Penicillium ochrochloron* has been showed strong antimicrobial activities against all tested microorganisms; gram positive, gram negative bacteria and the yeast *Candida albicans.* Two antimicrobial compounds were identified as (-)2,3,4-

trihydroxybutanamide and (-)erythritol (Rančić, Soković, Karioti, Vukojević & Skaltsa, 2006). The metabolite extracts from the fungus *Gliocladium* sp. using different solvents exhibited an interesting antibacterial activity against all bacteria tested and all fungi except *Candida albicans* (Liouane et al., 2009). Wang et al. (2007) have screened antimicrobial activities of endophytic fungi isolated from *Quercus variabilis.* The most active antifungal strain I(R)9-2, *Cladosporium* sp. was selected and fermented to obtain a secondary metabolite, brefeldin A.

Saprobic fungi is particularly an interest group of fungi to produce valuable commodities (Murphy & Horgan, 2005). Because during the decaying of organic material, these fungi also produce inhibitory compounds to defend other organisms. This may be potentially as biological resource in the discovery of new active compounds (Hyde, 2001a). In present study, saprobic fungi were selected for screening of bioactive compounds that effect antimicrobial activity.

1.5 Research Objectives

1.5.1 To study the diversity of saprobic fungi on leaves of *Magnolia liliifera* and *Cinnamomum iners* in the forests of northern Thailand.

1.5.2 To study morphological and molecular taxonomy of fungi in *Planistromellaceae* from type specimens.

1.5.3 To screen antimicrobial activities of some saprobic fungi

1.6 Research Contents

This thesis is divided into five chapters (see Fig. 1.4). Chapter 1 “Introduction”, describes and introduces the phylum Ascomycota, the objectives and scope of research, and a literature review discussing diversity of saprobic fungi and fungal metabolites from fungi.

Chapter 2 “Diversity of saprobic fungi on *Magnolia liliifera* and *Cinnamomum iners* in Doi Suthep-Pui forest, Chiang Mai, Thailand” comprises of materials and methods of the study and results on diversity of saprobic fungi in northern Thailand.

Chapter 3 “Possible new fungi discovered from this study” comprises of description and sequence blast data of possible new taxa

Chapter 4 “Study of type specimens on *Planistromellaceae*” comprises of morphological taxonomy and molecular data of type species in *Planistromellaceae*.

Chapter 5 “Antimicrobial activities of saprobic ascomycetes” comprises of materials and methods of the study and results on screening antimicrobial activities from saprobic fungi.

Finally, chapter 6 “Overall conclusion” concludes the major findings and advances of the study, its advantages and disadvantages and the further research needs.

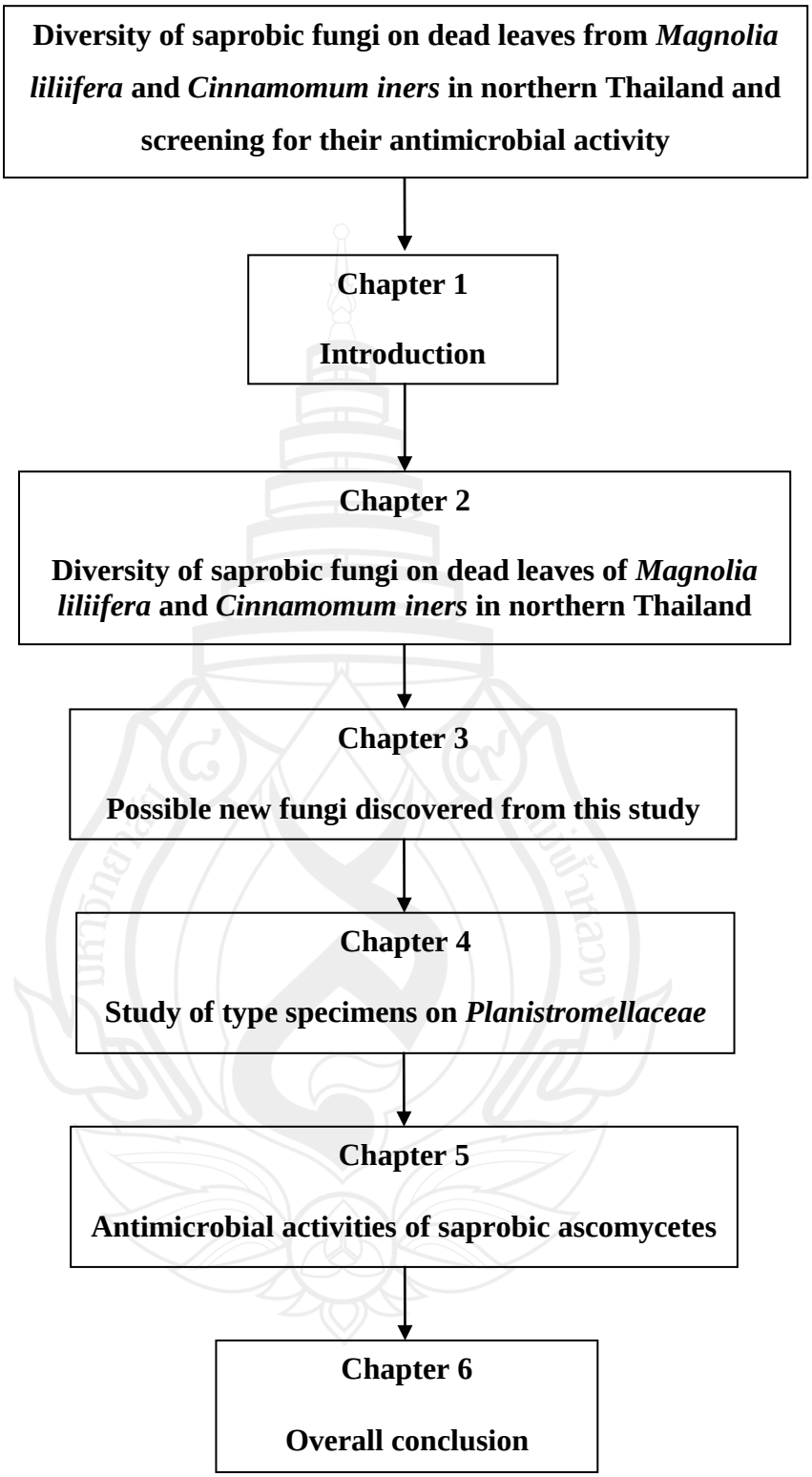


Figure 1.4 Diagrammatic Arrangements to Show the Content of Thesis

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CHAPTER 2

DIVERSITY OF SAPROBIC FUNGI ON DEAD LEAVES OF *Magnolia liliifera* AND *Cinnamomum iners* IN NORTHERN THAILAND

2.1 Introduction

Saprobic fungi function as decomposers of organic materials and recycle nutrients to other organisms (Cooke & Rayner, 1984). The diversity of saprobic fungi on various hosts has generally been poorly studied in both temperate and tropical regions (see Hyde et al. 2001; Kannangara et al., 2007; Lee et al., 2004; Parungao et al., 2002; Paulus et al., 2003; Paulus et al., 2006; Photita et al., 2001; Polishook et al., 1996; Przybył et al., 2007; Santana et al., 2005; Wong & Hyde, 2001). To help estimate numbers of fungal species, a greater number studies on fungal diversity are needed, especially in unexplored habitats, hosts or poorly studied countries and especially in tropical regions (Hawksworth & Rossman, 1997; Hyde, 2001b).

Forests of the northern part of Thailand have great plant diversity (Gardner et al., 2000) and several studies on their fungal diversity have been carried out in recent years. These include studies of fungi on monocotyledons (Bhilabutra et al., 2010; Bussaban et al., 2004; Photita et al., 2003; Thongkantha et al., 2008), on *Shorea obtusa* (*Dipterocarpaceae*) (Osono et al., 2009), on *Ficus* species (Wang et al., 2008a), on leaf and woody litter of *Magnoliaceae* (Kodsueb et al., 2008; Promputtha et al., 2002, 2004), on *Castanopsis diversifolia* (Duong et al., 2008) and several studies on basidiomycetes (Sanmee, Tulloss, Lumyong, Dell & Lumyong, 2008; Sysouphanthong, Thongkantha, Zhao, Soytong & Hyde, 2010; Van de Putte, Nuytinck, Stubbe, Le & Verbeken, 2010; Zhao, Desjardin, Soytong, Perry & Hyde,

2010). This has resulted in several taxonomic advances (see Boonmee et al., 2011, 2012; Fournier, Stadler, Hyde & Duong, 2010; Liu et al., 2011). Studies of saprobic fungi have provided further biodiversity data and many new taxa have been also described (Duong et al., 2004). However, only a few published studies have established the diversity of fungal communities and overlapping fungi on litter types in the tropics (Ananda & Sridhar, 2004; Dulymamode, Cannon & Peerally, 2001; Paulus et al., 2006; Yanna & Hyde, 2001; Yule & Gomez 2008).

Magnolia liliifera (L.) Baill. (*Magnoliaceae*) is an evergreen tree and in northern Thailand, is commonly located in Doi Suthep-Pui National Park (Gardner et al., 2000). *M. liliifera* leaves are large and thick and this host is a good source for saprobic fungi (Promputtha et al., 2004). Promputtha et al. (2002, 2004) studied saprobic fungi on *M. liliifera* leaves in the forest of Doi Suthep-Pui National Park during the wet season of 2001. They found many diverse fungal communities and undescribed species (e.g. *Anthostomella monthadoia*, *Dokmaia monthadangii*, *Hyponectria manglietiae*, *Pseudohalonectria suthepensis*). Saprobic fungi on decaying woody litter of *M. liliifera* in the forest of Doi Suthep-Pui National Park during the wet and dry seasons were reported by Kodsueb et al. (2008). The results showed that samples collected in the dry season provided greater species richness than samples collected in the wet season.

Cinnamomum iners Reinw. ex Blume (*Lauraceae*) is an evergreen tree and has essential oils in the leaves, which are used for flavoring sweets and confectionery (Jantan et al., 1995). It has been reported that leaf extracts can produced some biological and pharmaceutical activity (Jantan et al., 1992; Zaridah et al., 2006). In Thailand, there have been some studies concerning endophytic fungi from *Cinnamomum* species (Lumyong et al. 2002; Suwannarach et al., 2010; Worapong et al., 2001, 2003). Worapong et al. (2003) and Suwannarach et al. (2010) discovered new fungal species and strains that can produce volatile compounds. However, there has been no study on the fungal diversity on decaying *C. iners* leaves. It is therefore interesting to study these fungi to understand the relationship between fungi and host.

Most previous studies on saprobic fungi are based on the wet season when the number of fungi on the forest floor is abundant (Hyde et al., 2001; Parungao et al., 2002; Paulus et al., 2006). This study focused on saprobic fungi on *M. liliifera* and *C.*

iners in the dry season at Doi Suthep-Pui, Chiang Mai, Thailand. The purposes of the present study were to examine fungal communities and compare fungal species between two hosts and to establish whether fungal saprobes are host-specific or host-recurrence (Zhou & Hyde 2001). In addition, the saprobic fungal communities and patterns on each host were evaluated for the effect of different stages of decomposition, incubation periods or decaying times and parts of leaf (leaf lamina, midrib and petiole) or tissue specificity.

2.2 Materials and Methods

2.2.1 Study Site and Sample Collection

The study site was located in an evergreen forest in Doi Suthep-Pui, Chiang Mai, northern Thailand (N 18º 48’ 18.73”, E 98 º 54’ 47.28”, elev., 107 m) and samples were collected in the dry season between November 2009 and April 2010, when there was low humidity; the forest floor was damp but not wet. Ten decaying leaves were randomly collected from each individual tree of *M. liliifera* and *C. iners* in 100 m^2 plots and returned to the laboratory.

2.2.2 Fungal Examination and Isolation

Leaves were divided into two stages of decomposition (five leaves for each stage); stage I were recently green or yellow fallen leaves and stage II were mostly decaying brown leaves (Fig. 2.1). Five leaves of each stage were observed. Samples were incubated in 15 cm diameter sterile petri dishes with a tissue paper moistened with sterile distilled water at room temperature (Manoch, 2004). Leaves were examined for fungi after one week, three weeks and six weeks of incubation and fungi occurring on leaf lamina, midrib or petiole were recorded. Fungi were identified based on morphological characters using relevant references (e.g. Carmichael, Kendrick, Conners & Sigler, 1980; Ellis, 1971, 1976; Hanlin 1990, 1998a, 1998b; Nag Raj, 1993; Seifert, Morgan-Jones, Gams & Kendrick, 2011; Sivanesan, 1984; Sutton, 1980). Single spore isolation method was used in fungal isolation (Choi, Hyde & Ho, 1999; Chomnunti et al., 2011). All cultures were grown on potato dextrose agar

(PDA) and malt extract agar (MEA) and deposited in MFLU Culture Collection (MFLUCC) and BIOTEC Culture Collection (BCC).

2.2.3 Data Analysis

The percentage of occurrence of fungi was calculated and fungal taxa with a percentage occurrence higher than 10 are regarded as 'common species' in this study. The formula of fungal percentage occurrence was measured by using the following formula.

$$\text{Percentage occurrence} = \frac{\text{Number of leaves on which fungus was detected}}{\text{Total number of leaves examined}} \times 100$$

Species diversity were compared in each stage of decomposition (stage I and II), three incubation periods (week 1, 3 and 6) and different parts of leaf (leaf lamina, midrib and petiole) using diversity indices to demonstrate the result of species diversity of a community (Shannon & Weaver, 1949). Species richness means the number of fungal species in a community and species evenness means the contribution (relative equability) of individuals (McCune & Grace, 2002). The Shannon index (H') refers to the abundance of species diversity of a community and the Shannon evenness (E') refers to the equability of species diversity, which ranges from 0 to 1. If the Shannon evenness is equal to 1, then every species in the community has the same frequency of occurrence. The Shannon index is calculated according to the equation:

$$H' = -\sum_{i=1}^{n} P_i \log_e P_i \quad \text{, and} \quad P_i = \frac{N_i}{N}$$

Where, N_i is individual number of *i* species, N is individual number of all species, P_i is the proportion of *i* species, n is the number of species.

The Shannon evenness is calculated according to the equation:

E' = H/lnS (S: total species number).

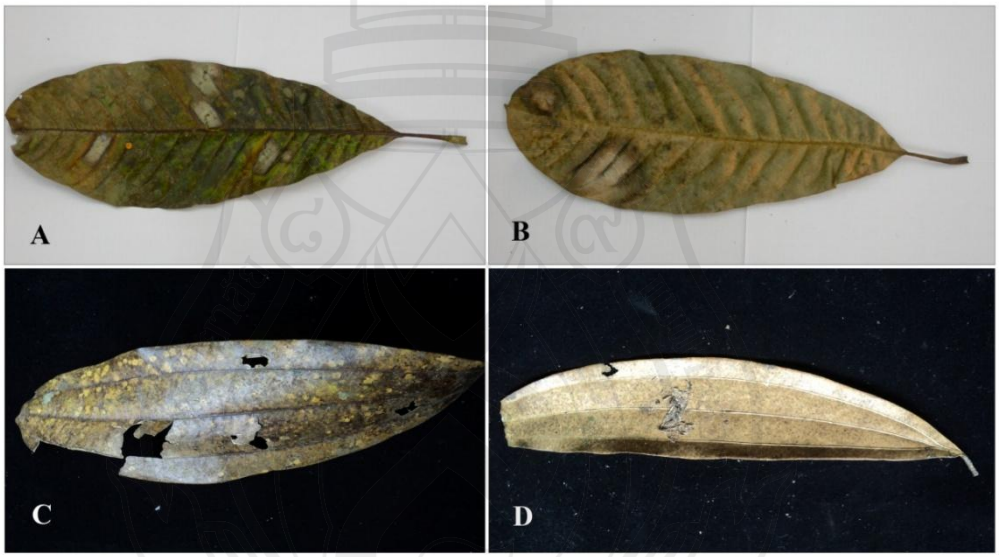
Sørensen's index of similarity was used to compare the similarity of the species on different hosts (Magurran, 1988).

Sørensen's similarity index = 2c/a + b

Where a = the number of species in host A, b = the number of species in host B, c = the number of species in common in both hosts. Similarity is expressed with values between 0 (no similarity) and 1 (absolute similarity).

The overlap fungi from different hosts were calculated using the Sørensen quotient:

$$\text{Overlap (\%)} = \frac{\text{Number of taxa shared between host A and host B}}{\text{Total number of taxa observed in host A and host B}} \times 100$$



Note. (A) Stage of decomposition I of *M. liliifera* leaf. (B) Stage of decomposition II of *M. liliifera* leaf. (C) Stage of decomposition I of *C. iner*s leaf. (D) Stage of decomposition II of *C. iners* leaf.

Figure 2.1 *Magnolia liliifera* and *Cinnamomum iners* Leaves at Each Stage of Decomposition

2.3 Results and Discussion

2.3.1 Diversity of Saprobic Fungi on *Magnolia liliifera* and *Cinnamomum iners*

In this study, fungal diversity on decaying leaves of *M. liliifera* and *C. iners* in the dry season in northern Thailand were investigated. A total of 139 fungal collections were made and 35 taxa were identified from decaying leaves of *M. liliifera*. This comprised 8 ascomycetes (representing 23% of all taxa) and 27 anamorphic fungi (77%) including 11 coelomycetes (31%) and 16 hyphomycetes (46%). The number of fungal collections and their percentage occurrence are shown in Table 2.1. The most abundant species were *Sporidesmium* sp. (80%), *Colletotrichum fructicola* (70%), *Stachybotrys parvispora* (70%), *Dicyma pulvinata* (60%), *Lasiosphaeria*-like sp. 1 (60%) and *Volutella consors* (60%). Diversity indices of fungi on decaying leaves of *M. liliifera* are shown in Table 2.3.

Fungal diversity of *M. liliifera* in the dry season can be compared with previous studies in the wet season (Promputtha et al., 2002, 2004). Number of fungi recorded on a succession study were 22 taxa from 110 leaf samples (Promputtha et al., 2002) and recorded on naturally occurring decaying leaves were 37 taxa from 90 leaf samples (Promputtha et al., 2004). In the present study, 35 fungal taxa were identified from 10 leaf samples. This sample number was shown to be a threshold for studying fungal diversity in *M. liliifera* (Dr. I Promputtha, personal communication). It seems that leaves of *M. liliifera* in the dry season support more fungal taxa than in the wet season. Similar results were reported by Kodsueb et al. (2008), Seephueak et al. (2010) and Seephueak et al. (2011) who found that samples collected in the dry season had greater species richness and higher Shannon diversity index than samples collected in the wet season. There were no seasonal effects of fungal communities on palms in Hong Kong (Yanna & Hyde, 2001) or on *Pandanus penetrans* in Thailand (Thongkantha et al., 2008). The reason is still unclear. Pinnoi et al. (2006) showed that the spore germination and reproduction of fungi required quite high humidity. Rayner and Todd (1979) reported that the wettest period had an unsuitable ratio between moisture content and aeration of wood with relative high moisture and low

aeration. Fungal composition and dominant species reported by Promputtha et al. (2002, 2004) were different from this study. Only a few overlapping genera occurred in both studies (*Colletotrichum*, *Phaeosphaeria*, *Phomopsis*, *Stachybotrys* and *Volutella*) While *S. parvispora* is the only overlapping species between the two studies. Nevertheless, the given name of taxa may be different following sexual-asexual states which were presented on samples. *Clonostachys rosea* was found in this study, whereas *Bionectria ochroleuca*, which is linked to be sexual states of this fungus (Schroers, 2001), was reported by Promputtha et al. (2002, 2004). However, differences in the number of samples and the years of collecting, which varied in temperature, humidity and rainfall make comparisons difficult.

In decaying leaves of *Cinnamomum iners*, 58 fungal collections were made and 17 taxa were identified. This comprised two ascomycetes (representing 11% of all taxa) and 15 anamorphic fungi (89%) including 6 coelomycetes (33%) and 9 hyphomycetes (56%). The number of fungal collections and their percentage occurrence are shown in Table 2.2. The most abundant species were anamorph of *Eutypa* sp. 2 (60%), *Pleurophragmium* sp. (60%), *Acremonium* sp. (40%), *Colletotrichum* sp. (40%) and Hyphomycetes sp. 2 (40%). Diversity indices of fungi on decaying leaves of *C. iners* are shown in Table 2.4.

Only 17 fungal taxa were identified from *C. iners* leaves and most were anamorphic fungi. Lumyong et al. (2002) isolated endophytic fungi from *C. iners* collected from Doi Suthep-Pui National Park, Thailand. The genera of endophytes (Lumyong et al. 2002) were not the same as in our study except for *Colletotrichum* sp. Fungal diversity on *M. liliifera* leaves provides greater species richness and Shannon diversity indices than fungi on *C. iners* leaves (see Table 2.3–2.4). A reason for this may be that leaves of *M. liliifera* are larger than those of *C. iners*. Larger leaves are likely to provide higher species diversity than smaller leaves (Wong & Hyde, 2001). This is consistent with previous studies on hosts such as banana, palm and *M. liliifera*, which supported higher diversity and more diverse taxa (Photita et al., 2001, 2003; Promputtha et al., 2002, 2004; Yanna & Hyde, 2001). Chemical composition of leaves may affect fungal numbers. Leaves of *C. iners* contain essential oils such as eugenol and cinnamaldehyde as major bioactive compounds (Jantan et al., 1992). Reports have shown antimicrobial activities of essential oils from *Cinnamomum*

species (Jantan et al., 1992; Mustaffa, Indurkar, Ismail, Shah & Mansor, 2011; Ranasinghe, Jayawardena & Abeywickrama, 2002). Antifungal tests demonstrated that cinnamaldehyde and eugenol had strong inhibitory effect against wood decay fungi (Cheng, Liu, Hsui & Chang, 2006; Wang, Chen & Chang, 2005), while Yen and Chang (2008) concluded that the synergy of cinnamaldehyde with eugenol could alter cell wall structure of fungi, reduce cell wall synthesis, and the addition of radical scavenging. Such factors may lead to a reduced number of fungi on *C. iners* leaves.

2.3.2 Host Specificity

Overlapping genera on both hosts are anamorph of *Eutypa*, *Colletotrichum*, *Lasiodiplodia* and *Ophioceras*. Only one species, *Lasiodiplodia theobromae*, overlapped on both hosts. Sørensen's similarity index, overlap (%) of fungi from the two hosts are shown in Table 2.5. Few taxa overlapped between two hosts, and this resulted in a low similarity index suggesting that saprobic fungi may be host-specific. Fungal composition between hosts in the different family has been shown to be varied (Wong & Hyde, 2001) and *M. liliifera* and *C. iners* are in different families. Saprobic fungi were less specific at host species level (Wang et al., 2008a). Other factors such as season, location and number of samples might also affect to the host specific of saprobic fungi. Further studies in wet season with other hosts and/or other locations should be done to determine host-specificity of saprobic fungi.

Table 2.1 Number of Decaying Leaves of *Magnolia liliifera* and Percentage Occurrence of Fungi Occurring on Two Stages of Decomposition during Three Incubation Periods and Different Parts of Leaf

Fungal species	Stage I					Stage II					Overall	Overall	Part of leaf					
	W1	W3	W6	TL	%	W1	W3	W6	TL	%	TL	%	L	%-L	M	%-M	P	%-P
Anamorph of *Eutypa* sp. 1						1	2		3	60	3	30	1	10	3	30		
Ascomycetes sp. 1							1		1	20	1	10			1	10	1	10
Ascomycetes sp. 2							1		1	20	1	10	1	10				
Beltrania rhombica		1	1	2	40						2	20			1	10	2	20
Botryosphaeria sp.		1		1	20		1		1	20	2	20			1	10	1	10
Canalisporium caribense							1		1	20	1	10	1	10				
Ceolomycetes sp. 1		1		1	20						1	10	1	10	1	10	1	10
Ceolomycetes sp. 2							1	1	1	20	1	10					1	10
Ceolomycetes sp. 3								1	1	20	1	10			1	10		
Cladosporium sp. 1	1			1	20						1	10	1	10				
Cladosporium sp. 2							1		1	20	1	10			1	10		
Clonostachys compactiuscula		1		1	20						1	10	1	10				
Clonostachys rogersoniana		1		1	20						1	10	1	10	1	10	1	10
Clonostachys rosea		1		1	20						1	10	1	10	1	10	1	10
Colletotrichum fructicola	4	1		4	80	3		1	3	60	7	70	3	30	5	50	2	20
Dicyma pulvinata	5	1		5	100	1			1	20	6	60	6	60				
Fusicoccum aesculi		2	2	2	40		1	1	1	20	3	30			2	20	3	30

Table 2.1 (continued)

Fungal species	Stage I					Stage II					Overall	Overall	Part of leaf					
	W1	W3	W6	TL	%	W1	W3	W6	TL	%	TL	%	L	%-L	M	%-M	P	%-P
Hyphomycetes sp. 1		1		1	20						1	10	1	10				
Lasiodiplodia theobromae		1	2	3	60						3	30			2	20	1	10
Lasiosphaeria-like sp.	4	1		4	80	2			2	40	6	60	6	60				
Montagnula sp.							1	1	2	40	2	20	2	20				
Nodulisporium sp.							1		1	20	1	10					1	10
Ophioceras cf. *leptosporum*		1	1	1	20		2	2	3	60	4	40			1	10	3	30
Pestalotiopsis sp.	1			1	20						1	10	1	10				
Phaeosphaeria sp.	2	1		2	40						2	20	2	20				
Phialophora sp.							1	1	1	20	1	10			1	10	1	10
Phoma sp. 1	1	1	1	3	60	2			2	40	5	50	4	40	1	10	1	10
Phoma sp. 2			1	1	20						1	10	1	10				
Phomatospora sp.			1	1	20						1	10	1	10	1	10		
Phomopsis sp.			1	1	20						1	10					1	10
Sporidesmium sp.		4	5	5	100	1	3	1	3	60	8	80	4	40	6	60	4	40
Stachybotrys parvispora	2	2	3	4	80	2	2	1	3	60	7	70	7	70	3	30	2	20
Stachylidium bicolor							1		1	20	1	10					1	10
Volutella consors		3	3	5	100		1		1	20	6	60	1	10	4	40	2	20
Zygosporium sp.						2			1	20	1	10	1	10	1	10		

Note. W= Week, L = Leaf lamina, M = Midrib, P = Petiole, TL = Total number of leaves, **%** = Percentage occurrence

Table 2.2 Number of Decaying Leaves of *Cinnamomum iners* and Percentage Occurrence of Fungi Occurring on Two Stages of Decomposition during Three Incubation Periods and Different Parts of Leaf

Fungal species	Stage I					Stage II					Overall	Overall	Part of leaf					
	W1	W3	W6	TL	%	W1	W3	W6	TL	%	TL	%	L	%-L	M	%-M	P	%-P
Acremonium sp.		2	2	3	60			1	1	20	4	40	1	10			3	30
Anamorph of *Eutypa* sp. 2	1	2		2	40	1	3	1	4	80	6	60	1	10			6	60
Chaetomium sp.		1		1	20						1	10	1	10				
Coelomycetes sp. 4	1			1	20						1	10	1	10				
Coelomycetes sp. 5		2		2	40						2	20			2	20		
Colletotrichum sp.	1	1	1	2	40		2		2	40	4	40			1	10	3	30
Ellisiopsis occulta	1			1	20						1	10			1	10		
Gliocladium sp.		1		1	20						1	10			1	10		
Graphium penicillioides		1	1	1	20						1	10	1	10				
Graphium sp.							1	2	2	40	2	20	2	20				
Hyphomycetes sp. 3						1	1		1	20	1	10	1	10				
Hyphomycetes sp. 2	2			2	40	2			2	40	4	40	3	30	1	10		
Lasiodiplodia theobromae	1	1	1	2	40	1			1	20	3	30	2	20			1	10
Ophioceras cf. *commune*						1			1	20	1	10	1	10				
Pleurophragmium sp.	3			3	60	3			3	60	6	60	6	60				
Pyrenochaeta sp.		1	1	1	20						1	10	1	10	1	10		
Pyricularia costina	2	1		2	40		1				2	20	1	10			1	10

Note. W= Week, L = Leaf lamina, M = Midrib, P = Petiole, TL = Total number of leaves, **%** = Percentage occurrence

Table 2.3 Diversity Indices of Fungi from *M. Liliifera* at Two Stages of Decomposition, Three Incubation Periods and Different Parts of Leaf

	Stage I				Stage II				L	M	P
	W 1	**W 3**	**W 6**	**Total**	**W 1**	**W 3**	**W 6**	**Total**			
Species richness	8	18	11	23	8	15	9	21	22	20	19
Species evenness	20	25	21	88	14	20	10	44	48	38	30
Shannon indices	1.9	2.75	2.22	2.84	2	2.62	2.2	2.9	2.79	2.75	2.81
Shannon evenness	0.84	0.9	0.83	0.74	0.93	0.92	0.97	0.84	0.73	0.8	0.88

Note. W= Week, L = Leaf lamina, M = Midrib, P = Petiole

Table 2.4 Diversity Indices of Fungi from *C. iners* at Two Stages of Decomposition, Three Incubation Periods and Different Parts of Leaf

	Stage I				Stage II				L	M	P
	W 1	**W 3**	**W 6**	**Total**	**W 1**	**W 3**	**W 6**	**Total**			
Species richness	8	10	5	14	6	5	3	9	13	6	5
Species evenness	12	13	6	24	9	8	4	17	22	7	24
Shannon indices	1.98	2.25	1.56	2.56	1.67	1.5	1.04	2.1	2.32	1.75	1.4
Shannon evenness	0.9	0.94	0.95	0.92	0.9	0.9	0.94	0.9	0.8	0.95	0.81

Note. W= Week, L = Leaf lamina, M = Midrib, P = Petiole

Table 2.5 Sørensen's Similarity Index and Overlap (%) of Fungi from *M. liliifera* and *C. iners*

	Overlapping genera	**Overlapping species**
Sørensen's similarity index	0.04	0.15
Overlap (%)	1.92	7.69

2.3.2 Effect of Decomposing Stages and Incubation Periods on Fungal Communities

The decomposition process by saprobic fungi follows three succession stages: the pioneer (early) stage, mature (middle) stage and the impoverished (later) stage (Dix & Webster, 1985; Yanna & Hyde, 2002). Pioneer communities are generally of low fungal diversity and have few species occurring at high percentage occurrence (Dix & Webster, 1985). Mature communities have high fungal diversity and have many species occurring at high percentage occurrence and some species become obviously dominant (Dix & Webster, 1985; Promputtha et al., 2002). Eventually, the species diversity and the number of species declines in impoverished communities (Dix & Webster, 1985). This hypothesis is supported by our results showing different fungal communities between two hosts on each stage of decomposition and incubation periods. The fungi present at each stage of decomposition of *M. liliifera* are listed in Table 2.6. On *M. liliifera*, dominant species at stage I of decomposition were *D. pulvinata* (100%), *Sporidesmium* (100%) and *V. consors* (100%). The most abundant species at stage II of decomposition were anamorph of *Eutypa* sp. 1 (60%), *C. fructicola* (60%), *Sporidesmium* (60%), *Ophioceras* cf. *leptosporum* (60%) and *S. parvispora* (60%). The fungi present at each stage of decompositions on *C. iners* are shown in Table 2.7. On *C. iners*, the most abundant species at stage I of decomposition were *Acremonium* sp. (60%), *Pleurophragmium* sp. (60%), anamorph of *Eutypa* sp. 2 (40%), Coelomycetes sp. 5 (40%), *Colletotrichum* sp. (40%), Hyphomycetes sp. 2 (40%), *L. theobromae* (40%) and *Pyricularia costina* (40%). The most abundant species in stage II of decomposition were anamorph of *Eutypa* sp. 2

(80%), *Pleurophragmium* sp. (60%), *Colletotrichum* sp. (40%), *Graphium* sp. (40%) and Hyphomycetes sp. 2 (40%).

On *M. liliifera* in both stages of decomposition, the highest number and diversity of fungi were found after 3 weeks incubation (Table 2.3). On the other hand, on *C. iners*, number and diversity of fungi were distinct on each stage of decomposition. Stage I of decomposition showed a trend of abundant fungi on different incubation periods same as in *M. liliifera.* However, on stage II of decomposition, the highest number and diversity of fungi were found after 1 week of incubation and declined after 3 and 6 weeks of incubation (Table 2.4). From these results, it can be assumed that decaying process of *C. iners* leaves might be slower than *M. liliifera* leaves. However, this study used moist chamber method for examination of fungal diversity, and the leaves did not come from natural habitat as in other succession studies. So, in forest ecosystem, saprobic fungi also need environmental factors, such as pH, temperature and moisture for growing (Osono, Fukasawa. & Takeda, 2003).

The replacement of fungal species composition sequentially throughout decomposing process relies on the capability of decomposers to utilize organic matter and nutrients, which are particular to each substrate or host (Frankland, 1992; Tang et al., 2005). In early stage, decomposer fungi might switch their roles from endophyte and pathogen (Lodge & Cantrell, 1995; Duong et al., 2008). The evidence that endophytic fungi change to be saprobes has been reported in many previous studies (Koide et al., 2005; Osono et al., 2004; 2009; Promputtha et al., 2010; Purahong & Hyde, 2011; Tang et al., 2005). Promputtha et al. (2010) also proved that endophytes can produce various degrading enzymes in succession process, which is an important activity for their adaptation to saprobic lifestyle. The primary enzymes degrade small soluble carbon-based molecules, such as hemicelluloses and the most complex cellulose and lignin are then degraded at the late stage of decomposition (McClaugherty & Berg 1987; Promputtha et al., 2010). In addition, soilborne and airborne fungi can colonize fallen leaves (Duong et al., 2008) so that leaves which have been on the forest floor for a long time might have higher fungal diversity than leaves which have recently fallen. In this study, decaying leaves of *M. liliifera* and *C. iners* in stage I of decomposition supported greater fungal diversity than in stage II of

decomposition (Table 2.3–2.4) suggesting that endophytic fungi might be play an important role in the decay of dead leaves and these fungi are different at different stages of decay. Shanthi and Vitthal (2010) studied leaf litter fungi of *Pavetta indica* on freshly fallen senescent leaves (grade 1) and on leaves already undergoing active decomposition (grade 2) and found more taxa on grade 1 than on grade 2 litter. Seephueak et al. (2010, 2011) studied fungi on different stages of decaying leaf and branch litter of the rubber tree. They showed that the number of taxa on middle stage decaying branches was higher than new and old decaying fallen branches

Table 2.6 Fungal Species Appearing on Each Stage of Decomposition on *M. liliifera* Leaves

Fungal species appearing on both stages of decomposition	**Fungal species appearing on stage I of decomposition**	**Fungal species appearing on stage II of decomposition**
Botryosphaeria sp.	*Beltrania rhombic*	*Phialophora* sp.
Colletotrichum fructicola	Ceolomycetes sp. 1	Anamorph of *Eutypa* sp. 1
Dicyma pulvinata	*Phoma* sp. 2	Ascomycetes sp. 1
Fusicoccum aesculi	*Cladosporium* sp. 1	Ascomycetes sp. 2
Lasiosphaeria-like sp.	*Clonostachys rogersoniana*	*Canalisporium caribense*
Ophioceras cf. *leptosporum*	*Lasiodiplodia theobromae*	Ceolomycetes sp. 2
Phoma sp. 1	Hyphomycetes sp. 1	Ceolomycetes sp. 3
Sporidesmium sp.	*Clanostachys compactiuscula*	*Nodulisporium* sp.
Stachybotrys parvispora	*Pestalotiopsis* sp.	*Cladosporium* sp. 2
Volutella consors	*Phaeosphaeria* sp.	*Montagnula* sp.
	Phomatospora sp.	*Stachylidium bicolor*
	Phomopsis sp.	*Zygosporium* sp.
	Clonostachys rosea	
Number species = 10	**Number species = 13**	**Number species = 12**

Table 2.7 Fungal Species Appearing on Each Stage of Decomposition on *C. iners* Leaves

Fungal species appearing on both stages of decomposition	**Fungal species appearing on stage I of decomposition**	**Fungal species appearing on stage II of decomposition**
Acremonium sp.	*Chaetomium* sp.	*Graphium* sp.
Anamorph of *Eutypa* sp. 2	Coelomycetes sp. 4	Hyphomycetes sp. 3
Colletotrichum sp. 1	Coelomycetes sp. 5	*Ophioceras* cf. *commune*
Hyphomycetes sp. 2	*Ellisiopsis occulta*	
Lasiodiplodia theobromae	*Gliocladium* sp. 1	
Pleurophragmium sp.	*Graphium penicillioides*	
	Pyrenochaeta sp.	
	Pyricularia costina	
Number species = 6	**Number species = 8**	**Number species = 3**

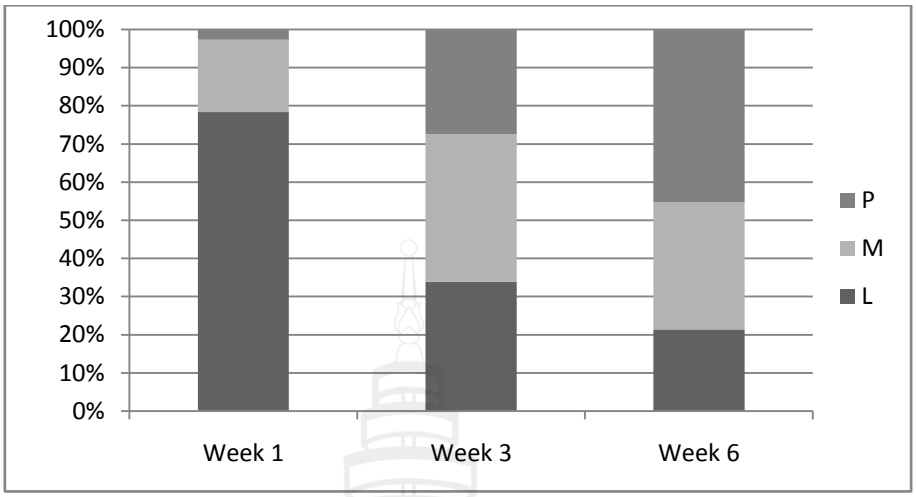
2.3.3 Effect of leaf tissue types on fungal communities

On *M. liliifera*, the most abundant species on the leaf lamina were *S. parvispora* (70%), *D. pulvinata* (60%), *Lasiosphaeria-like* sp. 1 (60%), *Sporidesmium* (40%) and *Phoma* sp. 1 (40%). The most abundant species in the midribs were *Sporidesmium* (60%), *C. fructicola* (50%), *V. consors* (40%), anamorph of *Eutypa* sp. 1 (30%) and *S. parvispora* (30%). The most abundant species on the petioles were *Sporidesmium* (40%), *Fusicoccum aesculi* (30%) and *Ophioceras* cf. *leptosporum* (30%). On *C. iners*, most abundant species on the leaf lamina were *Pleurophragmium* (60%) and Hyphomycetes sp. 2 (30%). The most abundant species in the midribs were Coelomycetes sp. 5 (20%). The most abundant species in the petioles were anamorph of *Eutypa* sp. 2 (60%), *Acremonium* sp. (30%) and *Colletotrichum* sp. (30%).

Abundance of fungi on different parts of leaf were distinct on each host. Leaf lamina provides the largest surface area and may support a greater number of fungal species than other parts of leaf (Promputtha et al., 2002). As expected, leaf lamina provided the highest number and diversity of fungi on *M. liliifera* and *C. iners* (Table

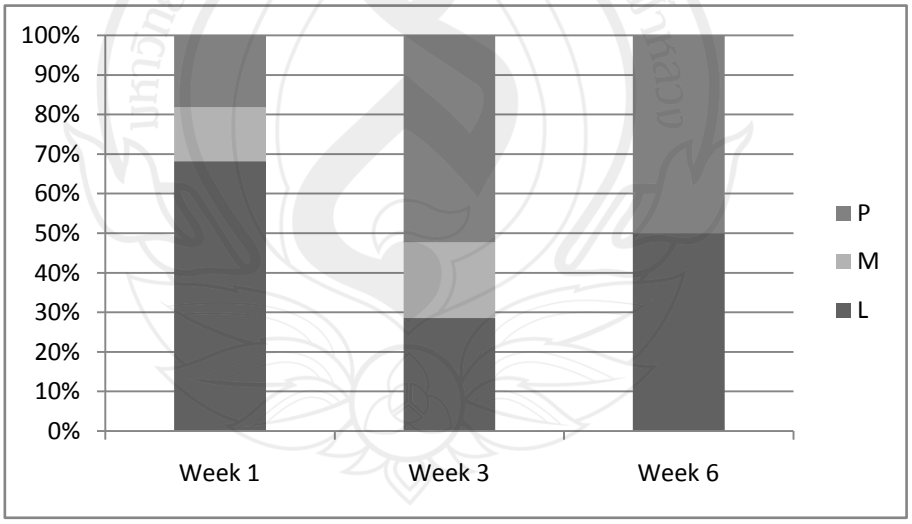
2.3-2.4). In addition, number of fungi occurring on each incubation periods was different among part of leaf (Fig. 2.2–2.3). In early week of incubation, leaf lamina provided the higher number of fungi than midribs and petioles. Then, number of fungi on the leaf lamina decreased, while number of fungi on midribs and petioles increased in later week of incubation of *M. liliifera* (Fig. 2.2). But in later week of *C. iners*, number of fungi on the leaf lamina still increased (Fig. 2.3). It may be assumed that primary fungal saprobes prefer to colonize leaf lamina, and later fungi are able to colonize midrib and petiole. Structure and moisture content of different tissue types may influence the presence of saprobic fungi. The leaf lamina contains thin-walled parenchymatous cells and little moisture. Midrib and petiole have more sclerenchyma cells that are thick-walled supporting more nutrients for fungal growth (Pinnoi et al., 2006). Petioles contain vascular bundles, which may keep moisture for a longer time (Fisher, Tan & Toh, 2002). This may result in more fungi on midribs and petioles in the later period.

Some fungi can grow in every part of the leaf, some grow only on leaf lamina and/or midrib and/or petiole. Some fungi are restricted to a single tissue type. For example, on *M. liliifera*, Ceolomycetes sp. 3 and *Cladosporium* sp. 2 were found only on midrib. Coelomycetes sp. 2, *Nodulisporium* sp., *Phomopsis* sp. and *Stachylidium bicolor* were found only on petiole. On *C. iners*, Coelomycetes sp. 5, *Ellislopsis occulta* and *Gliocladium* sp. were found only on midrib. These results indicate that saprobic fungi are specific to tissue type. The recurrence of saprobic fungi on certain tissues has been also observed with other hosts (Duong et al., 2008; Photita et al., 2001, 2003; Pinnoi et al., 2006; Promputtha et al., 2002, 2004; Yanna & Hyde, 2001). Yanna and Hyde (2001) found that different frond parts of palms supported distinct fungal communities on most samples. The tissue specificity of saprobic fungi may be due to differing nutritional requirements and enzymatic capabilities to utilize different substrates (Adaskaveg, Blanchette & Gilbertson, 1991; Photita et al., 2003; Yanna & Hyde, 2001).



Note. P = Petioles, M = Midrib, L = Leaf lamina

Figure 2.2 Percentage of Fungi Occurring on Different Parts of Leaf of *M. liliifera* at Three Incubation Periods



Note. P = Petioles, M = Midrib, L = Leaf lamina

Figure 2.3 Percentage of Fungi Occurring on Different Parts of Leaf of *C. iners* at Three Incubation Periods

2.5 Conclusion

Different host species supported different fungal communities and number of fungal taxa. *M. liliifera* had greater diversity of leaf litter fungi than *C. iners*. Due to a low number of overlapping taxa between two hosts, this result suggested that saprobic fungi may be host-specific. The stages of decomposition, incubation periods and parts of leaf also appeared to affect fungal communities on each host. For both hosts, leaves on early stage of decomposition supported higher fungal diversity than leaves on later stage of decomposition. Saprobic fungi were specific to different tissues types (leaf lamina, midrib, petiole). This study provided an insight on biodiversity data of saprobic fungi in Thailand.

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CHAPTER 3

POSSIBLE NEW FUNGI DISCOVERED FROM THIS STUDY

3.1 Introduction

Systematics is the study of biological diversity and classification of organisms which include many subjects such as nomenclature, taxonomy and phylogeny (Kirk et al., 2008; Rossman & Palm-Hernández, 2008). Generally, individual organism is grouped into taxonomic ranks comprising Kingdom, Division, Class, Order, Family, Genus and Species. The importance of systematics are evident in conservation, environmental monitoring, agriculture, biotechnology and geological prospecting (Hawksworth, 1988). Fungal systematics is traditionally based on morphological characters. Nowadays, gene sequencing and molecular data are increasingly being used to study the phylogeny of fungi and to determine concepts for species and relationships among taxa. Combination of morphological and genetic data has been successfully used in describing new genera and species (Rossman & Palm-Hernández, 2008). Nomenclature is a part of systematics that defines the correct scientific name for a taxon. The naming of fungi is currently governed by the International Code of Botanical Nomenclature for algae, fungi, and plants (ICN). According to the International Botanical Congress in Melbourne in July 2011, anamorphic and teleomorphic names would be revised based on the "one name one fungus" concept (Hawksworth, 2011).

In this study, the diversity of fungi on leaf litter of *Magnolia liliifera* and *Cinnamomum iners* in Doi Suthep-Pui forest were investigated. The study revealed 52 taxa and three taxa are probably new to science. These taxa are described and illustrated in this chapter. Sequence data of these strains were also made and blasted

in GenBank to reveal the closest matches. The new taxa mentioned in this chapter will remain invalid as they are not named to species level (Seifert & Rossman, 2010).

3.2 Materials and methods

Fungi were isolated from leaf litter of *Magnolia liliifera* and *Cinnamomum iners*. For methodology refer to the previous section in chapter 2. Type specimens were deposited in MFLU Herbarium, Mae Fah Luang University, Thailand.

Genomic DNA was extracted from the fungal mycelium (grown on PDA for 7 days at 28 ^{o}C in the dark) by using modified CTAB protocol as previously described by Guo, Hyde and Liew (2000). The amplification of the internal transcribed spacers (ITS) and the large subunit rDNA (LSU) were carried out using primers ITS5/ ITS4 and LROR/ LR6 (Vilgalys & Hester, 1990; White, bruns, Lee & Taylor, 1990). The PCR reaction was carried out in 50 µl consisting of 35.7 µL sterilized water, 5 µL 10X buffer, 4 µL of 10 mM dNTPs, 2 µL of each forward and reverse primer, 0.3 µL of 5U/ µL Taq Polymerase, and 1 µL DNA template. The PCR conditions used initial denaturation at 95 ^{o}C for 3 mins, followed by 30-35 cycles of 30 sec denaturation at 95°C, 30 sec primer annealing at 52°C-54°C, 1 min extension at 72°C and a final extension step of 72°C for 10 mins. The PCR products were estimated visually by staining with red gel on 1% agarose gel electrophoresis for purity and sent for sequencing by the Sinogenomax Company, Beijing, China. The nucleotide sequences of ITS and LSU region were submitted to GenBank and blast searches were made to reveal the closest matches in GenBank.

3.3 Results

3.3.1 Ascomycete sp. 1

Ascomata solitary, immersed to semi-immersed, becoming erumpent, perithecial, black, papillate. *Papilla* central, black, conical. *Paraphyses* widest at the base up to 8.0 µm and narrowest at the end up to 2.7 µm, septate, unbranched. *Asci*

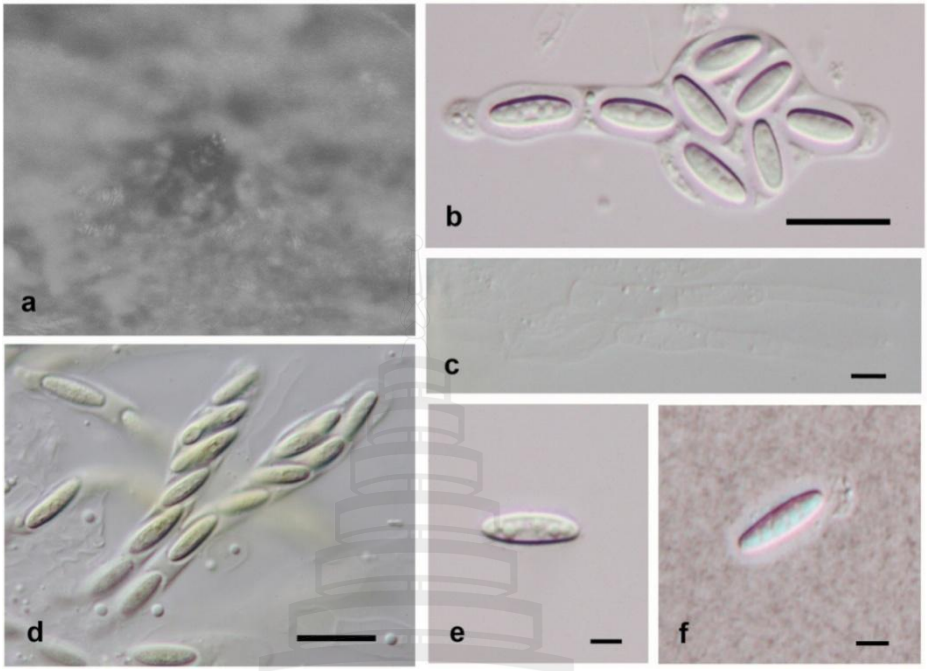
47-61 × 5 -10 µm ($\bar{x}$ = 52.9 × 7.8 µm), 8-spored, unitunicate, cylindrical, clavate, swollen in the centre in squash mount, with a short pedicel, no apical ring observed in Melzer's reagent. *Ascospores* 8.1-11.3 × 2.4-3.4 µm ($\bar{x}$ = 2.8 × 9.2 µm), uniseriate at the base, overlapping uniseriate to biseriate in main part, cylindrical with slightly narrowing ends, hyaline, one-celled, with several guttulates, rough-walled, surrounded by a gelatinous sheath, 1.5- 2.4 thick ($\bar{x}$ = 1.9 µm).

Asexual morph: present on cultures

Cultures: MFLUCC0282

Colonies on potato dextrose agar effuse, mostly superficial, white, 10 mm diam in 15 days at 28 ºC. *Mycelium* mucoid, irregular, crenated edge, no pigment diffusing into agar, produce anamorphic stage. *Conidiophore* hyaline, branching, tightly aggregate along the lengh. *Conidiogenous cell* polyphialidic. *Conidia* broadly ellipsoidal, concave in the middle and thickening toward the end, one cell, hyaline, formed in chain.

Holotype: Thailand: Chiang Mai, Doi Suthep-Pui forest, on dead leaves of *Magnolia liliifera*, 11 January 2010, Jutamart Monkai, (MFLU12-2222).



Note. (a) Ascoma on the host surface. Note the papilla. (b) Ascus in wet mount slide by squashing. (c) Paraphyses. (d) Asci in Melzer's reagent with no visible apical ring. (e) Ascospore. (f) Ascospore in India ink. Scale bars: (b)-(d) = 10 µm, (c) = 5 µm, (e)-(f) = 3 µm

Figure 3.1 Ascomycete sp. 1

3.3.1 Hyphomycete sp. 2

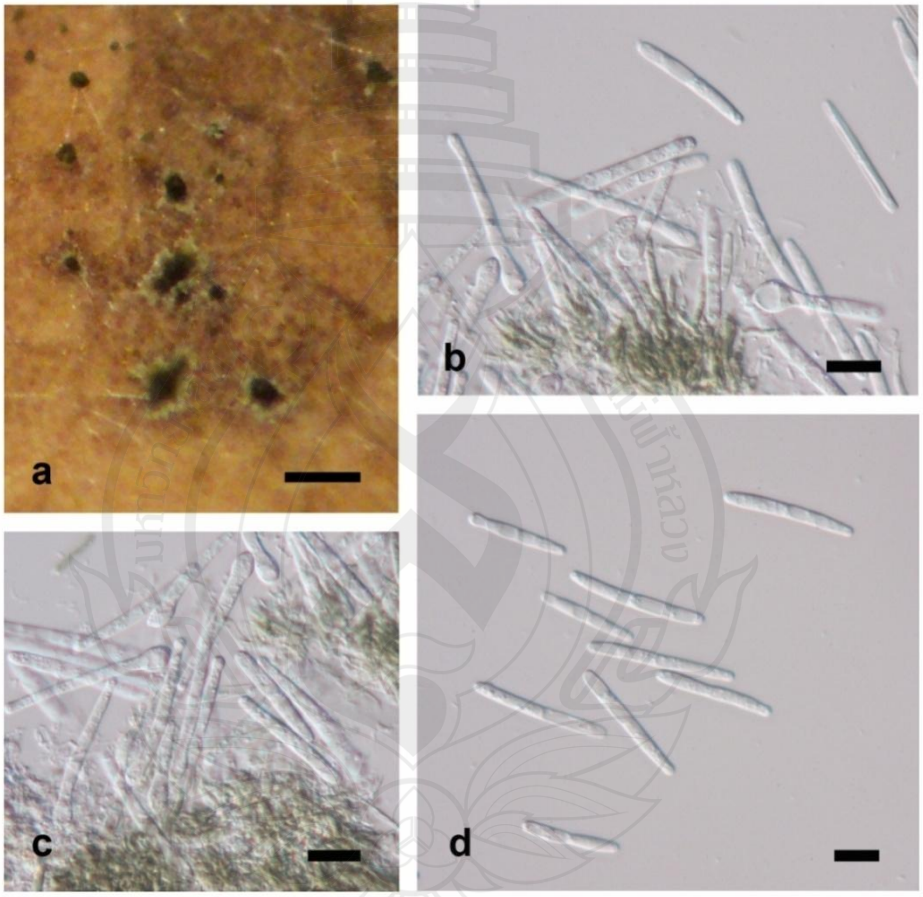
Colonies on natural substrate in the form of sporodochia, 100-214 × 29-157 µm ($\bar{x}$ = 161.8 × 80.9 µm) and 14-42 µm high ($\bar{x}$ = 27.4 µm), superficial, solitary to gregarious, irregular, mucous, blackish green to black, surrounded by a white zone . *Mycelium* immersed, green to blackish green. *Conidia* 19-46 × 2-4 µm ($\bar{x}$ = 30.3 × 3.1 µm), some swollen at the base 28-44 long × 3-4 wide µm ($\bar{x}$ = 34.9 × 3.2 µm), 4-7 µm wide at the base ($\bar{x}$ = 5.5 µm), filiform to cylindrical, hyaline, one celled, guttulate, verrucose.

Sexual morph: unknown

Cultures: MFLUCC10-0750

Colonies on potato dextrose agar effuse, mostly superficial, white and pale brown, 5 cm diameter in 7 days at 28 ºC. Mycelium circular, edge fimbriate, thinly hairy in the center, no pigment diffusing into agar. Conidiophores hyaline, septate, branching. Conidiogenous cell enteroblastic, monotretic. Conidia variously shaped, ellipsoid, lemoniform, fusiform, have distinct pointed ends, one celled, hyaline.

Holotype: Thailand: Chiang Mai, Doi Suthep-Pui forest, on dead leaves of *Cinnamomum iners*, 24 April 2010, Jutamart Monkai, (MFLU12-2223)



Note. (a) Sporodochium on natural substrate. (b)-(c) Squash mount of conidiophores and conidia. (d) Conidia. Scale bars: (a) = 150 µm, (b)-(d) = 10 µm

Figure 3.2 Hyphomycete sp. 2

3.3.3 Hyphomycetes sp. 3

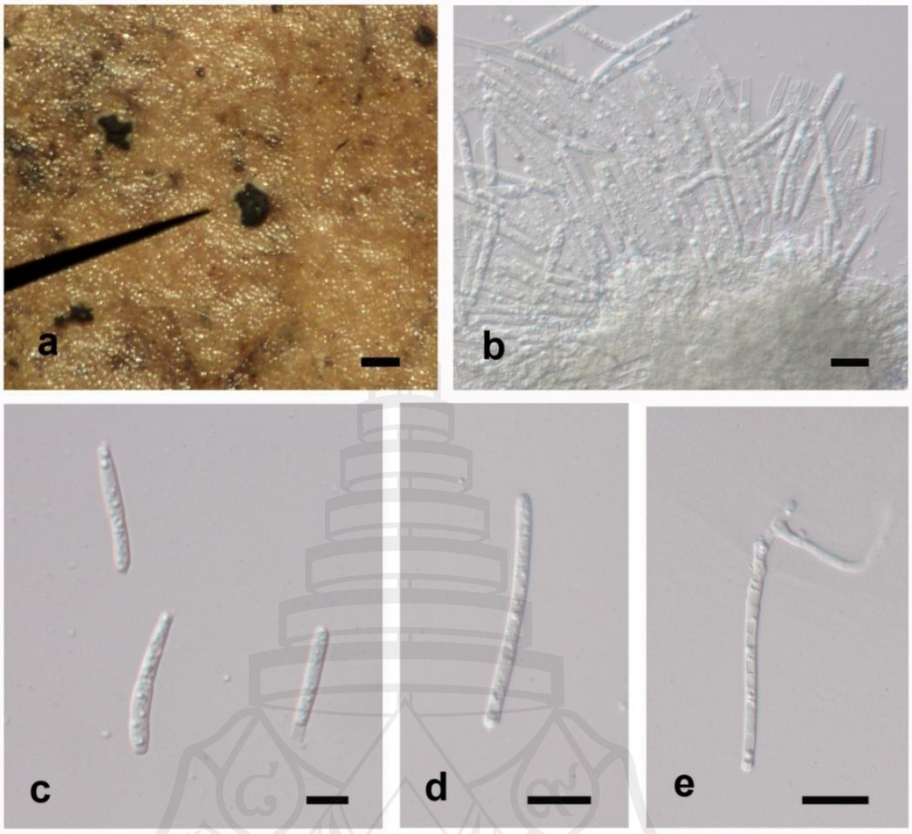
Colonies on natural substrate in the form of sporodochia, 100 -200 × 57-128.5 µm ($\bar{x}$ = 146.4 × 89.3 µm) and 14 × 43 µm high ($\bar{x}$ = 29.5 µm), superficial, solitary to gregarious, irregular, mucous, black , surrounded by a white zone. *Mycelium* immersed, grey to black. *Conidia* 25.3-43.9 × 2.1-3.5 µm ($\bar{x}$ = 33.3 × 2.9 µm), filiform to cylindrical, hyaline, one cell, many guttulate, verrucose.

Sexual morph: unknown

Cultures: MFLUCC10-0755

Colonies on potato dextrose agar effuse, mostly superficial, white and pale brown, 5 cm diameter in 7 days at 28 ºC. Mycelium circular, fimbriate edge, thinly hairly, no pigment diffusing into agar.

Holotype: Thailand: Chiang Mai, Doi Suthep-Pui forest, on dead leaves of *Cinnamomum iners*, 24 April 2010, Jutamart Monkai, (MFLU12-2224).



Note. (a) Sporodochium on natural substrate. (b) Squash-mount of conidiophores and conidia. (c)-(d) Conidia. (e) Germinating conidium. Scale bars: (a) = 150 µm, (b)-(e) = 10 µm

Figure 3.3 Hyphomycete sp. 3

In addition, the sequences of the ITS and LSU of these fungi were obtained as shown in Table 3.1. Blast analysis was performed to reveal the closely related species of these fungal strains (Table 3.1).

Table 3.1 The blast searches results of sequences data on ITS and LSU gene of new fungal strains (show only the closest matches results)

Strains	GenBank accession number		Blast match (% Identity) (Accession number)
	ITS	LSU	
Ascomycete sp.1 (MFLUCC 10-0282)	KC775774		*Acremonium furcatum* strain AM01 (99%) (JN596334.1) *Acremonium furcatum* strain JZ-48 (99%) (HQ637291.1) *Acremonium furcatum* strain P266_D2_27 (99%) (JF311939.1) *Acremonium furcatum* strain P266_D1_10 (99%) (JF311914.1) Hypocreales sp. r146 (99%) (HQ649868.1) Hypocreales sp. r382 (99%) (HQ649873.1) *Acremonium furcatum* culture-collection CABI:IMI212109 (99%) (JQ647429.1) *Acremonium furcatum* CBS 122.42 (99%) (AY378154.1) Hypocreales sp. r335 (99%) (HQ649871.1) Hypocreales sp. r174 (99%) (HQ649869.1) Hypocreales sp. r394 (99%) (HQ649874.1) Hypocreales sp. r180 (99%) (HQ649870.1) *Acremonium furcatum* strain CBS 116550 (99%) (DQ825975.1) Hypocreales sp. r354 (99%) (HQ649872.1) *Acremonium furcatum* isolate 21/1.3.1 (99%) (DQ865092.1) *Acremonium furcatum* strain AM04 (99%) (JN596337.1) Acremonium sp. LX M8-2 (99%) (HE664157.1)

Table 3.1 (continued)

Strains	GenBank accession number		Blast match (% Identity) (Accession number)
	ITS	LSU	
Ascomycete sp.1 (MFLUCC 10-0282)		KC775775	*Gibellulopsis nigrescens* strain DAOM 226890 (98%) (GU180648.1) *Acremonium nepalense* strain CBS 971.72 (98%) (HQ231970.1) *Acremonium cucurbitacearum* strain CBS 683.88 (98%) (HQ231968.1) *Plectosphaerella* sp. REF168 (98%) (JN859490.1) *Plectosphaerella cucumerina* strain DAOM 226828 (98%) (GU180647.1)
Hyphomycete sp. 2 (MFLUCC 10-0750)	KC775776		*Myrothecium setiramosum* strain CBS 534.88 (91%) (AY254156.1) *Myrothecium* sp. P066 (90%) (EF423537.1) *Myrothecium roridum* strain BCRC 34581 (88%) (GU929191.1) *Myrothecium lachastrae* strain IMI 273160 (88%) (AY254159.1) *Myrothecium roridum* strain MA-20 (88%) (JF724152.1) *Myrothecium roridum* strain A-332 (88%) (KC140224.1) *Myrothecium roridum* strain CGMCC 3.1962 (88%) (FJ231214.1)
Hyphomycete sp. 2 (MFLUCC 10-0750)		KC775777	*Didymostilbe echinofibrosa* strain AR2824 (97%) (AY489706.1) *Albosynnema elegans* GB3101 (97%) (AF193226.1) *Peethambara spirostriata* strain CBS110115 (97%) (AY489724.1) *Peethambara sundara* CBS646.77 (97%) (AF193245.1) *Peethambara sundara* strain CCFC52196 (97%) (AY283546.1)

Table 3.1 (continued)

Strains	GenBank accession number		Blast match (% Identity) (Accession number)
	ITS	LSU	
Hyphomycete sp. 3 (MFLUCC 10-0755)	KC775778		*Myrothecium setiramosum* strain CBS 534.88 (91%) (AY254156.1) *Myrothecium* sp. P066 (90%) (EF423537.1) *Myrothecium roridum* strain BCRC 34581 (88%) (GU929191.1) *Myrothecium lachastrae* strain IMI 273160 (88%) (AY254159.1) *Myrothecium roridum* strain MA-20 (88%) (JF724152.1) *Myrothecium roridum* strain A-332 (88%) (KC140224.1) *Myrothecium roridum* strain CGMCC (88%) (FJ231214.1)
Hyphomycete sp. 3 (MFLUCC 10-0755)		KC775779	*Myrothecium setiramosum* strain CBS 534.88 (91%) (AY254156.1) *Myrothecium* sp. P066 (90%) (EF423537.1) *Myrothecium roridum* strain BCRC 34581 (88%) (GU929191.1) *Myrothecium lachastrae* strain IMI 273160 (88%) (AY254159.1) *Myrothecium roridum* strain MA-20 (88%) (JF724152.1) *Myrothecium roridum* strain A-332 (88%) (KC140224.1) *Myrothecium roridum* strain CGMCC (88%) (FJ231214.1)

3.4 Conclusion

The three unidentified fungi show different morphological characters from other fungal genera, so these fungi might be possible new taxa. Ascomycete sp. 1 has perithecial ascomata, unitunicate asci with no apical apparatus and gelatinous sheath ascospore, which are most similar to the member of *Nectriaceae* (Rossman, Samuels, Rogerson & Lowen, 1999). Hyphomycete sp. 2 and Hyphomycete sp. 3 are most similar to *Myrothecium* in having sporodochia, with mucous, and green to black masses of conidia which is usually surrounded by a white zone (Ellis, 1971). But *Myrothecium* has branched and penicillately arranged conidiophores (Ellis, 1971), whereas Hyphomycete sp. 2 and Hyphomycete sp. 3 did not show coniodiophores clearly on the leaf samples. Therefore, it is generally difficult to identify these fungi to the genus level. The nucleotide blast results are still not enough to use for identification. Molecular phylogenetic is needed to help identify these fungal taxa in the future.

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CHAPTER 4

STUDY OF TYPE SPECIMENS ON *Planistromellaceae*

4.1 Introduction

The class *Dothideomycetes* contains the largest species numbers and is the most phylogenetically diverse group in the phylum *Ascomycota*. Development in this group is ascolocular and asci are bitunicate (Kirk et al., 2008). Previously, the classification of *Dothideomycetes* was determined using morphological characters such as ascomatal characters, the type of pseudoparaphyses and anamorphic states (Arx Von & Müller, 1975; Barr, 1987; Boonmee et al., 2011, 2012; Eriksson, 1981; Liu et al., 2011, 2012; Luttrell, 1955; Zhang et al., 2011). Several studies have focused on molecular phylogenies of *Dothideomycetes* to elucidate the confusing classification which has resulted from using morphological characters (Berbee, 1996; Chomnunti et al., 2011, 2012b; Lindemuth, Wirtz & Lumbsch, 2001; Lumbsch & Lindemuth, 2001; Schoch et al., 2009; Silva-Hanlin & Hanlin, 1999; Zhang, Crous, Schoch & Hyde, 2012). These studies showed that the *Dothideomycetes* is not monophyletic. Recent phylogenetic analyses using multigene data have shown that *Dothideomycetes* comprise several lineages among a class wide context (Chomnunti, Bhat, Jones, Chukeatirote & Bahkali, 2012a; Chomnunti et al., 2011, 2012b; Schoch et al., 2006, 2009;).

The *Planistromellaceae* was introduced by Barr (1996) with the generic type *Planistromella*. Presently, the genera *Comminutispora*, *Eruptio*, *Loratospora*, *Microcyclus*, *Mycosphaerellopsis*, *Planistroma* and *Planistromella* are included in this family (Lumbsch & Huhndorf, 2010). Species of these genera usually grow on living or dead leaves or on stems of various plants, and are mostly saprobes, but some species, especially in the asexual genera, are pathogens (Barr, 1996; Evans, 1984;

Kohlmeyer & Volkmann-Kohlmeyer, 1993; Lieberei, 2007; Minnis, Kennedy, Grenier, Palm & Rossmana, 2012; Ramaley, 1991, 1992, 1993, 1995, 1998; Sivanesan & Shivas, 2002). The important morphological characters of *Planistromellaceae* were deemed to be multi- or uniloculate ascostromata, locules opening by cracking or splitting of host tissue, or periphysate ostioles, and bitunicate asci interspersed with interthecial tissues. Asexual genera included *Aposphaeria*-like, *Fusicladium*, *Hyphospora*, *Kellermania*, *Lecanosticta*, *Pazschkeella* and *Piptarthron* (Wijayawardene, Mckenzie & Hyde, 2012). These taxa were thought to be similar to members of the *Pseudosphaeriaceae* in having multi or uniloculate ascostromata and in lacking any true peridial structure. In *Pseudosphaeriaceae*, the locules opening by a simple, lysigenous pore or by dehiscence of a cap-like structure. The *Mycosphaerellaceae* also resembles genera of *Planistromellaceae* especially ascus, ascospore and anamorph morphology. The classification of *Eruptio*, *Loratospora*, *Microcyclus* and *Planistroma* is confused as morphological characters are quite similar to other related genera. Recently, molecular phylogeny has validated some members of the family. However, molecular data is only available for a few genera including *Comminutispora* (Tsuneda, Isuneda & Currah, 2004), *Eruptio* (Verkley, Starink-Willemse, Van Iperen & Abeln, 2004), *Hyphospora* (Sterflinger, Hoog & Haase, 1999), *Kellermania* (Minnis et al., 2012), *Lecanosticta* (Crous et al., 2009a, 2009b; Crous, Kang & Braun, 2001), *Loratospora* (Suetrong et al., 2009), *Microcyclus* (Chee & Holiday, 1986; Le Guen et al., 2004) and *Piptarthron* (Minnis et al., 2012).

The purpose of the present study is to re-examine the type specimens of the sexual states of this family. Sequence data from GenBank were downloaded to provide a tree that shows taxonomic placement of the members of this family. A new treatment for this family, re-describe the genera and link the asexual states were provided where possible, and the excluded genera previously placed in the family by Lumbsch and Huhndorf (2010) were relocated to other families.

4.2 Materials and methods

4.2.1 Examination of Herbarium Materials

Type specimens of genera were obtained from the Herbaria BPI, DAOM, IMI, K, S and UC. The herbarium specimens were rehydrated in 5% KOH prior to examination. Ascomata were sectioned by free-hand under a Motic SMZ 168 Series microscope. Morphological characters were studied using a Nikon ECLIPSE 80i microscope with a Canon 450D digital camera. The measurements were made using Tarosoft (R) Image Frame Work program (Liu et al., 2010).

4.2.2 Phylogenetic Analysis

The reference nucleotide sequences of ITS and LSU regions of various taxa were obtained from GenBank (Table 4.1). Fungal members from different genera of the *Botryosphaeriales* and related orders were included in the analyses. Sequences were aligned using Bioedit (Hall, 1999) and ClustalX v. 1.83 (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997). The alignments were checked visually and improved manually where necessary. Phylogenetic analyses were performed by using PAUP v. 4.0b10 (Swofford, 2002) for Maximum-parsimony (MP) and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003) for Bayesian analyses.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull, 1993). The phylogram with bootstrap values above the branches is presented in Fig. 4.1 by using graphical options available in TreeDyn v. 198.3 (Chevenet, Brun, Bañuls, Jacq & Christen, 2006).

A maximum likelihood analysis was performed at the CIPRES webportal (Miller, Pfeiffer & Schwartz, 2010) using RAxML v. 7.2.8 as part of the “RAxML-

HPC2 on TG" tool (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008). A general time reversible model (GTR) was applied with a discrete gamma distribution and four rate classes. Fifty thorough maximum likelihood (ML) tree searches were done in RAxML v. 7.2.7 under the same model, with each one starting from a separate randomised tree and the best scoring tree selected with a final in value of -13974.356237. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution.

The model of evolution was estimated by using MrModeltest 2.2 (Nylander, 2004). Posterior probabilities (PP) (Rannala & Yang, 1996; Zhaxybayeva & Gogarten, 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist, 2001). Six simultaneous Markov chains were run for 1000000 generations and trees were sampled every 100th generation (resulting in 10000 total trees). The phylogram is presented in Fig. 4.2.

4.3 Results and Discussion

4.3.1 DNA Sequencing - Combined LSU and ITS Gene Phylogenies

DNA sequence data from the LSU and ITS gene regions were combined, and the data set consists of 108 taxa, with *Dothidea insculpta* and *D. sambuci* as the outgroup taxa. The dataset consists of 1486 characters after alignment. Of the included bases, 634 sites (42.66%) are parsimony-informative. A heuristic search with random addition of taxa (1000 replicates) and treating gaps as missing characters generated six equally parsimonious trees. All trees were similar in topology and not significantly different (data not shown). The first of 1000 equally most parsimonious trees is shown in Fig. 4.1 (TL = 2247, CI = 0.495, RI = 0.832, RC = 0.412). Bootstrap support (BS) values of MP and ML (equal to or above 50% based on 1,000 replicates) are shown on the upper and lower branches. A phylogenetic tree derived from a Bayesian analysis is shown in Fig. 4.2. Values of the Bayesian posterior probabilities (PP) from MCMC analyses are shown.

No protein genes such as EF1-α and β-tubulin sequences are available for the genus *Kellermania*. Therefore, LSU and ITS were chosen for combined phylogenetic analysis; the positions of some genera of *Botryosphaeriales* are not stable, especially the complex sections such as *Aplosporella*, *Auerswaldia*, *Diplodia*, *Lasiodiplodia*, *Macrophomina*, *Melanops*, *Phaeobotryon* and *Saccharata*. All strains of *Kellermania* clustered in a strong clade (100% MP, 1.00 PP) in both maximum parsimonious and Bayesian analysis. Within this clade, 15 strains divided into three groups in the parsimonious tree (Fig. 4.1): 1) Group A represented the type of *Piptarthron* (*Kellermania macrospora = Piptarthron macrospora*); 2) Group B represented the type of *Planistroma* (*Planistroma yuccigenum*); 3) group C represented the type of *Kellermania* (*K. yuccifoliorum*). In Bayesian analysis (Fig. 4.2), the taxa also clustered into three groups, representing the type species of *Piptarthron, Planistroma* and *Kellermania* however, two strains, *K. anomala* CBS 132218 and *K. nolinifoliorum* CBS 131718 were unstable and clustered differently at the base of Group C.

Table 4.1 Fungal Isolates Used in This Study

Taxon	Culture Accession No.[1]	GenBank Accession No.	
		LSU	ITS
Amniculicola lignicola	CBS 123094	EF493861	-
Aplosporella yalgorensis	MUCC 512	EF591944	EF591927
Aplosporella yalgorensis	MUCC 511 [T]	EF591943	EF591926
Auerswaldia dothiorella	MFLUCC 11-0438 [T]	JX646813	JX646796
Auerswaldia lignicola	MFLUCC 11-0435 [T]	JX646814	JX646797
Auerswaldia lignicola	MFLUCC 11-0656	JX646815	JX646798
Barriopsis fusca	CBS 174.26 [T]	DQ377857	EU673330
Botryobambusa fusicoccum	MFLUCC 11-0143 [T]	JX646809	JX646792
Botryobambusa fusicoccum	MFLUCC 11-0657	JX646810	JX646793
Botryosphaeria melanops	CBS 118.39	DQ377856	FJ824771
Botryosphaeria agaves	MFLUCC 10-0051	JX646807	JX646790
Botryosphaeria agaves	MFLUCC 11-0125 [T]	JX646808	JX646791
Botryosphaeria corticis	CBS 119047 [T]	EU673244	DQ299245

Table 4.1 (continued)

Taxon	Culture Accession No.[1]	GenBank Accession No.	
		LSU	ITS
Botryosphaeria corticis	ATCC 22927	EU673245	DQ299247
Botryosphaeria dothidea	CMW 8000 [T]	AY928047	AY236949
Botryosphaeria dothidea	CBS 110302	EU673243	AY259092
Capnodium coffeae	CBS 147.52	DQ247800	-
Cophinforma eucalypti	MFLUCC 11-0425 [T]	JX646817	JX646800
Cophinforma eucalypti	MFLUCC 11-0655	JX646818	JX646801
Diplodia cupressi	CBS 168.87 [T]	EU673263	DQ458893
Diplodia cupressi	CBS 261.85	EU673264	DQ458894
Diplodia mutila	CBS 112553 [T]	AY928049	AY259093
Diplodia mutila	CBS 230.30	EU673265	DQ458886
Diplodia scrobiculata	CBS 113423	EU673267	DQ458900
Diplodia scrobiculata	CBS 109944	EU673268	DQ458899
Dothidea insculpta	CBS 189.58	DQ247802	AF027764
Dothidea sambuci	DAOM 231303	AY544681	DQ491505
Dothidotthia symphoricarpi	CPC 12929 [T]	EU673273	-
Dothiorella iberica	CBS 115041 [T]	AY928053	AY573202
Dothiorella iberica	CBS 113188	EU673230	AY573198
Dothiorella sarmentorum	IMI 63581b [T]	AY928052	AY573212
Dothiorella sarmentorum	CBS 115038	DQ377860	AY573206
Falciformispora lignatilis	BCC 21117	GU371826	-
Falciformispora lignatilis	BCC 21118	GU371827	-
Gloniopsis subrugosa	CBS 123346	FJ161210	-
Guignardia bidwellii	CBS 111645	DQ377876	FJ824766
Guignardia citricarpa	CBS 102374	DQ377877	FJ824767
Guignardia philoprina	CBS 447.68	DQ377878	FJ824768
Hysterium angustatum	CBS 123334	FJ161207	-
Kellermania anomala	CBS 132218	JX444869	JX444853
Kellermania confusa	CBS 131723	JX444870	JX444854
Kellermania macrospora	CBS 131716	JX444874	JX444858
Kellermania nolinifoliorum	CBS 131718	JX444877	JX444861
Kellermania ramaleyae	CBS 131722	JX444879	JX444863
Kellermania rostratae	CBS 131721	JX444880	JX444864

Table 4.1 (continued)

Taxon	Culture Accession No.[1]	GenBank Accession No.	
		LSU	ITS
Kellermania uniseptata	CBS 131725	JX444881	JX444866
Kellermania yuccifoliorum	CBS 131726	JX444882	JX444867
Kellermania yuccigena	CBS 131727	JX444883	JX444868
Lasiodiplodia crassispora	CBS 110492	EU673251	EF622086
Lasiodiplodia crassispora	CBS 118741 [T]	DQ377901	DQ103550
Lasiodiplodia gonubiensis	CBS 115812 [T]	DQ377902	DQ458892
Lasiodiplodia gonubiensis	CBS 116355	EU673252	AY639594
Lasiodiplodia pseudotheobromae	CBS 447.62	EU673255	EF622081
Lasiodiplodia pseudotheobromae	CBS 116459 [T]	EU673256	EF622077
Lasiodiplodia theobromae	CBS 124.13	AY928054	DQ458890
Lasiodiplodia theobromae	CBS 164.96 [T]	EU673253	AY640255
Lasiodiplodia theobromae	CAA 006	EU673254	DQ458891
Leptosphaerulina australis	CBS 939.69	EU754167	-
Macrophomina phaseolina	CBS 227.33	DQ377906	-
Macrophomina phaseolina	CBS 162.25	DQ377905	-
Macrophomina phaseolina	CPC 11108	DQ377912	-
Macrophomina phaseolina	CPC 11085	DQ377910	-
Macrophomina phaseolina	CPC 11106	DQ377911	-
Melanops tulasnei	CBS 116805 [T]	FJ824764	FJ824769
Melanops tulasnei	CBS 116806	FJ824765	FJ824770
Murispora rubicund	IFRD-2017	FJ795507	-
Mycosphaerella punctiformis	AFTOL-942	DQ470968	-
Neodeightonia palmicola	MFLUCC 10-0822 [T]	HQ199222	HQ199221
Neodeightonia palmicola	MFLUCC 10-0823	HQ199225	HQ199224
Neodeightonia phoenicum	CBS 169.34	EU673259	EU673338
Neodeightonia phoenicum	CBS 122528 [T]	EU673261	EU673340
Neodeightonia phoenicum	CBS 123168	EU673260	EU673339
Neodeightonia subglobosa	CBS 448.91	DQ377866	EU673337
Neofusicoccum luteum	CBS 110299 [T]	AY928043	AY259091
Neofusicoccum luteum	CBS 110497	EU673229	EU673311
Neofusicoccum parvum	CMW 9081 [T]	AY928045	AY236943
Neofusicoccum parvum	CBS 110301	AY928046	AY259098

Table 4.1 (continued)

Taxon	Culture Accession No.[1]	GenBank Accession No.	
		LSU	ITS
Neoscytalidium dimidiatum	CBS 251.49	DQ377923	FM211430
Neoscytalidium dimidiatum	CBS 499.66	DQ377925	FM211432
Neoscytalidium novaehollandiae	WAC 12691 [T]	EF585548	EF585543
Neoscytalidium novaehollandiae	WAC 12688	EF585549	EF585542
Phaeobotryon mamane	CPC 12440 [T]	EU673248	EU673332
Phaeobotryon mamane	CPC 12442	DQ377899	EU673333
Phaeobotryon mamane	CPC 12443	EU673249	EU673334
Phaeobotryosphaeria citrigena	ICMP 16812 [T]	EU673246	EU673328
Phaeobotryosphaeria citrigena	ICMP 16818	EU673247	EU673329
Phaeobotryosphaeria eucalypti	MFLUCC 11-0579 [T]	JX646819	JX646802
Phaeobotryosphaeria eucalypti	MFLUCC 11-0654	JX646820	JX646803
Phaeobotryosphaeria visci	CBS 186.97	DQ377868	EU673325
Phaeobotryosphaeria visci	CBS 100163	DQ377870	EU673324
Pleospora herbarum	CBS 191.86 [T]	DQ247804	GU238232
Planistroma dasylirionicola	CBS 131720	JX444872	JX444856
Planistroma dasylirionis	CBS 131715	JX444873	JX444857
Planistroma kellermaniae	CBS 131717	JX444876	JX444860
Planistroma micranthae	CBS 131724	JX444875	JX444859
Planistroma nolinae	CBS 131714	JX444871	JX444855
Planistroma yuccigenum	CBS 131719	JX444878	JX444862
Pseudofusicoccum adansoniae	WAC 12689 [T]	EF585554	EF585534
Pseudofusicoccum adansoniae	WAC 12718	EF585555	EF585533
Pseudofusicoccum stromaticum	CBS 117448	DQ377931	AY693974
Pseudofusicoccum stromaticum	CBS 117449	DQ377932	DQ436935
Psiloglonium simulans	CBS 206.34	FJ161178	-
Saccharata capensis	CBS 122693 [T]	EU552130	EU552130
Saccharata proteae	CBS 115206	DQ377882	AF452560
Spencermartinsia viticola	CBS 117006	EU673236	AY905555
Spencermartinsia viticola	CBS 112870	DQ377872	AY343376
Spencermartinsia viticola	CBS 117009 [T]	DQ377873	AY905554
Trematosphaeria pertusa	CBS 122368 [T]	FJ201990	FJ201991
Trematosphaeria pertusa	CBS 122371	FJ201992	FJ201993

Note. [1]AFTOL = Assembling the Fungal Tree of Life, AR = Culture collection of Amy Rossman, housed at U.S. National Fungus Collections (BPI), Beltsville, MD, USA, ATCC = American Type Culture Collection, Virginia, USA, BCC = BIOTEC Culture Collection, Bangkok, Thailand, CAA = A. Alves, Universidade de Aveiro, Portugal, CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa, CPC = Collection of Pedro Crous housed at CBS, DAOM = Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada, ICMP = International Collection of Micro-organisms from Plants, Landcare Research, New Zealand, IFRDCC = Culture Collection, International Fungal Research & Development Centre, Chinese Academy of Forestry, Kunming, China, IMI = International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K, MFLUCC = Mae Fah Luang University Culture Collection, ChiangRai, Thailand, MUCC = Murdoch University Algal Culture Collection, Murdoch, Western Australia, STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa, WAC = Department of Agriculture Western Australia Plant Pathogen Collection, South Perth, Western Australia, T = ex-type/ex-epitype isolates.

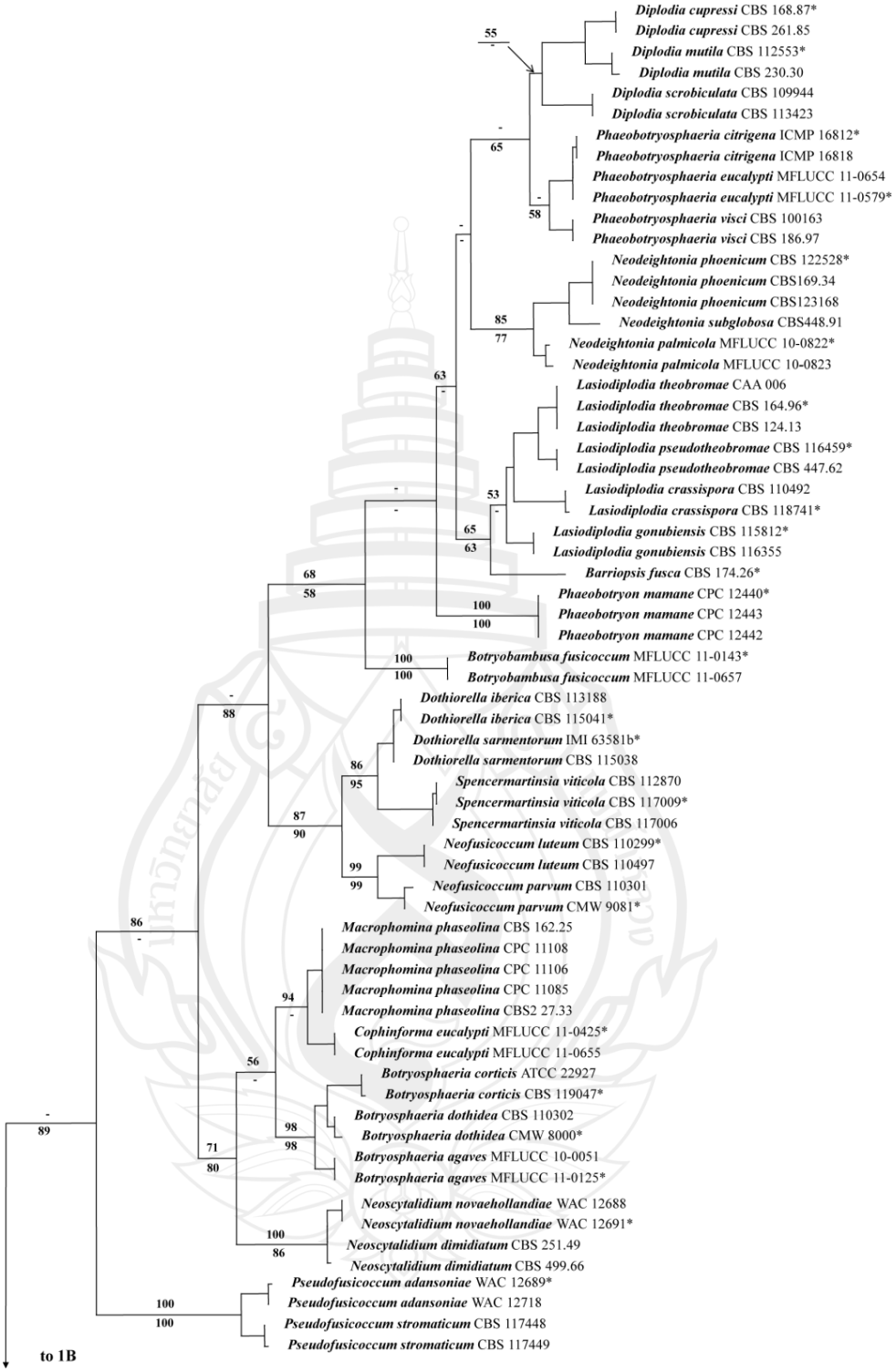
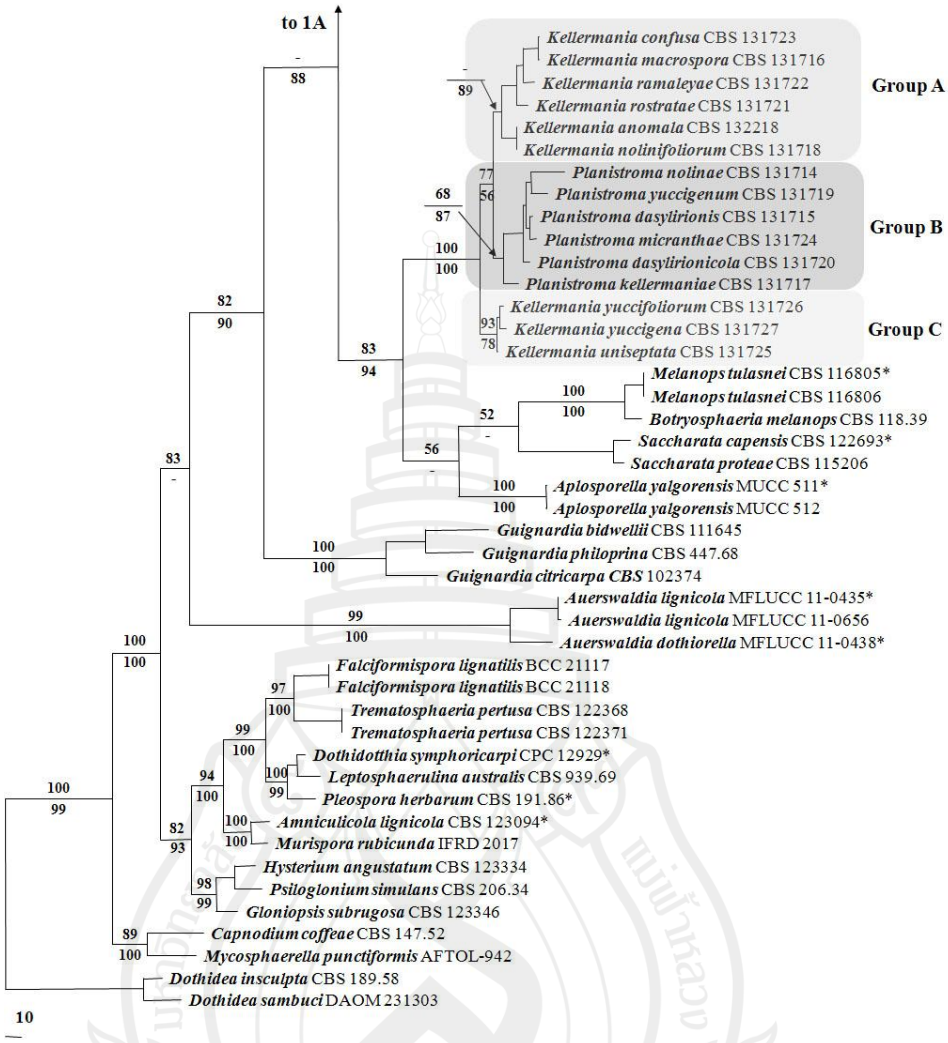


Figure 4.1 A–B The First of 1000 Equally Most Parsimonious Trees Obtained from A Heuristic Search with 1000 Random Taxon Additions of the Combined Dataset of LSU and ITS Sequences Alignment Using PAUP v. 4.0b10.



Note. The scale bar shows 10 changes. Bootstrap support values for maximum parsimony (MP) and maximum likelihood (ML) greater than 50 % above and under the nodes. Hyphen ("–") indicates a value lower than 50 % (BS). The original isolate numbers are noted after the species names, ex-type/ex-epitype isolates are marked by an asterisk "*". The tree is rooted to *Dothidea insculpta* and *Dothidea sambuci*

Figure 4.1 A–B The First of 1000 Equally Most Parsimonious Trees Obtained from A Heuristic Search with 1000 Random Taxon Additions of the Combined Dataset of LSU and ITS Sequences Alignment Using PAUP v. 4.0b10.

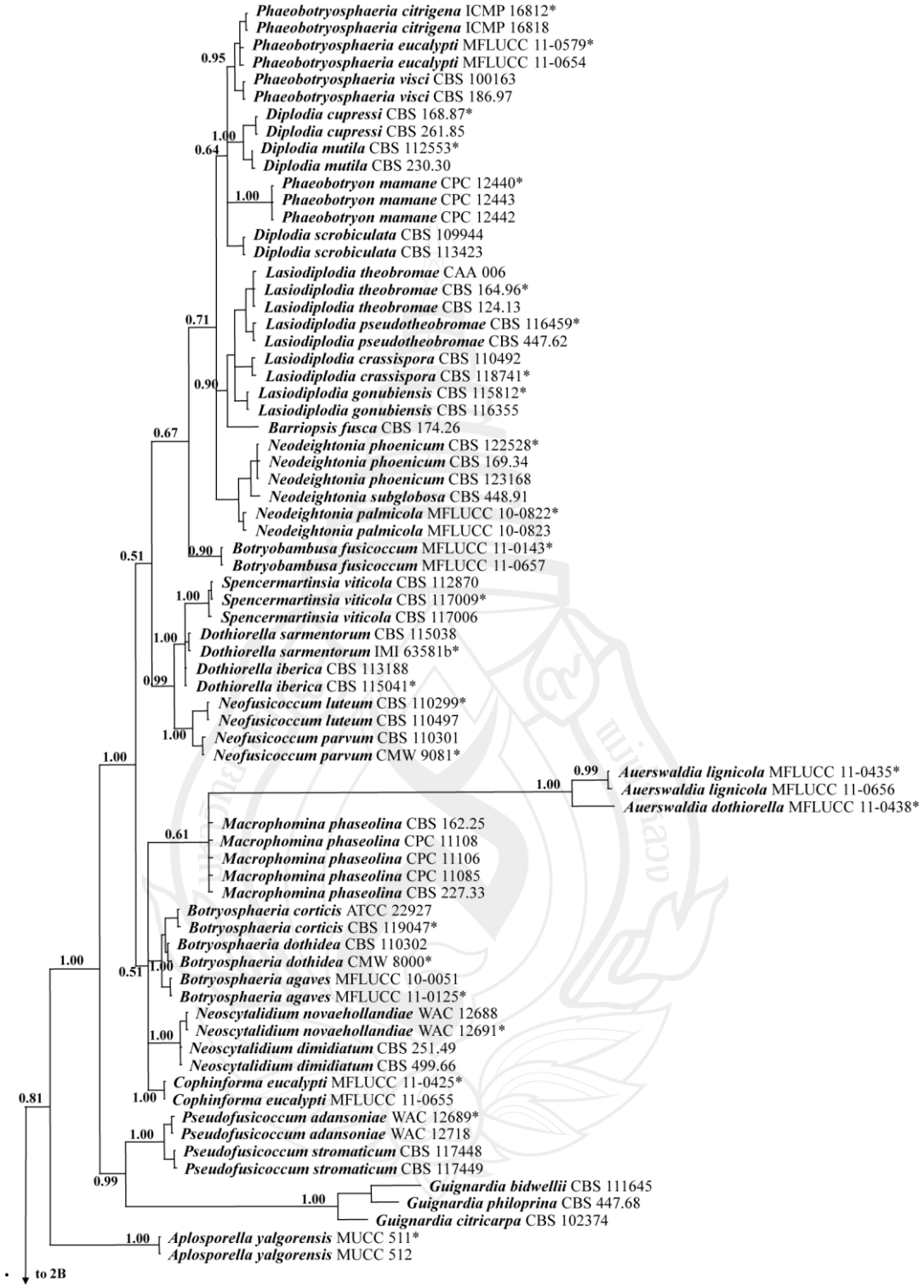
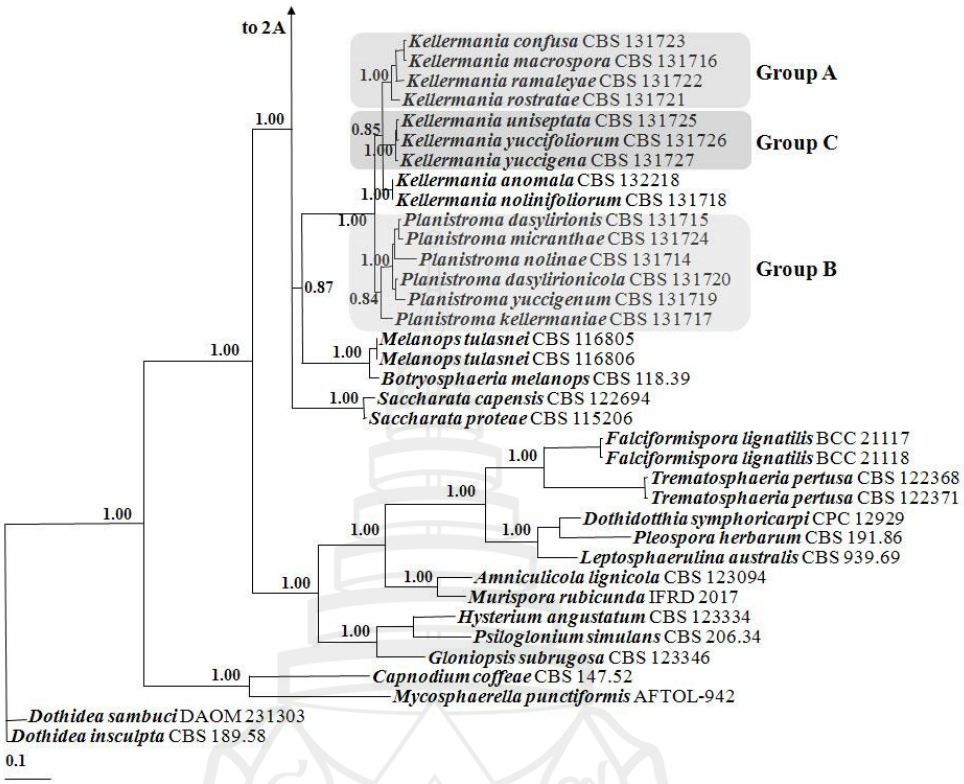


Figure 4.2 A–B Phylogenetic Tree Derived from A Bayesian Analysis of an Alignment of LSU and ITS Sequences



Note. The scale bar shows 0.1 changes. The original isolate numbers are noted after the species names, ex-type/ex-epitype isolates are marked by an asterisk "*".

Figure 4.2 A–B Phylogenetic Tree Derived from A Bayesian Analysis of an Alignment of LSU and ITS Sequences (continued)

4.3.2 Taxonomy

Botryosphaeriales

For characters of this order see Liu et al. (2012)

Planistromellaceae M.E. Barr, Mycotaxon 60: 437 (1996)

MycoBank: MB 81919

Biotrophic, *hemibiotrophic* or *saprobic* on leaves and stems of various plants in terrestrial habitats. *Ascostromata* multi- or uniloculate, immersed to erumpent through cracking or splitting of the host tissue, solitary to gregarious, with periphysate

ostioles, with or without papillate. *Cells of ascostromata* thick-walled, composed of several layers of dark brown cells, arranged in a *textura angularis*. *Locules* ovoid to globose, developing in the same stroma of the conidiogenous and/or spermatogenous locules, collapsing with the empty locule which previously producing conidia or spermatia or both, periphysate ostiole. *Peridium* of locules composed of a few layers of hyaline to light brown flattened cells. *Hamathecium* lacking pseudoparaphyses, interascal cells abundant even at maturity. *Asci* 8-spored, bitunicate, fissitunicate, oblong, clavate to nearly cylindrical, with a pedicel and with an ocular chamber, forming in a basal layer, often interspersed with and covered by cellular remnants of interthecial tissues. *Ascospores* overlapping 1-3-seriate, hyaline or lightly pigmented, yellowish to brownish, ellipsoid to broadly obovoid, aseptate or one to two transverse septa; wall thin, with or without gelatinous sheath, contents guttulate. *Conidiomata* subepidermal, dark, immersed to erumpent, solitary to gregarious, pycnidia, locules or acervuli in a stroma or bearing conidia over stroma surface prior to locule formation, ostiolate. *Conidiomata walls* comprising several layers with cells of *textura angularis*, the outer layers composed of dark thick-walled cells, lighter towards the inner layers of hyaline cells. *Conidiogenous cells* short cylindric, conidiogenesis holoblastic, hyaline, smooth. *Conidia* oblong, ellipsoid-cylindric, aseptate or one to several transversely septate, hyaline to brown, wall smooth or verruculose, with or without one or more apical appendages at times. *Spermatial state* developing in the same or separate locules. *Spermatogenous cells* discrete or integrated, phialidic, cylindric to elongate-conical, determinate, hyaline, smooth. *Spermatia* bacillary, hyaline, aseptate, smooth.

Included genera: *Kellermania* (= *Planistromella* and ?*Piptarthron*), *Planistroma* and *Mycosphaerellopsis*

Notes: The *Planistromellaceae* is reduced to include *Kellermania* and *Planistroma* based on molecular data. *Mycosphaerellopsis* is tentatively included, based on morphology, however there is no molecular data to support this. The family belongs in the *Botryosphaeriales* (Minnis et al., 2012). The type species, *Kellermania yuccifoliorum* is characterized by subepidermal, immersed, multilocular ascostromata with periphysate ostioles, bitunicate, slightly clavate or nearly cylindric asci, and smooth, hyaline, septate ascospores. The asexual state of *K. yuccifoliorum* is

characterized by uniloculate conidiomata, which develop in the same stroma as the ascogenous locules and has 2-septate conidia with a unique apical appendage (Ramaley, 1993). Molecular data indicates that *Comminutispora* does not belong in *Botryosphaeriales*, as it clusters and is more typical of *Capnodiales* (Crous et al., 2009a; Hambleton, Tsuneda and Currah, 2004; Schoch et al., 2009). *Eruptio* is typical of the *Mycosphaerellaceae* and this is confirmed by molecular data (Crous, Kang & Braun, 2001; Crous et al., 2009a, 2009b; Verkley et al., 2004). Molecular data (Suetrong et al., 2009) places *Loratospora* in the family *Phaeosphaeriaceae* and the characters of this genus are also in concordance with this finding. *Microcyclus* may be a member of *Mycosphaerellaceae* based on its morphology.

Family type: *Kellermania* Ellis & Everh.

Kellermania Ellis & Everh., J. Mycol. 1(12):153 (1885) (Figs. 4.3-4.4)

Synonyms

Piptarthron Mont. ex Höhn., Hedwigia 60: 203 (1918)

Alpakesa Subram. & K. Ramakr., J. Indian Bot Soc. 33: 204 (1954)

Septoplaca Petr., Sydowia 17: 271 (1964,‘1963’)

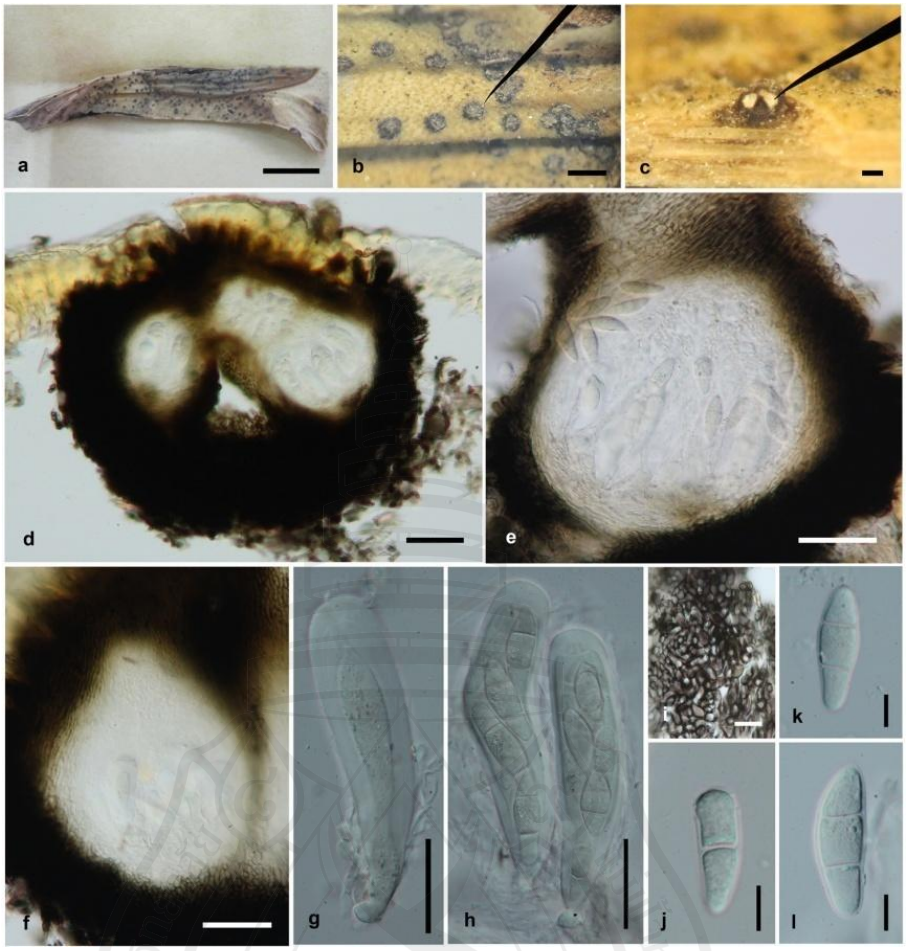
Planistromella A.W. Ramaley, Mycotaxon 47: 260 (1993)

MycoBank: MB 22437

Biotrophic, *hemibiotrophic* and *saprotrophic* on leaves and stems. *Ascostromata* subepidermal, immersed, becoming erumpent, solitary to gregarious, multilocular, subglobose to ovoid, dark brown to black, thick-walled. *Cells of ascostromata* composed of several layers of dark brown cells, *textura angularis.* The upper part of the ascostromata comprises columns of elongated cells attached with the host epidermis. *Locules* ovoid to globose, the collapsed locule producing conidia or spermatia or both, periphysate ostiole. *Peridium* of locules composed of a few layers of hyaline to light brown flattened cells. *Hamathecium* lacking pseudoparaphyses when mature, interascal cells abundant, filamentous. *Asci* 8-spored, bitunicate, fissitunicate, clavate to nearly cylindrical, with a short knob-like pedicel and an ocular chamber. *Ascospores* overlapping 1-2-seriate, ellipsoid and slightly curved with bluntly rounded ends, hyaline, 1-2-septate, guttulate. *Conidiomata* subepidermal, dark, immersed, erumpent by remaining at the rim covered by epidermis, solitary to

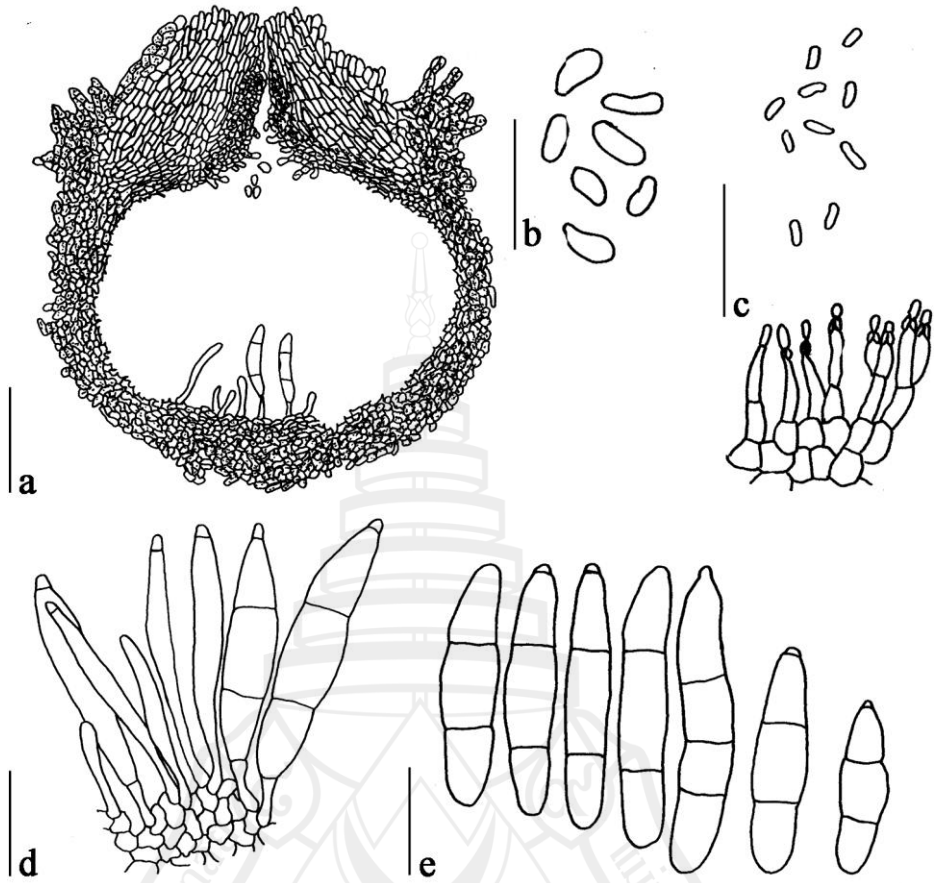
gregarious, unilocular, ostiolate. *Conidiomata walls* comprising several layers with cells of *textura angularis*, the outer layers composed of 6-12 layers of dark, thick-walled cell, lighter toward the inner layers composed of 2-3 layers of hyaline cells. *Conidiogenesis* holoblastic. *Conidiophores* absent. *Macroconidiogenous cells* short cylindric, hyaline, smooth, each forming acrogenous holoblastic conidia. *Macroconidia* narrowly ellipsoid-cylindric, the base bluntly rounded, the apex more pointed and often surrounded by an appendage, mostly 2-septate. *Microconidiogenous cells* arising on the upper wall of conidioma and in ostiolar channel. *Microconidia* more or less cylindric, aseptate, smooth-walled, hyaline. *Spermatia* formed in the central locule of a stroma or in the locule in the vertical column of the lateral walls of some conidiomata. *Spermatogenous cells* discrete or integrated on one-celled conidiophores, phialidic, cylindric to elongate-conical. *Spermatia* bacillary, hyaline, smooth (asexual morph description follows Ramaley, 1993).

Notes: The type species of *Kellermania* (*K. Yuccifoliorum* ≡ *Planistromella yuccifoliorum*) differs from other genera in *Planistromellaceae* in having 1-2 septate ascospores (Ramaley, 1993; Barr, 1996). The asexual state of *K. yuccifoliorum* is characterized by uniloculate conidiomata, which develops in the same stroma as the ascogenous locules and has 2-septate conidia with a unique apical appendage (Ramaley, 1993). Several other species of *Kellermania* are illustrated by Minus et al., (2012) and range from having 0 to several trans-septa and with or lacking appendages. Species in this genus are known from the genera *Agave* and *Nolina* (*Asparagaceae*). There are 13 species recorded in Index Fungorum (n.d.). Five species have been reported with both sexual and asexual states (Barr, 1996; Ramaley, 1993, 1995, 1998). Phylogenetic analyses based on combined LSU and ITS gene data show that the type of *Kellermania* which is also the type of "Planistromella", cluster in Group C and contains three *Kellermania* species which are linked to "Planistromella" sexual states (Figs. 4.1, 4.2). However, the other two strains, which are linked with "Planistromella" sexual states, i.e. *Kellermania anomala* (CBS 132218) and *K. nolinifoliorum* (CBS 131718), cluster in Bayesian analysis as a sister group to the other three species (Fig. 4.2).



Note. (a) – (b) Ascostromata on the host surface. (c) – (d) Section of ascostroma. (e) – (f) Ascoma. (g) Immature ascus. (h) Mature asci. (i) Cells of ascostroma. (j) Immature ascospore. (k) – (l) Mature ascospores. Scale bars: (a) = 1 cm, (b) = 1000 µm, (c)= 200 µm, (d) = 100 µm, (e) – (f)= 50 µm, (g) – (h) = 30 µm, (i) = 20 µm, (j) – (l) = 10 µm

Figure 4.3 *Kellermania yuccifoliorum* (Holotype of *Planistromella yuccifoliorum*) on Leaves of *Yucca brevifolia*



Note. (a) Conidioma. (b) Microconidia. (c) Spermatia and spermatiogenesis. (d) Conidiogenesis andappearance of apical appendage. (e) Conidia. Scale bars: (a) = 80 µm, (b) – (d) = 27 µm, (e) = 40 µm

From Ramaley, A. W. (1993). New fungi from Yucca: *Planistromella yuccifoliorum*, gen. et sp. nov., and its anamorph *Kellermania yuccifoliorum*, sp. nov., and *Planistromella uniseptata*, sp. nov., the teleomorph of *Kellermania yuccigena*. **Mycotaxon, 47,** 259–274.

Figure 4.4 *Kellermania yuccifoliorum*

The generic type of *Piptarthron* (= *K. macrospora*) (Group A) clusters separately from *Kellermania* (Group C) and *Planistroma* (Group B), with strong support in both maximum-parsimony and Bayesian analyses (Figs 4.1, 4.2). This may indicate that *Piptarthron* may prove to be a good genus once the tree includes more taxa. Minnis et al. (2012) combined *Piptarthron, Planistroma* and *Planistromella* under *Kellermania* as they could find little molecular support for retaining this entities. In this study, *Planistroma* was accepted as both molecular and morphological data show this genus to be different. However, following Minnis et al. (2012) *Piptarthron* was treated as a synonym of *Kellermania* because the separation is only supported by molecular data with no obvious morphological similarities.

This work tried to establish groupings based on morphology. Group A which includes the type of *Piptarthron* mostly has conidia with 1-5 septa which lack appendages. Group B which contains the type of *Planistroma* has conidia lacking septa and appendages, while Group C includes the type of *Planistromella*, mostly have conidia with 1-3 septa and appendages.There are however exceptions. For example, conidia of *Kellermania nolinae* (Minus et al., 2012, Fig 4.5g) has three septa and appendages but clusters in group B. Part of the problem may lie with the fact that some of the links between the sexual and asexual states are based on the fact that the taxa developed on the same samples (Ramaley, 1993, 1995, 1996). Isolates were made from both the conidia or ascospores from separate conidiomata and ascomata and thus may have not have been related. Another problem is that the cultures used by Minnis et al. (2012) were not general type strains. This group obviously needs further study with many more taxa being sequenced and carefully isolated from fresh material to establish their correct identification.

Generic Type

Kellermania yuccifoliorum A.W. Ramaley, Mycotaxon 47: 262 (1993)

(Figs. 4.3-4.4)

≡ *Planistromella yuccifoliorum* A.W. Ramaley, Mycotaxon 47: 261 (1993)

MycoBank: MB 360149

Biotrophic, hemibiotrophic and *saprotrophic* on leaves and stems. *Ascostromata* 0.4-0.6 mm diam., up to 0.5 mm high, subepidermal, immersed, becoming erumpent, solitary to gregarious, multilocular, subglobose to ovoid, dark brown to black, with 1-5 locules, thick-walled (Fig. 4.3b-e). *Cells of ascostromata* 84.5-116 µm wide, composed of several layers of dark brown cells, *textura angularis.* The upper part of the ascostromata comprises columns of elongated cells attached with the host epidermis(Fig. 4.3d,i). *Locules* 150 µm wide × 90-244 µm high, ovoid to globose, the collapsed locule producing conidia or spermatia or both, periphysate ostiole (Fig. 4.3e, f). *Peridium* of locules composed of a few layers of hyaline to light brown flattened cells (Fig. 4.3e-f). *Hamathecium* lacking pseudoparaphyses when mature, interascal cells abundant, filamentous (Fig. 4.3g-h). *Asci* 93-153 × 25-35 µm ($\bar{x}$ = 120.8 × 29.3 µm, n = 13), 8-spored, bitunicate, fissitunicate, clavate to nearly cylindrical, with a short knob-like pedicel up to 9 µm wide × 7 µm high, and with an ocular chamber up to 2.5 µm wide × 5 µm high (Fig. 4.3g-h). *Ascospores* 32-40 × 11-14 µm ($\bar{x}$ = 36.3 × 12.6 µm, n = 15), overlapping 1-2-seriate, ellipsoid and slightly curved with bluntly rounded ends, hyaline, 2-septate, young ascospore with 1-septate, distoseptate, small guttules, granulate ornamentation (Fig. 4.3j-l). *Conidiomata* subepidermal, dark, immersed, erumpent by remaining at the rim cover by epidermis, solitary to gregarious, 250-600(-800) µm diam., up to 500 µm thick, unilocular, ostiolate (Fig. 4.4a). *Conidiomata walls* comprising several layers with cells of *textura angularis*, the outer layers composed of 6-12 layers of dark, thick-walled cell, lighter toward the inner layers composed of 2-3 layers of hyaline cells (Fig. 4.4a). *Conidiogenesis* holoblastic (Fig. 4.4d). *Conidiophores* absent. *Macroconidiogenous cells* short cylindric, hyaline, smooth, each forming acrogenous holoblastic conidium (Fig. 4.4d). *Macroconidia* narrowly ellipsoid-cylindric, the base bluntly rounded, the apex more pointed and often surmounted by an appendage up to 5 µm long, mostly 2-septate, 50-100 × (8-)13-14(-16) µm (Fig. 4.4e). *Microconidiogenous cells* arising on the upper wall of conidioma and in ostiolar channel (Fig. 4.4a). *Microconidia* more or less cylindric, aseptate, smooth-walled, hyaline 5-10 × 2.5-4 µm (Fig. 4.4b). *Spermatia* formed in the central locule of a stroma or in the locule in the vertical column of the lateral walls of some conidiomata. *Spermatogenous cells* discrete or integrated on one-celled conidiophores, phialidic, cylindric to elongate-conical, 8-16

× 2-3.5 µm. *Spermatia* bacillary, hyaline, smooth, 3-7 × 1.5-2.5 µm (Fig. 4.4c) (asexual morph description follows Ramaley, 1993).

Material examined: USA: California, San Bernardino County, Roadside 20 miles east of Baker (Hwy. 91/466), on leaves of *Yucca brevifolia* Engelmann, 14 April 1960, Isabelle Tavares No.466 (UC 1202973, holotype).

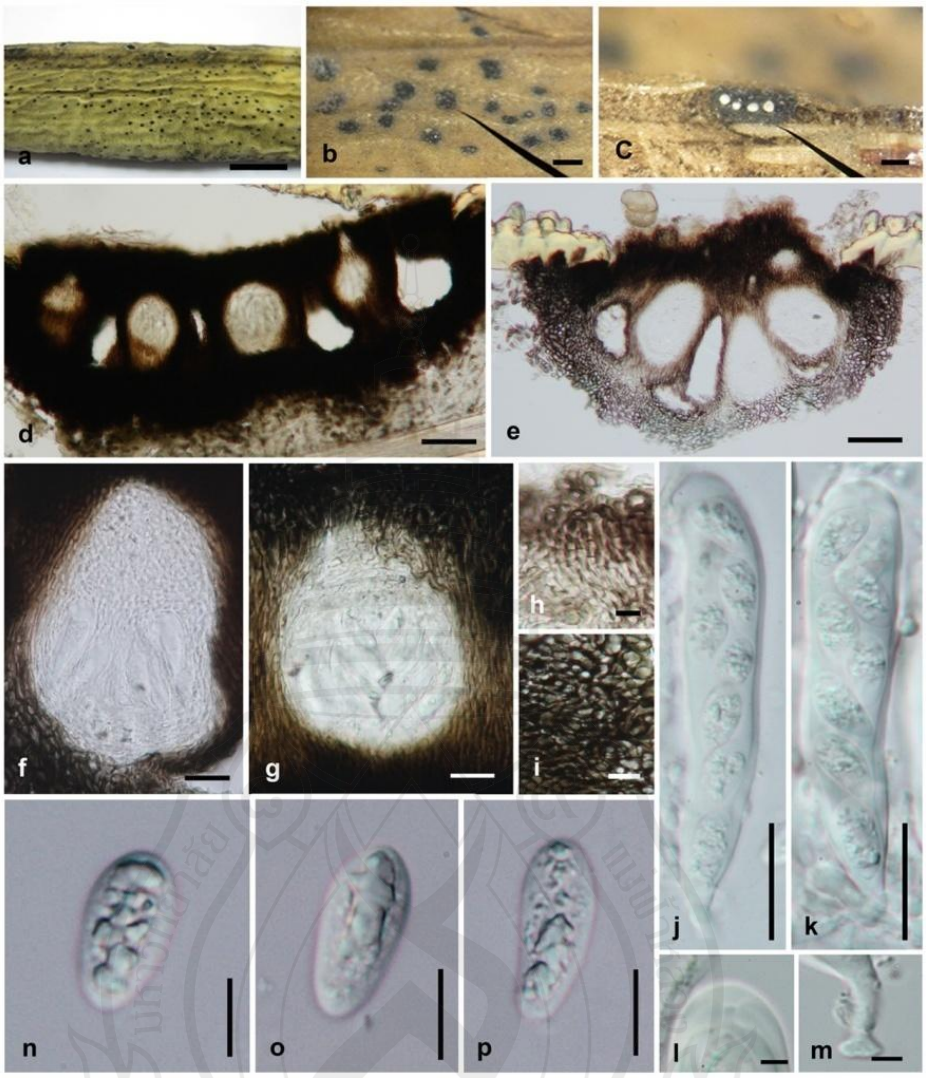
Planistroma A.W. Ramaley, Mycotaxon 42: 69 (1991) (Fig. 4.5)

MycoBank: MB 25358

Biotrophic or *saprotrophic* on leaves and stems. *Ascostromata* subepidermal, immersed, partially erumpent remaining at the rim covered by epidermis, solitary to gregarious, multilocular, hemispherical, dark brown to black, thick-walled. *Cells of ascostromata* at the side composed of several layers of dark brown columns of elongate cells, reaching from the base to the top, at the base composed of several layers of dark brown cells, *textura angularis*. *Locules* ovoid to globose, developing in the same stroma of the conidiogenous and/or spermatogenous locules, collapsing with the empty locule which previously producing conidia or spermatia or both, periphysate ostiole. *Peridium of locules* composed of a few layers of hyaline to light brown flattened cells. *Hamathecium* lacking pseudoparaphyses when mature, filamentous, cylindrical or irregularly curved interascal cells which are swollen at the ends. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical, with a long fan-shaped pedicel and with an ocular chamber. *Ascospores* overlapping 1-2-seriate, ellipsoid, hyaline, aseptate, many guttules, rough, sometimes surrounded by a slime layer. *Stromata* subepidermal, black, immersed, erumpent by remaining at the rim cover by epidermis, solitary to gregarious, hemispherical, multilocular. *Stromata wall* composed of dark brown, thick-walled cells of *textura angularis*, lighter colored toward the interior, the top layers of the stromata composed of columns of elongated cells, extending from the base and the sides to the top surface of the stromata. *Conidiophores* absent. *Microconidiogenous cells* form on the wall of locules, short cylindric, hyaline, smooth, and each forming acrogenous holoblastic conidium. *Macroconidia* fusiform, curved or bent, tapering toward the apex or the base, base truncate, mostly aseptate, smooth, hyaline. *Microconidia* cylindric, irregularly swelled or bent, aseptate, smooth-walled, hyaline. *Spermatia* formed on a part of walls of a

macroconidiogenous locule or in one or more separate locules in a stroma. *Spermatogenous cells* phialidic, cylindric to elongate-conical, discrete or integrated on one-celled conidiophores, determinate, hyaline, smooth, forming acrogenous spermatia. *Spermatia* bacillary, hyaline, aseptate, smooth (asexual morph description follows Ramaley, 1991).

Notes: Planistroma was established by Ramaley (1991), and similar to *Kellermania* in ascostromata characters, but *Planistroma* differs with aseptate ascospores (Barr, 1996). *Planistroma* species inhabit the *Agavaceae*, with four species: *P. kellermania, P. nolinae, P. obtusilunatum* and *P. yuccigenum* listed in Index Fungorum (n.d.). All species have been connected to asexual states which are characterized by thick-walled ostiolate conidiomata, holoblastic conidiogenous cell and conidia lacking appendages (Ramaley, 1991, 1992, 1995, 1998). The asexual states of *Planistroma* resemble with asexual morphs of *Kellermania* excepted in having apically appendaged conidia in the latter genus (Ramaley, 1995). Phylogenetic analysis of *Kellermania* and *Piptarthron* based on SSU, ITS and LSU genes showed that both genera group in the same clade and Minnis et al. (2012) synonymised *Piptarthron*, *Planistroma*, *Planistromella* under *Kellermania.* However, from this study based on molecular data from LSU and ITS genes, the phylogenetic tree shows that most species of *Planistroma* which are linked to *Piptarthron* are included in Group B.



Note. (a) – (b) Ascostromata on the host surface. (c) – (e) Section of ascostroma. (f) Young ascoma. (g) Mature ascoma. (h) Cells of ascostroma at the side. (i) Cells of ascostroma at the base. (j) – (k) Asci. (l) Ocular chamber (m) Pedicel. (n) – (p) ascospores. Scale bars: (a) = 1 cm, (b) = 1 mm, (c) = 0.5 mm, (d) – (e) = 100 µm, (f) – (g) = 25 µm, (h) – (i) = 10 µm, (j) – (k) = 20 µm, (l) – (m) = 5 µm, (n) – (p) = 10 µm

Figure 4.5 *Planistroma yuccigenum* (Holotype of *Planistroma yuccigenum*) on Leaves of *Yucca baccata*

Generic Type

Planistroma yuccigenum A.W. Ramaley, Mycotaxon 42: 69 (1991) (Fig. 4.5)

≡ *Piptarthron pluriloculare* A.W. Ramaley, Mycotaxon 42: 63 (1991)

≡ *Kellermania plurilocularis* (A.W. Ramaley) Minnis & A.H. Kenn., Persoonia 29: 21 (2012)

MycoBank: MB 358836

Biotrophic or *saprotrophic* on leaves and stems. *Ascostromata* black ellipsoid to subcircular on surface of leaves, subepidermal, immersed, partially erumpent remaining at the rim covered by the epidermis, solitary to gregarious, multilocular, hemispherical, 0.5-0.7 mm diam., forming elongate stroma up to 1 mm long, up to 0.5 mm thick, dark brown to black, 2-10 locules, variable arrangement of locules, thick-walled (Fig. 4.5b-e). *Cells of ascostromata* 48-127 µm wide at the side, composed of several layers of dark brown columns of elongate cells, reaching from the base to the top, 81.5-197 µm wide at the base, composed of several layers of dark brown cells *textura angularis* (Fig. 4.5e, h-i). *Locules* 43-153 µm wide × 56-161 µm high, ovoid to globose, developing in the same stroma of the conidiogenous and/or spermatogenous locules, collapsing with the empty locule which previously producing conidia or spermatia or both, periphysate ostiole (Fig. 4.5d- g). *Peridium of locules* composed of a few layers of hyaline to light brown flattened cells (Fig. 4.5f, g). *Hamathecium* lacking pseudoparaphyses when mature filamentous, cylindrical or irregularly curved interascal cells which are swollen at their tips. *Asci* 73-111 × 14-18 µm ($\bar{x}$ = 89.3 × 16 µm, n = 30), 8-spored, bitunicate, fissitunicate, cylindrical, with a long fan-shaped pedicel 7-8 µm up to 15 µm high, at the stipe 5.5 µm wide, and with an ocular chamber up to 3 µm wide × 3 µm high (Fig. 4.5j-m). *Ascospores*15.5-22 × 7-10.5 µm ($\bar{x}$ = 18.8 × 8.5 µm, n = 30), overlapping 1-2-seriate, ellipsoid with broadly rounded ends at the apex and narrowly rounded ends or tapering toward the base, hyaline, aseptate, irregularly many guttules, rough, sometimes surrounded by a slime layer (Fig. 4.5n-p). *Stromata* subepidermal, black, immersed, erumpent by remaining at the rim covered by the epidermis, solitary to gregarious, hemispherical, 0.4-1 mm diam, up to 0.5 mm thick, multilocular, 4-30 or more locules in stromata. *Stromata wall* 70-100 µm thick, composed of dark brown, thick-walled cells of

textura angularis, lighter colored toward the interior, the top layers of the stromata composed of columns of elongated cells, extending from the base and the sides to the top surface of the stromata. *Conidiophores* absent. *Microconidiogenous cells* form on the wall of locules, short cylindric, hyaline, smooth, 6.5-14.5 × 3.5-5.5 µm, each forming an acrogenous holoblastic conidium. *Macroconidia* fusiform, curved or bent, tapering toward the apex or the base, base truncate, mostly aseptate, smooth, hyaline, (48-)59-76(-98) × (4-)5.5-7(-8) ($\bar{x}$ = 67.8 × 6.5) µm. *Microconidia* cylindric, irregularly swelled or bent, aseptate, smooth-walled, hyaline 5.5-25 × 2.5-3.5 µm. *Spermatia* formed on a part of walls of a macroconidiogenous locule or in one or more separate locules in a stroma. *Spermatogenous cells* phialidic, cylindric to elongate-conical, ca 8-16 × 1.5-3 µm, discrete or integrated on one-celled conidiophores, determinate, hyaline, smooth, forming acrogenous spermatia. *Spermatia* bacillary, hyaline, aseptate, smooth, 3.5-5.5 × 1.5 µm (asexual morph description follows Ramaley, 1991).

Material examined: USA: Colorado, La Plata County, Durango, below Fort Lewis College, Roadside, on leaves of *Yucca baccata*, 26 September 1990, Annette W. Ramaley, (UC 1475061, holotype).

Notes: Ramaley (1991) introduced *Piptarthron pluriloculare* as the anamorph of *Planistroma yuccigenum* by confirming the anamorph-teleomorph connection.

Taxonomic Changes

Planistroma dasylirionicola (Minnis & A.H. Kenn) Monkai, J.K Liu & K.D. Hyde, comb. nov.

MycoBank: MB803335

Basionym: *Kellermania dasylirionicola* Minnis & A.H. Kenn., Persoonia 29: 21 (2012)

Planistroma dasylirionis (A.W. Ramaley) Monkai, J.K Liu & K.D. Hyde, comb. nov.

MycoBank: MB 803009

Basionym: *Piptarthron dasylirionis* A.W. Ramaley, Mycotaxon 55: 263 (1995)

Synonym: *Kellermania dasylirionis* (A.W. Ramaley) Minnis & A.H. Kenn., Persoonia 29: 21 (2012)

Planistroma kellermaniae A.W. Ramaley, Mycotaxon 66: 510 (1998)

Basionym: *Bartalinia nolinae* Pollack, Mycologia 39: 620 (1947)

Synonym: *Alpakesa nolinae* (Pollack) Morgan-Jones, Nag Raj & W.B. Kendr., Canad. J. Bot. 50: 879 (1972)

Synonym: *Kellermania nolinae* (Pollack) Nag Raj, in Nag Raj, Coelomycetous anamorphs with appendage-bearing conidia: 442 (1993)

Planistroma micranthae (Minnis & A.H. Kenn) Monkai., J.K Liu & K.D. Hyde, comb. nov.

MycoBank: MB803336

Basionym: *Kellermania micranthae* Minnis & A.H. Kenn., Persoonia 29: 21 (2012)

Planistroma nolinae A.W. Ramaley, Mycotaxon 55: 258 (1995)

Basionym: *Piptarthron crassisporum* A.W. Ramaley, Mycotaxon 55: 261 (1995)

Synonym: *Kellermania crassispora* (A.W. Ramaley) Minnis & A.H. Kenn., Persoonia 29: 21 (2012)

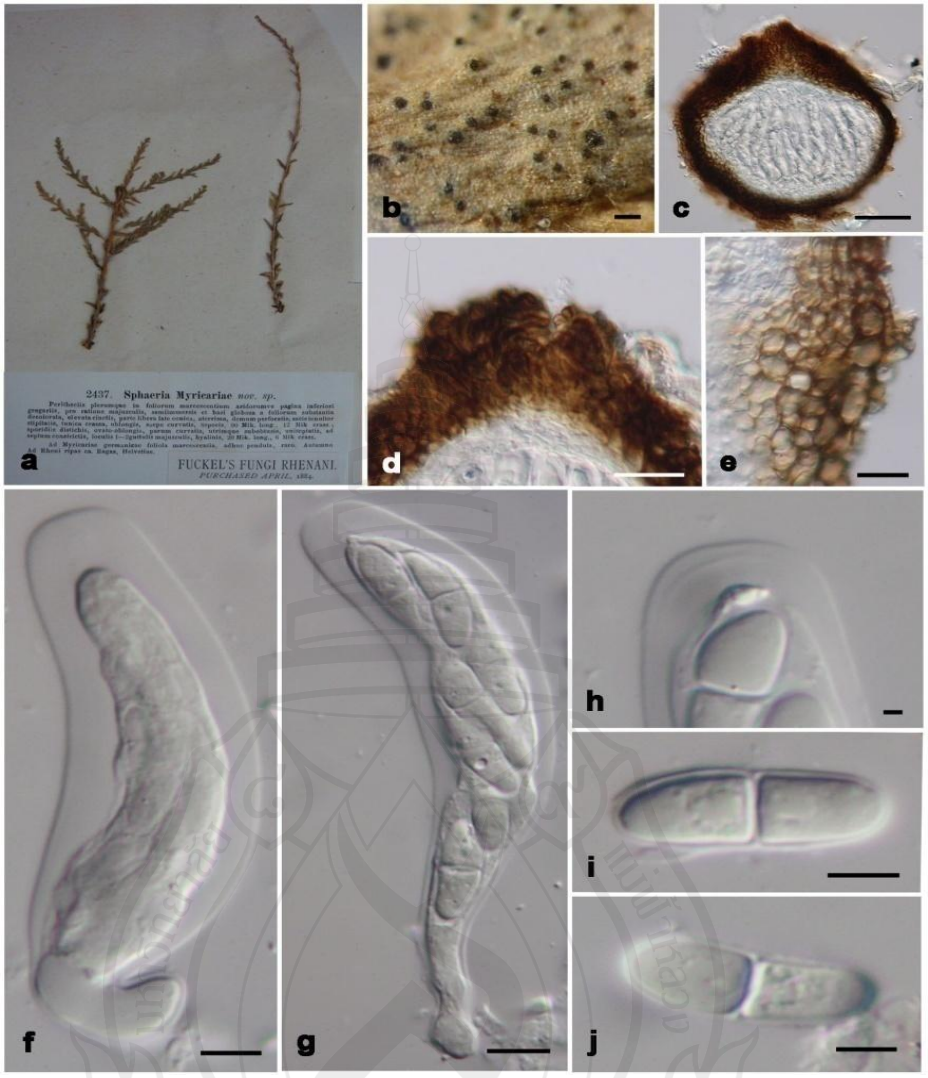
Mycosphaerellopsis Höhn., Annales Mycologici 16(1/2): 157 (1918) (Fig. 4.6)

MycoBank: MB 3345

Biotrophic or *saprotrophic* on leaves. *Ascostromata*, uniloculate, solitary, gregarious, immersed to semi-immersed, globose to subglobose, brown to dark brown. *Cells of ascostromata* composed of brown-walled cells arranged in a *textura globulosa*. *Ostioles* periphysate. *Hamathecium* lacking pseudoparaphyses at maturity. *Asci* 8-spored, bitunicate, fissitunicate, oblong to saccate, with a long pedicel, and an ocular chamber. *Ascospores* 1-3 seriate and partially overlapping, ellipsoid to broadly obovoid, broadly rounded at the apex, narrowly rounded at the base, hyaline, 1-septate, constricted at the central septum, with one guttule in each cell, slightly

roughened. *Conidiomata* pycnidia. *Conidiogenous cells* short, holoblastic. *Conidia* oblong, uniseptate, hyaline (asexual morph description follows Barr, 1996).

Notes: There are two *Mycosphaerellopsis* species recorded *M. myricariae* and *M. moravica* (Index Fungorum, n.d.). *Mycosphaerellopsis* differs from other genera in *Planistromellaceae* in having uniloculate ascomata and 1-septate, broadly obovoid ascospores. Although this study place this genus in *Planistromellaceae* its inclusion must be tentative until confirmation with molecular data. The term ascostromata have used for the fruiting body of this species, however this is rather a loose term and if the genus proved to not belong in *Planistromellaceae* these could be termed ascomata. The characters are typical of *Botryosphaeriales* and fresh material is required for sequencing and placement of this genus.



Note. (a) – (b) Ascostromata on the host surface. (c) Section of an ascostroma. (d) Close up of the ostiole. Note periphyses. (e) Section of the peridium. (f) Immature ascus. (g) Mature ascus. (h) Close up of ocular chamber. (i) – (j) Ascospores. Scale bars: (b) = 200 µm, (c) = 30 µm, (d) = 10 µm, (e) = 5 µm, (f) = 3 µm, (g) = 5 µm, (h) = 1 µm, (i) – (j) = 3 µm

Figure 4.6 *Mycosphaerellopsis myricariae* (Holotype of *Sphaeria myricariae*) on Dead Leaves of *Myricariae germanicae*

Generic Type

Mycosphaerellopsis myricariae (Fuckel) Höhn, Annales Mycologici 16(1/2):157 (1918) (Fig. 4.6)

≡ *Sphaeria myricariae* Fuckel, Jb. nassau. Ver. Naturk. 27-28: 22 (1874) [1873-74]

MycoBank: MB 499606

Biotrophic or *saprotrophic* on leaves. *Ascostromata* 77-146 µm high × 93-156 µm ($\bar{x}$ = 110 × 117.3 µm, n = 25) diam., uniloculate, solitary, gregarious, immersed to semi-immersed, globose to subglobose, brown to dark brown. *Cells of ascostromata* 12-22 µm wide, composed of 3-6 layers of brown-walled cells arranged in a *textura globulosa* (Fig. 4.6b-e). *Ostioles* periphysate (Fig. 4.6d). *Hamathecium* lacking pseudoparaphyses at maturity. *Asci* 45.5-64 × 10-13 µm ($\bar{x}$ = 53.7 × 11.5 µm, n = 25), 8-spored, bitunicate, fissitunicate, oblong to saccate, with a 4-5 µm long pedicel, and a 1-3 µm wide × 0.5-2 µm high ocular chamber (Fig. 4.6f-h). *Ascospores* 10-12 × 4-5 µm ($\bar{x}$ = 10.9 × 4.2 µm, n = 25), 1-3 seriate and partially overlapping, ellipsoid to broadly obovoid, broadly rounded at the apex, narrowly rounded at the base, hyaline, two-celled, constricted at the central septum, with one guttule in each cell, slightly roughened (Fig. 4.6i-j). *Conidiomata* pycnidia. *Conidiogenous cells* short, holoblastic. *Conidia* oblong, uniseptate, hyaline (asexual morph description follows Barr, 1996).

Material examined: UK: England, Kew, Royal Botanic Gardens, on *Myricariae germanicae*, April 1884, Fuckel's fungi rhenani (2437, holotype).

Capnodiales genera *incertae sedis*

Comminutispora A.W. Ramaley, Mycologia 88(1):132 (1996) (Figs. 4.7-4.8)

MycoBank: MB 27576

Synonym

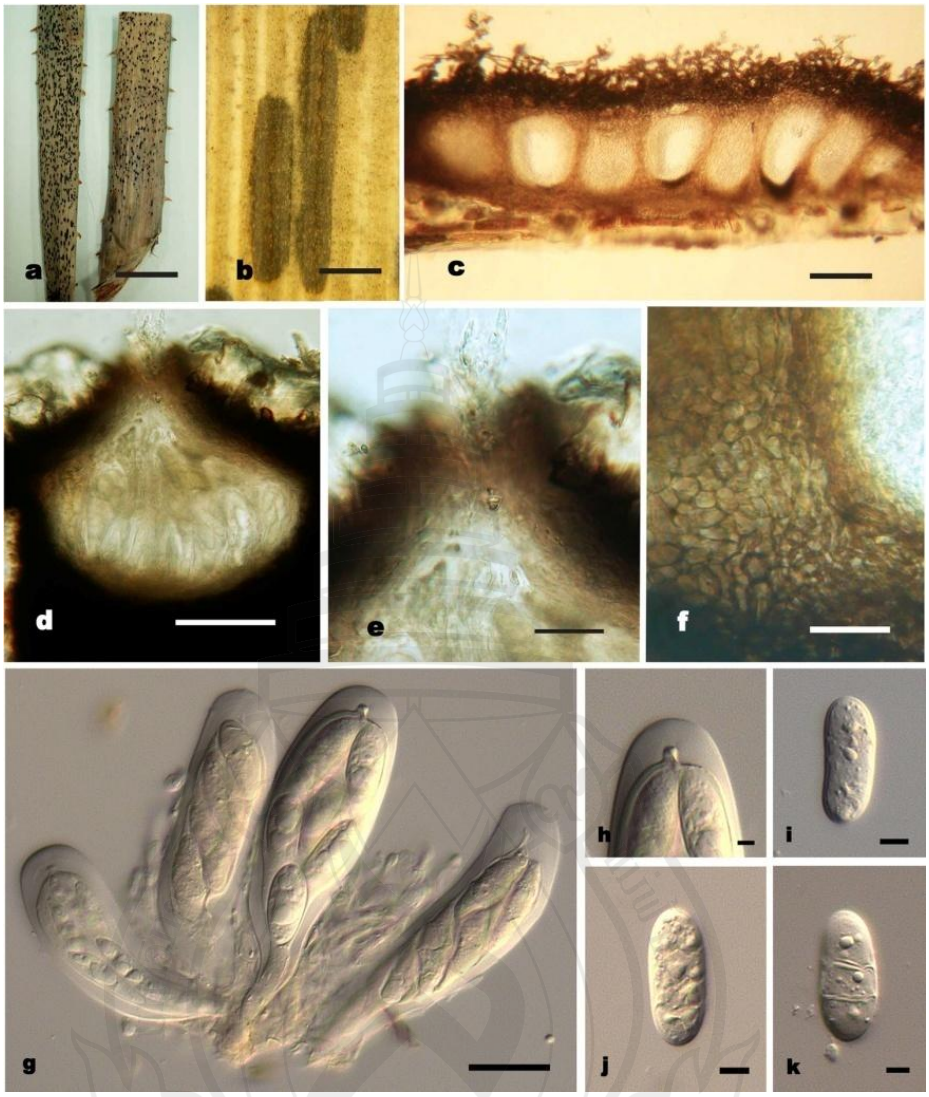
Hyphospora A.W. Ramaley, Mycologia 88(1):133 (1996)

Saprobic on dead leaves of *Asparagaceae*. *Ascostromata* apothecial and elongate hysterothecium, scattered to loosely, immersed, dark brown to black, rather dull, carbonaceous, thick-walled, composed of *textura angularis*, opening by a sunken

longitudinal slit. *Asci* 8-spored, bitunicate, fissitunicate, saccate, clavate or cylindrical, with a long pedicel, apically rounded, apex wall thick, with a large ocular chamber. *Ascospores* biseriate and overlapping in ascus, obovoid, ellipsoid to fusoid, with broadly to narrowly rounded ends, 0-3 septate when immature, transversely 1-5 septate and many longitudinal septate with ultimately forming tiny secondary ascospores when mature, roughened, with thin gelatinous coat. *Colonies* growing on MA (Malt Agar), flat to slightly effuse, radiating, edge fimbriate, hyphae generally dark brown in surface view, outward hyphae pale brownish to hyaline, darkened interior, slightly raised hairy, partly superficial and immersed. The small conidiogenous cells differentiated from vegetative hyphae developing endoconidia of dictyochlamydospores, elongation or long chains, variable shape, with transverse and longitudinal septation, dark brown constricted at the septa, smooth or slightly verrucose.

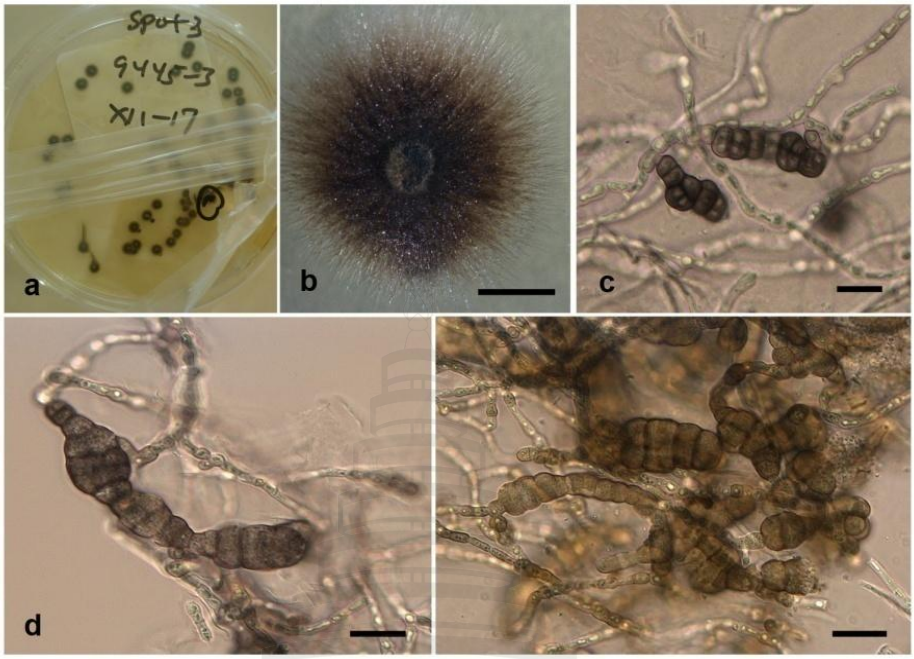
Notes: *Comminutispora* was established by Ramaley (1996) as a monotypic genus based on *C. agavacearum* and *Hyphospora agavaciensis* as an asexual morph. *C. agavacearum* is characterized by unilocular ascomata, immersed, ostiolar canal periphysate and forming transversely and longitudinally septate ascospores (Ramaley, 1996). Ramaley (1996) placed *Comminutispora* in the *Dothidiales sensu* Lindau 1897. However, following the classification by Lumbsch and Huhndorf (2010) *Comminutispora* is a member of the family *Planistromellaceae*.

Molecular analysis of the type species; *C. agavacearum* (strain CBS 619.95) was studied by Hambleton et al. (2003) and Schoch et al. (2009) on the basis of SSU and LSU genes. DNA sequence data for SSU showed that *C. agavacearum* falls in a large clade of *Capnodiales* (Hambleton et al., 2003) and thus is unrelated to *Planistromellaceae* based on SSU and LSU genes (Schoch et al., 2009). *C. agavacearum* formed a poorly supported clade with two strains of *Racondium rupestre* in the *Capnodiales* (Schoch et al., 2009), while in a larger sample of species in the order these taxa were designated *incertae sedis* (Crous et al., 2009a). Furthermore, Crous et al. (2009a) regarded the saprobes *Comminutispora* and *Phaeotheca* as an ancestral assemblage of taxa in the *Capnodiales*.



Note. (a) – (b) Ascostromata on host surface. (c) Ascostromata. (d) – (e) Close-up of ascostroma locule and ostiole. (f) Close-up of ascostromata wall. (g) Asci and pseudoparaphyses. (h) Close-up of ocular chamber. (i) – (k) Immature ascospores. Scale bars = (a) – (c) = 100 µm, (d) – (f) = 10 µm, (g) = 25 µm, (h) – (k) = 5 µm

Figure 4.7 *Comminutispora agavacearum* (Holotype of *Comminutispora agavacearum*) on Dead Leaves of *Dasylirion leiophyllum*



Note. (a) Material on dried cultures. (b) Colonies on MA. (c) – (e) Dictyochlamydospores. Scale bars: (b) = 500 µm, (c)-(e) = 20 µm

Figure 4.8 *Hyphospora agavaciensis* (Holotype of *Hyphospora agavaciensis*) on Dried Cultures at 1 Week

Generic type

Comminutispora agavacearum A.W. Ramaley [as 'agavaciensis'], Mycologia 88(1): 133 (1996) **(**Figs. 4.7-4.8)

≡ *Hyphospora agavacearum* A.W. Ramaley [as '*agavaciensis*'], Mycologia 133 (1996)

MycoBank: MB 414805

Saprobic on dead leaves. *Ascostromata* 0.6-2 mm length × 0.3-0.4 mm wide, measured at the surface of host, 300-600 µm high, and 250-300 µm diameter, within host tissue, apothecium and elongate hysterothecium, scattered to loosely, immersed, dark brown to black, rather dull, carbonaceous, thick-walled, composed of *textura*

angularis, opening by a sunken longitudinal slit (Fig. 4.7a-f). *Asci* 105-135×27.5-35 µm ($\bar{x}$ = 120.3×29.3 µm, n = 10), 8-spored, bitunicate, fissitunicate, saccate, clavate or cylindrical, with a 5-22.5 µm long pedicel, apically rounded, apex wall 5-7.5 µm thick, with an ocular chamber up to 5 µm wide × 2.5 µm high (Fig. 4.7g-h). *Ascospores* 22.5-35.5×7.5-12.5 µm ($\bar{x}$ = 27.5×9.1 µm, n = 20), biseriate and overlapping in ascus, obovoid, ellipsoid to fusoid, with broadly to narrowly rounded ends, 0-3 septate when immature, roughened, with thin gelatinous coat (Fig. 4.7i-k). Colonies growing on MA (Malt Agar), reaching 500 µm diam. in one week at 23-25 °C, flat to slightly effuse, radiating, edge fimbriate, hyphae generally dark brown on surface view, outward hyphae pale brownish to hyaline, darkened interior, slightly raised hairy, partly superficial and immersed (Fig. 4.8a-b). The small conidiogenous cells differentiated from vegetative hyphae developing endoconidia of dictyochlamydospores, elongation or long chains, variable shape, with (36.5-)93-150(-167) µm long × 15-23(-32.5) µm wide, with transverse and longitudinally septation, dark brown pigmented, constricted at the septa, smooth or slightly verrucose (Fig. 4.8c-e).

Material examined: USA: Texas, National Fungus Collections, on dead leaves of *Dasylirion leiophyllum*, October 1994 (BPI 802958, holotype of *Comminutispora agavacearum*), USA: Texas, Brewster, Big Bend National Park, on dead leaves of *Dasylirion leiophyllum*, 25 October 1994, Ramaley Annette 9445(BPI 802959, holotype of *Hyphospora agavacearum*).

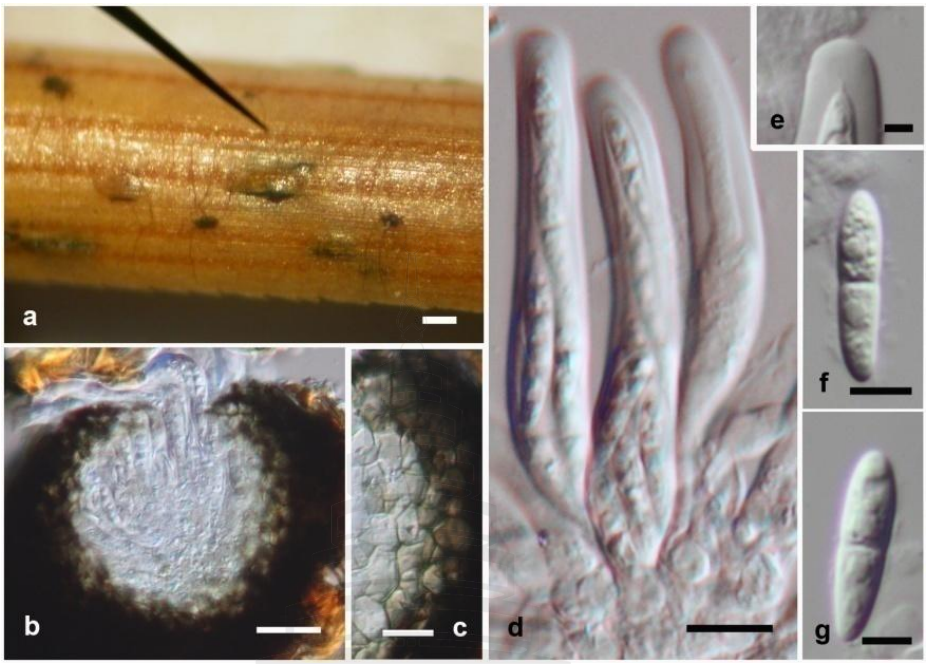
Notes: In the type specimen ascospores of *C. agavacearum* are immature and lack septa, while in the protologue they are reported to have transversely 1-5 septate and many longitudinal septate with ultimately forming tiny secondary ascospores (Ramaley, 1996); one ascospore (in Fig. 4.7k) has 3-septa.

Genera Transferred to *Mycosphaerellaceae incertae sedis*

Eruptio (Dearn.)M.E. Barr, Mycotaxon 60:437 (1996) (Fig. 4.9)

MycoBank: MB 27768

Biotrophic on leaves. *Ascostromata* uniloculate or multiloculate, linear, scattered to gregarious, immersed, erumpent through cracking or splitting of host tissues, globose to subglobose, black. *Locules* small, ovoid to globose, periphysate ostioles. *Cells of ascostromata* reddish brown to dark brown, composed pseudoparenchymatous cells of *textura globulosa*. *Hamathecium* not observed in herbarium material. *Asci* 8-spored, bitunicate, obclavate, usually wider in the base and narrower towards the apex, pedicellate, with an ocular chamber. *Ascospores* uniseriate at the top, biseriate in the middle and triseriate at the base of asci with partially overlapping,1-septate, oblong to cuneate, bluntly rounded at one end, tapering fusiform at other, hyaline, rough, 4-guttulate. *Asexual states* are recorded as *Lecanosticta* and *Dothistroma*. *Lecanosticta* is characterized by stromata; elongate, erumpent, the locules opening widely, the bases lined with short hyaline conidiophores, conidia; brown, elongate, tapered at apex, blunt at base, 3-septate, bent, the wall roughened, microconidial state: locules in immature stromata contain microconidia, hyaline, 1-celled (asexual morph description follows Barr, 1972). *Dothistroma* is characterized by *conidiomata* predominantly occurring in red bands on the upper and lower needle surfaces, separate to aggregated, sub-epidermal, becoming erumpent, and splitting the needle surface with one or two longitudinal slits; at maturity acervular, black, lined internally with pseudoparenchymatous cells giving rise to conidiophores; these cells are brown, becoming paler at the point of conidiophore attachment. *Conidiophores* pale brown to hyaline, smooth, densely aggregated, subcylindrical to irregular, 1–4 septate, branched or simple. *Conidiogenous cells* integrated, hyaline, smooth, subcylindrical, tapering towards the bluntly rounded apices, proliferating sympodially or percurrently near the apex. *Conidia* aggregated in cream to pale brown, slimy masses; smooth, thin-walled, hyaline, subcylindrical to narrowly obclavate or irregular (asexual morph description follows Barnes, Crous, Wingfield & Wingfield, 2004).



Note. (a) Ascostromata on the host surface. (b) Section of ascostroma locule. (c) Cells of ascostroma. (d) Asci. (e) Ocular chamber. (f) – (g) Ascospore. Scale bars: (a) = 50 µm, (b) = 25 µm, (c) = 20 µm, (d) = 10 µm, (e) = 3 µm, (f) – (g) = 5 µm

Figure 4.9 *Eruptio acicola* (Holotype of *Eruptio acicola*) on Withered Needles of *Yucca brevifolia*

Notes: This fungus has a complicated history with many synonyms. *Oligostroma acicola* was established by Dearness (1926) on needles of *Pinus palustris*. Barr (1972) transferred the species to *Mycosphaerella* using locule and ascus development as generic concepts rather than the position of ascomata. Barr (1996) later introduced the family *Planistromellaceae* and included *Eruptio* based on multilocular pseudothecia, with ostioles forming through cracking or splitting of host tissues, and with periphysate ostioles. *Eruptio* differs from *Planistromellaceae* as ascospores are 1-septate, narrow, oblong to fusoid and are more typical of

Mycosphaerellaceae. Presently, *Eruptio* has three species; *E. acicola*, *E. pini* and *E. gaubae* (Index Fungorum, n.d.). The asexual states, *Lecanosticta acicola* (*E. acicola*) and *Dothistroma pini* (*E. pini*), cause pine needle blight (Evans, 1984). The asexual states form acervuli in the stromata, conidia are hyaline to brown, septate, and cylindrical. *Lecanosticta* species have 1-3 septate conidia and produce microconidia, whereas *Dothistroma* species have 1-5 septate conidia (Barnes et al., 2004; Barr, 1972). *Lecanosticta gaubae* (*E. gaubae*) was removed from *Mycosphaerella* by Crous (1999) because of the different ascomatal characters and no *Lecanosticta* species are known to have asexual states in *Mycosphaerella.* Molecular analysis of the type species *E. acicola* was carried out by Crous et al. (2001) using an ITS rDNA sequence. *Eruptio* clustered in the same clade as *Mycosphaerella.* This result has since been supported in several studies based on LSU rRNA gene data (Crous et al., 2009b; Verkley et al., 2004) and SSU, ITS, LSU rRNA gene data (Crous et al., 2009a). *Lecanosticta* clustered with other asexual morphs of *Mycosphaerella* in these studies. Therefore, *Eruptio* and its asexual morphs were placed in *Mycosphaerellaceae.*

Generic Type

Eruptio acicola (Dearn.) M.E. Barr, Mycotaxon 60: 438 (1996) (Fig. 4.9)

MycoBank: MB 436296

≡*Cryptosporium acicola* Thüm. (1878)

≡*Septoria acicola* (Thüm.) Sacc. (1884)

≡*Lecanosticta pini* H. Sydow apud Sydow & Petrak (1922)

≡*Lecanosticta acicola* (Thüm.) Syd., in Sydow & Petrak, Annls mycol. 22(3/6): 400 (1924)

≡*Oligostroma acicola* Dearn, Mycologia 18(5): 251 (1926)

≡ *Scirrhia acicola* (Dearn.) Sigg., Phytopathology 29: 1076 (1939)

≡ *Systremma acicola* (Dearn.) F.A. Wolf & Barbour, Phytopathology 31: 70 (1941)

≡*Dothidea acicola* (Dearn.) M. Morelet, Annales de la Société des Sciences Naturelles et d'Archéologie de Toulon et du Var 177: 9 (1968)

≡ *Mycosphaerella dearnessii* M.E. Barr, *Contr. Univ. Mich. Herb.* 9: 587 (1972)

Biotrophic on leaves. *Ascostromata* 0.1-0.4 mm diam., up to 1.5 mm. high, uniloculate or multiloculate, linear, scattered to gregarious, immersed, erumpent through cracking or splitting of host tissues, globose to subglobose, black (Fig. 4.9a, b). *Locules* 88-98 µm high × 61-83.5 µm diam., small, ovoid to globose, periphysate ostioles (Fig. 4.9b). *Cells of ascostromata* 24-28 µm thick, reddish brown to dark brown, composed pseudoparenchymatous cells of *textura globulosa* (Fig. 4.9c). *Hamathecium* not observed in herbarium material. *Asci* 40-59 × 9-14 µm ($\bar{x}$ = 50.9 × 10.4 µm), 8-spored, obclavate, usually wider in the base and narrower towards the apex, pedicellate, with an ocular chamber 0.5 µm wide (Fig. 4.9d, e). *Ascospores* 11-15 × 2-4 µm ($\bar{x}$ = 13.8 × 3.5 µm), uniseriate at the top, biseriate in the middle and triseriate at the base of asci with partially overlapping, 1-septate, oblong to cuneate, bluntly rounded at one end, tapering fusiform at other, hyaline, rough, 4-guttulate (Fig. 4.9f, g). *Asexual state* is *Lecanosticta acicola*: *stromata* elongate, erumpent, the locules opening widely, the bases lined with short hyaline conidiophores; *conidia* 20-28 × 2.5-3 µm, brown, elongate, tapered at apex, blunt at base, 3-septate, bent, the wall roughened. *Microconidial state* locules in immature stromata contain microconidia, these hyaline, 2-3 × 1 µm, 1-celled (asexual morph description follows Barr, 1972).

Material examined: CANADA: Ontario, Ottawa; on withered needles of *Pinus palustris*, 27 February 1919, G. G. Hedgcock: 32146 (D:5831, holotype).

Microcyclus Sacc., Syd. & P. Syd., in Sydow & Sydow, Annales Mycologici 2(2): 165 (1904) (Figs. 4.10-4.11)

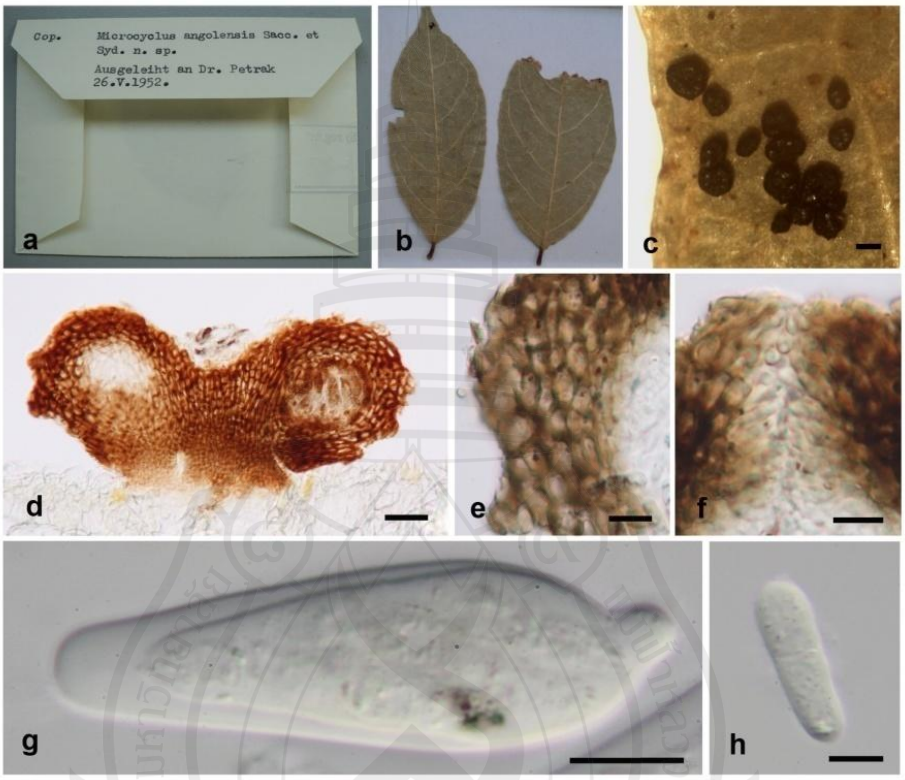
MycoBank: MB3160

Biotrophic on leaves and stems. *Ascostromata* pulvinate, irregularly shaped, developing from central basal hypostroma, superficial, multilocular, composed of pseudoparenchymatous cells; *textura angularis*, thick-walled, reddish brown. *Ostiole* papillate, periphysate. *Asci* 8-spored, thick-walled, bitunicate, fissitunicate, cylindrical to clavate, with an ocular chamber, with a long pedicel. *Ascospores*1-3 seriate partially overlapping, 1-septate, obovoid, upper cell shorter and wider than lower, not

or slightly constricted at the septum, smooth wall, granular, hyaline. Two asexual morphs have been reported for *Microcyclus* species including *Fusicladium* Bonorden and *Pazschkeella* Syd. & P. Syd. (Sivanesan, 1984). *Fusicladium* is described by *mycelium* immersed, sometimes subcuticular. *Stroma* often present, sometimes subcuticular. *Setae* absent. *Hyphopodia* absent. *Conidiophores* macronematous, mononematous, simple or occasionally once branched, often olivaceous brown, septate, usually fasciculate, bursting through the cuticle of the host plant. *Conidiogenous cells* integrated, terminal, polyblastic, sympodial, cicatrized; old conidial scars usually thickened, conspicuous and prominent, sometimes situated at the end of short lateral projections, numerous and often crowded, giving the conidiophore a nodular appearance. *Conidia* solitary or occasionally in short chains, dry, variable in shape but often tending to be broadly fusiform, truncate at the base and pointed at the apex, 0-3- (often 0- or 1-) septate, pale to mid olive or olivaceous brown, frequently minutely verruculose (Ellis, 1971). *Pazschkeella* is characterized by *conidiomata* pycnidial, solitary to gregarious, immersed to semi-immersed, becoming erumpent, thin dark brown to black wall surrounding with host tissue. *Conidiophores* hyaline, septate, cylindrical, smooth. *Conidiogenous cells* holoblastic, integrated, hyaline, cylindrical, producing a single apical conidium. *Paraphyses* non-septate, unbranched, not-anastomosed. *Conidia* hyaline, smooth, thin walled, aseptate, fusiform to cylindrical, sometimes irregular cylindrical, base obtuse, tapering toward apex attached with an appendage.

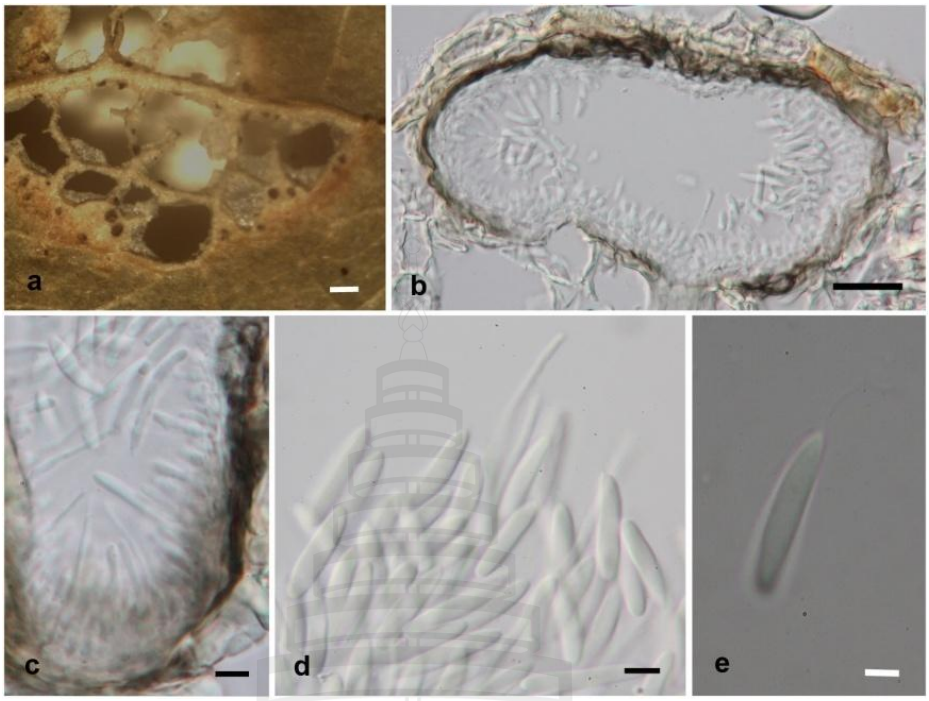
Notes: Barr (1996) arranged *Microcyclus* in the new family *Planistromellaceae* and distinguished this genus from others in the family based on its widely erumpent, multiloculate ascostromata, and 1-septate and obovoid ascospores.
The type species *M. angolensis* (Sacc.) Syd. & P. Syd. was described with periphysate ostioles by Theissen and Sydow (1915) and Müller and Sanwal (1954). Many species in this genus are biotrophic on leaves and stems of various plants in tropical and subtropical regions (Barr, 1996; Cannon, Carmarán & Romero, 1995). There are 36 species recorded in Index Fungorum (n.d.). One important species is *Microcyclus ulei* (Henn.) von Arx, an economically important pathogen that causes leaf blight on rubber trees in South America (Lieberei, 2007). The type of *Microcyclus* is similar to other genera of *Mycosphaerellaceae* in the form of its asci and ascospores, while

ascostromata are also found in *Cymadothea* (type, *C. trifolii* F.A. Wolf, Simon, Groenewald & Crous, 2009), *Euryachora* (type *E. sedii* Fuckel, genera presently placed in *Mycosphaerellaceae*, Lumbsch & Huhndorf, 2010).



Note. (a) Herbarium packet. (b) – (c) Ascostromata on the host surface. (d) Section of ascostromata. (e) Cells of ascostroma. (f) Periphysate ostiole. (g) Immature ascus. (h) Ascospore. Scale bars: (c) = 200 µm, (d) = 30 µm, (e) – (g) = 10 µm, (h) = 5 µm

Figure 4.10 *Microcyclus angolensis* (Holotype of *Microcyclus angolensis*) on Living Leaves of *Millettiae thonningii*



Note. (a) Conidiomata on the host surface. (b) Section of conidioma. (c) Conidiogenous cells. (d) Conidia and paraphyses. (e) Conidia with appendage. Scale bars: (a) – (b) = 100 µm, (c) – (d) = 5 µm, (e) = 3 µm

Figure 4.11 *Pazschkeella* sp. (Holotype of *Microcyclus angolensis*), on Living Leaves of *Millettiae thonningii*

Generic Type

Microcyclus angolensis (Sacc.) Syd. & P. Syd. , Annales Mycologici 2(2): 165 (1904) (Figs. 4.10-4.11)

MycoBank: MB 152201

Biotrophic on leaves and stems. *Ascostromata* 0.2-0.3 mm wide, 66.5-116 µm high × 58-109µm diam., pulvinate, irregularly shaped, developing from central basal hypostroma, superficial, multilocular, composed of pseudoparenchymatous cells; *textura angularis*, thick-walled, reddish brown (Fig. 4.10b-e). *Ostiole* papillate, periphyses (Fig. 4.10f). *Asci* 45-70 × 13-19 µm ($\bar{x}$ = 54.5 × 15.8 µm, n = 25), 8-

spored, thick-walled, bitunicate, fissitunicate, cylindrical to clavate,with an ocular chamber 1-1.5 µm wide × 0.5-1 µm high, with a pedicel, 4-6 µm long (Fig. 4.10g). *Ascospores*14-18 × 4-6 µm ($\bar{x}$ = 16.4 × 5.1 µm, n = 25), 1-3 seriate partially overlapping, 1-septate, obovoid, upper cell shorter and wider than lower, not or slightly constricted at the septum, smooth wall, granular, hyaline (Fig. 4.10h). An asexual morph is also present on the leaves as *Pazschkeella* sp.; *Conidiomata* 21-42.5 µm high × 49-76 µm diam., pycnidial, solitary to gregarious, immersed to semi-immersed, becoming erumpent, thin dark brown to black wall surrounded by host tissue (Fig. 4.11a-b). *Conidiophores* hyaline, septate, cylindrical, smooth. *Conidiogenous cells* 1-3 µm wide, holoblastic, integrated, hyaline, cylindrical, producing a single apical conidium (Fig. 4.11c). *Paraphyses* 1-1.5 µm wide, non-septate, unbranched, not-anastomosed (Fig. 4.11c-d). *Conidia* 13-15× 2-3 µm ($\bar{x}$ = 14 × 2.8 µm, n = 25), hyaline, smooth, thin-walled, aseptate, fusiform to cylindrical, sometimes irregular cylindrical, base obtuse, tapering toward apex attached with an appendage (7-8 µm long) (Fig. 4.11d-e).

Material examined: Angola, Africa; on living leaves of *Millettiae thonningii*, Welwitsch (F8592, F8593, holotype).

Genus Transferred to *Phaeosphaeriaceae*

Loratospora Kohlm. & Volkm.-Kohlm., Syst. Ascom. 12(1-2): 10 (1993) (Fig. 4.12)

MycoBank: MB 26473

Saprobic on dead culms of *Juncus roemerianus*. *Ascostromata* immersed in host tissue under a slightly raised darkened area, subglobose, solitary, gregarious, with periphysate ostioles. *Cells of ascostromata* composed of 4-5 layers of brown thick-walled, cuboid or angular cells. *Asci* 8-spored, bitunicate, fissitunicate, clavate to ovoid, with a short knob-like pedicel, apically rounded apex, and with ocular chamber. *Ascospores* 3-4 overlapping seriate, hyaline, narrowly obovoid, fusoid or clavate, 3-septate, slightly constricted septum, smooth-walled, surrounded by a thin mucilaginous sheath. *Asexual state* not established.

Notes: Loratospora is a monotypic genus represented by *L. aestuarii* (Index Fungorum, n.d.). This species is an obligate to facultative marine taxon and is characterized by unilocular ascostromata that are immersed in host tissue, with 3-septate, elongate ascospores (Jones, Sakayaroj, Suetrong, Somrithipol & Pang, 2009; Kohlmeyer & Volkmann-Kohlmeyer, 1993). Barr (1996) placed *L. aestuarii* in *Planistromellaceae* because its locules open through cracking or splitting of the host tissue and its periphysate ostioles. Combined multigene molecular analysis of *L. aestuarii* strain JK 5535D based on SSU, LSU andRPB2 gene data was carried out by Suetrong et al., (2009). *L. aestuarii* grouped in *Phaeosphaeriaceae* and this is followed here.

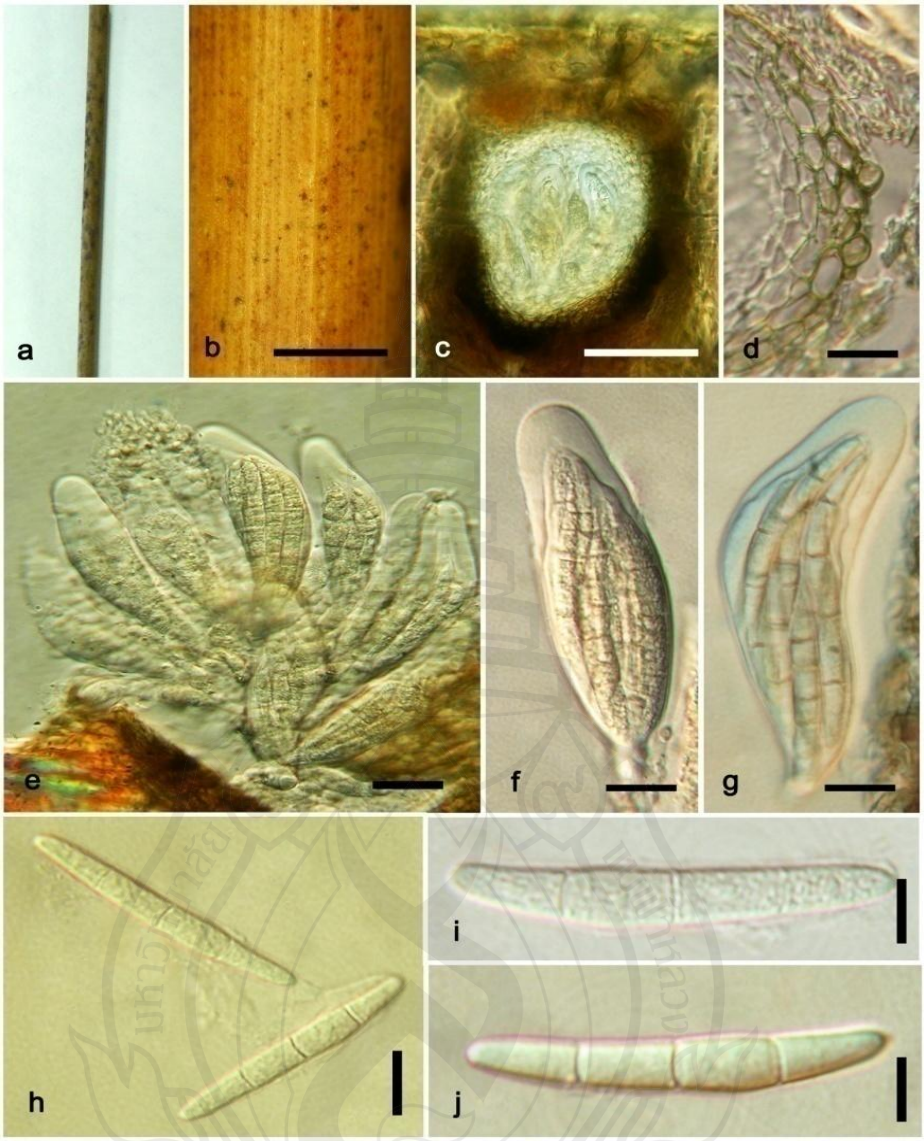
Generic Type

Loratospora aestuarii Kohlm. & Volkm.-Kohlm., Systema Ascomycetum. 12(1-2): 10 (1993) (Figs. 4.12)

MycoBank: MB 360815

Saprobic on dead culms of *Juncus roemerianus*. *Ascostromata* immersed in host tissue under a slightly raised darkened area (Fig 4.12a, b). *Locule*s 97.5-125 µm diam. × 117.5-175 µm high, subglobose, solitary, gregarious, with periphysate ostioles (Fig. 4.12c). *Cells of ascostromata* 17.5-18.8 µm thick ($\bar{x}$ = 17 µm, n = 5), composed of 4-5 layers of, brown thick-walled, cuboid or angular cells (Fig. 4.12d). *Asci* 75-137.5 µm × 20-32.5 µm ($\bar{x}$ = 95.5 × 26.3 µm, n = 20), 8-spored, bitunicate, fissitunicate, clavate to ovoid, with a short knob-like pedicel, apically rounded with 10-22.5 µm thick apex, and 0.5-1 µm wide ocular chamber (Fig. 4.12e-g). *Ascospores* 42.5-55-(-57.5) µm × 5-6 µm ($\bar{x}$ = 53.5 × 6.8 µm, n = 20), 3-4 overlapping seriate, hyaline, narrowly obovoid, fusoid or clavate, 3-septate, slightly constricted at septum, smooth-walled, surrounded by a thin mucilaginous sheath (Fig. 4.12h-j). *Asexual state* not established.

Material examined: USA, North Carolina, Broad Creek, on dead culms of *Juncus roemerianus*, 6 April 1993, J.K. 5505 (holotype: IMS).



Note. (a) – (b) Ascostromata forming in host tissue. (c) Vertical section through the ascostroma with asci. (d) Close-up of the peridium at the ascoma side. (note that the wall is not divided into distinct layers). (e) – (g) Asci 8-spored. (h) – (j) Ascospores 3-septate. Scale bars: (b) – (c) = 100 µm, (d) – (g) = 25 µm, (h) – (j) = 5 µm

Figure 4.12 *Loratospora aestuarii* (Holotype of *Loratospora aestuarii*) on Dead Culms of *Juncus roemerianus*

4.4 Conclusion

The *Planistromellaceae* is reduced to include *Kellermania* and *Planistroma* based on molecular data. *Mycosphaerellopsis* is tentatively included, based on morphology, however there is no molecular data to support this. The generic concept of this family represented by type species; *Kellermania yuccifoliorum* is subepidermal, immersed, multilocular ascostromata with periphysate ostioles, bitunicate, slightly clavate or nearly cylindric asci, and smooth, hyaline, septate ascospores. Further studies are expecially needed for sequencing and isolating many more taxa from fresh collections to establish the correct systematic of this group.

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CHAPTER 5

ANTIMICROBIAL ACTIVITIES OF SAPROBIC ASCOMYCETES

5.1 Introduction

For thousands of years bioactive compounds from microorganisms have provided many pharmaceutical aspects in various kinds of effective drug, antibiotic and vaccine (Berdy, 2005). However, with increasing of health problems in the world caused by the overuse of antibiotics, the resistance of antimicrobial agents and the emerging infectious diseases have become recurrent concern (Cohen, 2000; Livermore, 2004; Rochea & Guégan, 2011). In the past several years, the emergence and overgrowth of important infections caused by multi-drug resistant organisms have been more serious and become untreatable (Aksoy & Unal, 2008). Therefore, this is urgent need for searching new and effective antimicrobial agents.

Fungi serve as a significant source of bioactive compounds as diverse chemical structures and activities (Zhong & Xiao, 2009). Moreover, most secondary metabolites from fungi represent the drug-like structures which take more advantages of drug discovery (König et al., 2006; Strobel, 2003). On the basis of antibiotics discovery, the secondary metabolites from fungi have been possessed an enormous worldwide producers during the last decades (Berdy, 2005). In the beginning history of antibiotic discovery, there were many products derived from fungi such as penicillin produced by *Penicillium notatum*. (Fleming, 1929), griseofulvin isolated from *Penicillium griseofulvum* (Rehm, 1980), immunosuppressant cyclosporin produced by *Tolypocladium inflatum* (Dreyfuss et al., 1976) and the cholesterol biosynthesis inhibitor lovastatin isolated from *Aspergillus terreus* (Albert et al., 1980).

Recently the screening of secondary metabolites produced by microorganisms which isolated from unusual or unexplored habitats is highly increasing (Gunatilaka, 2006). Endophytic fungi and marine fungi are remarkable groups to search for new bioactive compounds (Firáková et al., 2007; Lin et al., 2005). There have been many previous researches on screening antimicrobial activities from endophytic fungi (Aly et al., 2008; Phongpaichit et al., 2006; Wang et al., 2007; Xing et al., 2011). Gu (2009) studied on screening antimicrobial activity of endophytic fungi isolated from *Malus halliana* and it was found that *Alternaria brassicicola* ML-P08 showing strong antimicrobial activity. Some studies found several new compounds which showed strong antimicrobial activities and these compounds were elucidated and analyzed for chemical structures (Adelin et al., 2011; Lin et al., 2005; Sun, Zhang, Zhang & Feng, 2011; Wang, 2012). For example, *Penicillium ochrochloron* showed strong antimicrobial activities against various tested microorganisms; gram positive, gram negative bacteria and the yeast *Candida albicans.* Two antimicrobial compounds were identified as (-)2,3,4-trihydroxybutanamide and (-)erythritol (Rančić et al., 2006).

Otherwise, the investigation of fungi isolated from plant debris or dead plant materials is an interesting alternative for finding novel bioactive compounds because these fungi can degrade organic materials in decaying process and might secrete secondary metabolites for promoting of their growth (Duong et al., 2004). There have been few studies about screening bioactive compounds from saprobic fungi, particularly in freshwater fungi (Wang et al., 2008b). The examples of some bioactive compounds which are derived from freshwater fungi are two new eudesmane sesquiterpenes (rhombidiol) isolated from *Beltrania rhombica* (Rukachaisirikul et al., 2005) and Ophiocerins A-D, ophioceric acid, an africane sesquiterpenoid isolated from *Ophioceras venezuelence* (Reategui et al., 2005). Another interesting research related to screening antimicrobial substances from saprobic fungi is Plectasin (small cysteine-rich peptides) isolated from *Pseudoplectania nigrella* showed active against *Streptococcus pneumoniae*, which the strains of this pathogen are resistant to conventional antibiotics (Mygind et al., 2005). The discovery of saprobic fungi as a new source of antimicrobial protein has been considered for development a human therapeutic (Mygind et al., 2005).

During the examination of the saprobic fungi from *Magnolia liliifera* and *Cinnamomum iners* leaves in northern Thailand, the studies isolated many species of saprobic fungi including some interesting and probably novel fungal species. These were selected for screening of bioactive compounds that have present antimicrobial activity. The compounds were extracted, separated and analyzed for initial chemical composition including detection for antimicrobial activity. The details of this study are presented in this chapter.

5.2 Materials and Methods

5.2.1 Antimicrobial Activities Screening from Non-Fractioned Extracts

5.2.1.1 Fungal Isolates

From a total of 32 fungi isolated from dead leaves (19 fungi from *Magnolia liliifera* and 13 fungi from *Cinnamomum iners*) as reported in chapter 2, only 10 isolates were selected for antimicrobial activities screening. A list of all fungi and their information are presented in Appendix B. These fungal isolates were selected since they are genera which have been reported on producing many bioactivities and/or were marked as probably new species (see Table 5.1). Fungi that are possible new to science were described and illustrated in chapter 3. The characteristics of other selected fungal isolates are showed in Appendix C (Fig. C1-C6).

5.2.1.2 Non-Fractioned Extracts Preparation

Inoculum was prepared by cutting agar of pure culture plate into discs, 4 mm. diameter, from the margin of a colony. The inoculum disc was inverted onto the centre of an 85 mm petri dish containing 20 ml of sabouraud dextrose agar (SDA). Inoculated plates were incubated for 30 days at 28 °C. Solid phase extraction method (Chamyuang, 2010) was adapted for fungal metabolites production. For each isolate, five replicate experiments were carried out. Fungal cultures with agar from each isolate were macerated with 30 ml ethyl acetate (EtOAc) and blended with a sterile

Table 5.1 Some Species of Selected Fungi which Have Been Reported on Producing Antimicrobial Compounds

Genera	Species	Metabolites	Activities	References
Beltrania	*B. rhombica*	rhombidiol (1) and rhombitriol (2)	antibacterial and antifungal	Rukachaisirikul et al. (2005)
Chaetomium	*Chaetomium* sp.	(-)-musanahol and 3-epi-aureonitol	antimicrobial	Marwah, Fatope, Deadman, Al-Maqbalib and Husbanda (2007)
	C. globosum	chaetoviridins A and B	against rice blast (*Magnaporthe grisea*)	Park et al (2005a)
Clonostachys	*Clonostachys* sp.	clonostachin	antifungal and antibacterial	Montesinos (2007)
	C. rosea	peptaibols	antifungal	Rodríguez, Cabrera, Gozzo, Eberlin and Godeas (2011)
Fusicoccum	*Fusicoccum* sp. (IMI CC No. 351573)	xanthofusin	antifungal	Breinholt, Demuth, Lange, Kjaer and Pedersen (1993)
	F. amygdali	fusicoccin	phytotoxic activity	Ballio et al. (1964)
Ophioceras	*O. venezuelense*	ophiocerins A-D	antifungal and antibacterial	Re tegui et al. (2005)
	O. dolichostomum	ophiocerol, isoamericanoic acid A and caffeic acid	antifungal	Dong et al. (2010)
Stachybotrys	*S. parvispora*	stachybotrin C and parvisporin	cell survival activity	Nozawa et al (1997)
	S. bisbyi	bisbynin	anti-pathogenic bacteria	De Silva et al. (1995)
	S. chartarum	cyclosporin family	immunosuppressant	Kazutoshi et al. (1993)

stainless blender. This extraction process was carried out three times. The agar slurry was left sitting overnight for the first EtOAc extraction, and eight hours each for the second and third extractions. All three EtOAc were combined and transferred to a pre-weighed vial and air dried to yield a crude extract. Crude extracts were extracts prepared to a concentration of 1 g/ml in methanol and stored in 4 °C in airtight bottles. The process of the solid phase extraction is showed in Fig. 5.1.

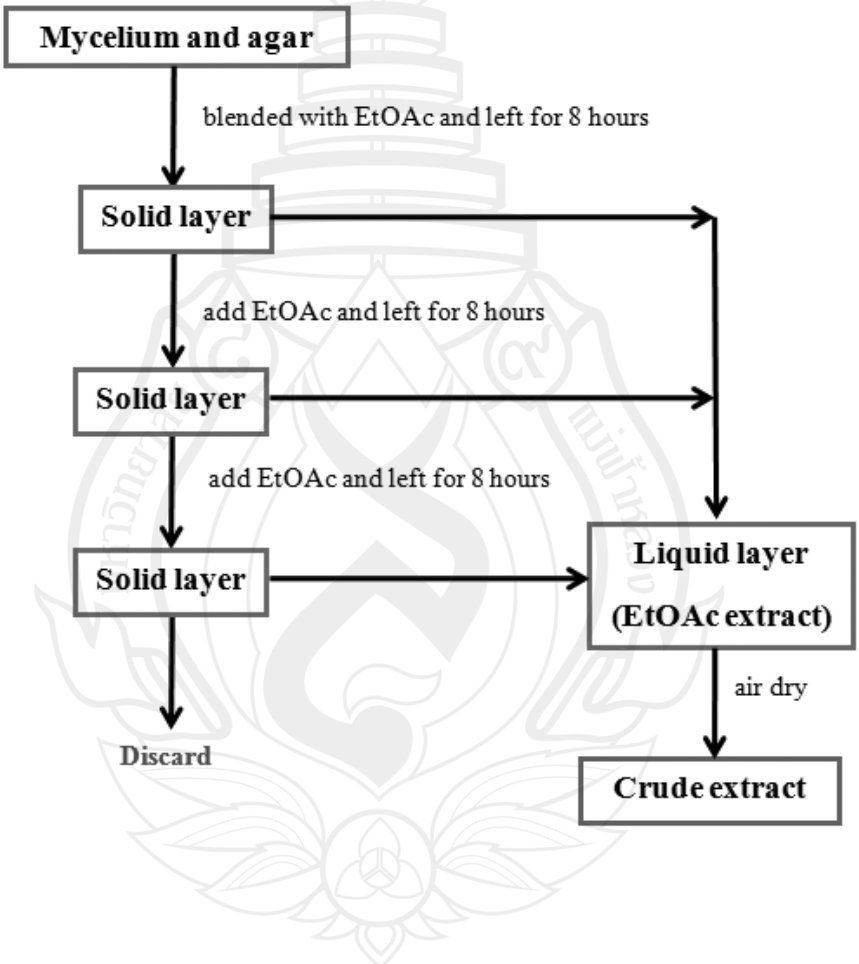


Figure 5.1 Non-Fractioned Extracts Preparation

5.2.1.3 Test Microorganisms

Five pathogenic bacteria and 4 pathogenic fungi were used as test microorganisms for antimicrobial activities assay of non-fractioned extracts. *Bacillus cereus* (TISTR 687), *Escherichia coli* (TISTR 780), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (TISTR 292), *Staphylococcus aureus* (TISTR 1466) and *Candida albicans* (TISTR 5779) were obtained from Biology and Biotechnology Laboratory of the Scientific and Technological Instruments Center, Mae Fah Luang University, Chiang Rai. *Colletotrichum fructicola* (MFLUCC10-0202), *Fusarium* sp. (MFUCC11-0219) and *Alternaria* sp. (MFUCC11-0123) were obtained from Mae Fah Luang University Culture Collection (MFUCC), Mae Fah Luang University, Chiang Rai.

5.2.1.4 Antimicrobial Activities Assay of Non-Fractioned Extracts

A modified paper disk method was used for antimicrobial activities assay (Gu, 2009; Wang et al, 2008b). Bacteria were grown in nutrient broth at 37 °C for 18 hr and yeast was grown in potato dextrose broth at 30 °C for 18 hr. After that, they were adjusted to approximately 10^8 colony-forming unit per milliliter (CFU/ml). Agar plates were swabbed uniformly by test bacteria on nutrient agar (NA) and yeast on potato dextrose agar (PDA). Whereas, other fungi were grown in PDA at 28 °C for 2-3 days until radius of colony are reached to approximately 20 mm. Sterile paper disks (6 mm diameter) each containing 20 µl of sample solution (0.1 g/ml) were dried thoroughly and placed on the surface of medium. The test plates were then incubated at 37 °C for 24 hr for bacteria and 28 °C for 1-4 days for fungi. Methanol was used as negative controls. Streptomycin sulphate (10 µg/ml) were used for bacteria as a positive control. For each test, five replicates were performed. The diameter (mm) of the growth inhibition are examined and measured.

5.2.2 Antimicrobial Activities Screening from Fractioned Extracts

5.2.2.1 Fungal Isolates

Nine fungal isolates following section 5.2.1.1 were used for antimicrobial activities screening. Furthermore, 4 fungi were provided from Department of Biochemistry and Microbiology, Institute of chemical technology, Prague, Czech

Republic to obtain more samples for antimicrobial activities test. These fungi were *Aspergillus oryzae* DBM4336, *Botrytis cinerea* DBM4208 and *Stachybotrys chartarum* DBM4297.

5.2.2.2 Fractioned Extracts Preparation

Firstly, fungal isolates was processed by solid phase extraction method using similar procedure as section 5.2.1.2. For each isolate, five replicate experiments were carried out. After incubation, mycelium was maximally scraped out from the surface of agar. Both mycelium and agar were mixed together using grinder. The extraction process was carried out three times. Ethanol was added into the mixtures of agar and mycelium with equal volume (1:1) at the first and the second extraction. At the third extraction, ethyl acetate was used with equal volume (1:1) to the mixture. The extraction was continued by shaking at 150 rpm for 24 hours. After the extraction had been finished, all extracts were combined and evaporated by rotary evaporator. To yield a crude extract, all extracts were air dried and collected into vial (Fig. 5.2).

Crude extracts were separated into four fractions by their hydrophilic and hydrophobic properties. The solvents of petroleum ether, acetone, ethanol and water were used to dissolve crude extracts respectively. To clean each fraction, insoluble solvent was used in a volume as much as possible by the centrifugation. Then all fractions were air dried, weighed and kept in small vial (Fig. 5.3).

5.2.2.3 Test Microorganisms

Six pathogenic bacteria and 1 pathogenic fungus were used as test microorganisms for antimicrobial activities assay of fractioned extracts. *Staphylococcus aureus* (DBM 3002), *Staphylococcus epidermidis* (DBM 3072), *Bacillus cereus* (DBM 3035), *Enterococcus faecalis* (DBM 3075), *Pseudomonas aeruginosa* (DBM 3081), *Enterobacter cloacae* (DBM 3126) and *Saccharomyces cerevisiae* (DBM 2101) were obtained from Department of Biochemistry and Microbiology, Institute of chemical technology, Prague, Czech Republic.

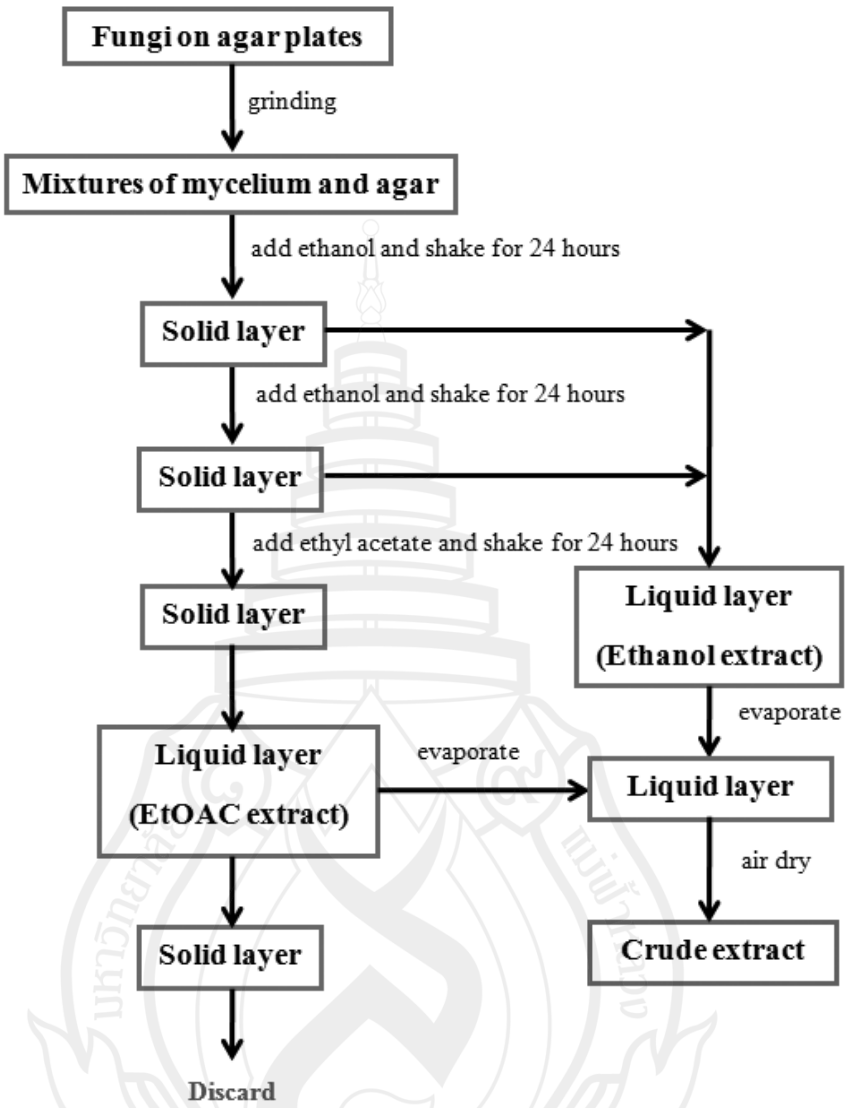


Figure 5.2 A Diagram Describing the Extraction of Crude Fungal Metabolites

5.2.2.4 Antimicrobial Activities Assay of Fractioned Extracts

The antimicrobial activities assay of fractioned extracts were used procedure in section 5.2.1.4. In case of fraction A, a needle was used to pick up extracts as small portion and spot on agar plate.

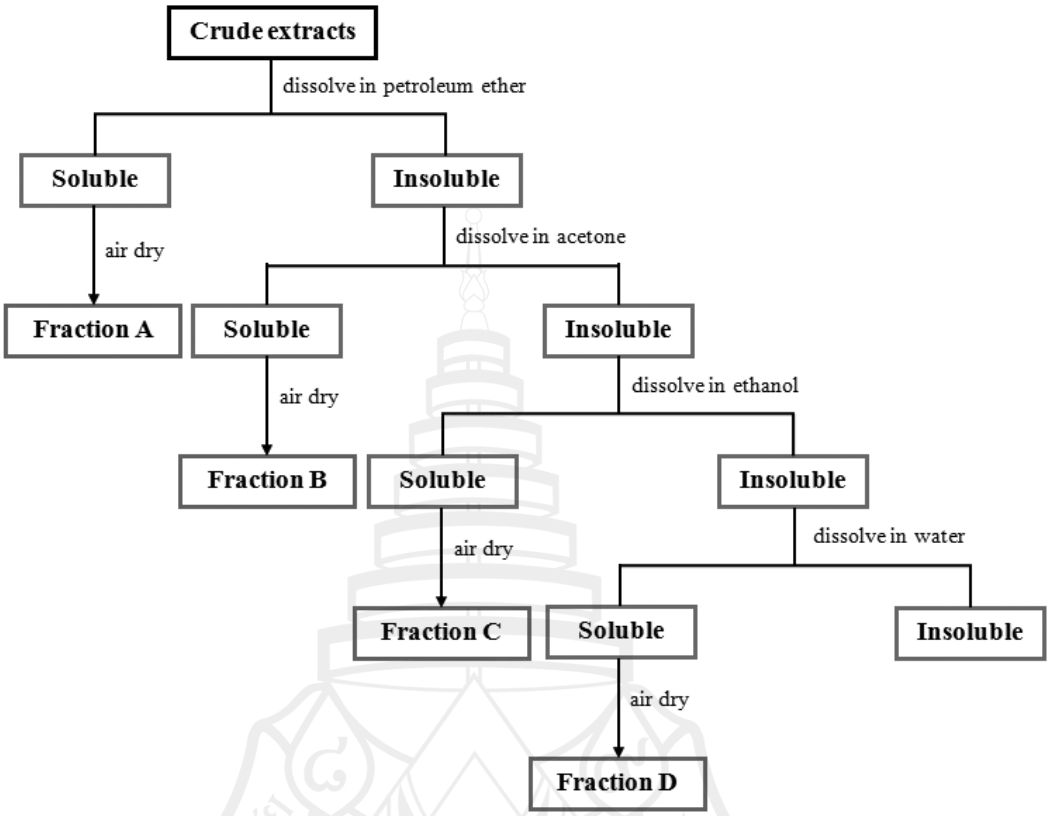
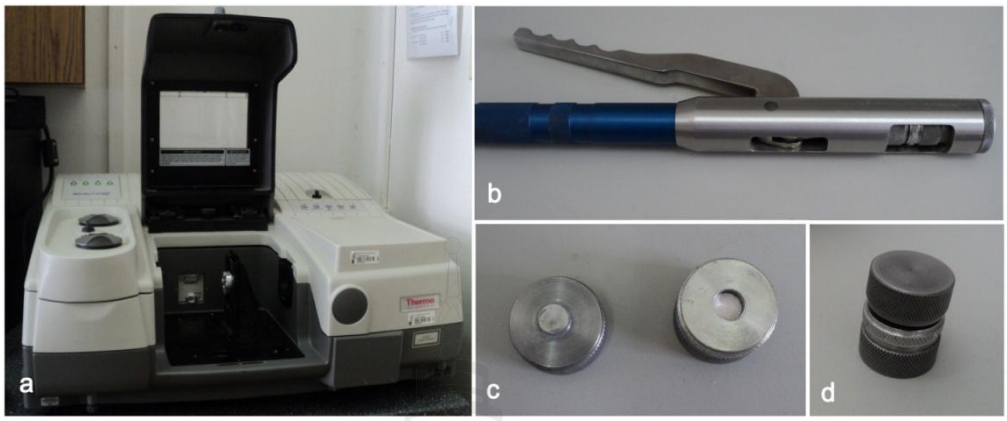


Figure 5.3 A Diagram Describing the Separation of Fungal Crude Extracts Using Different Solvents

5.2.3 FTIR Analysis of Fractioned Extracts

Crude extract about 1 mg was ground using a mortar and pestle with 100 mg of potassium bromide (for IR spectroscopy, MERCK) until fully mixed. The sample was put into the die set (Fig. 5.4c-d) and prepared into a thin tablet by using the hand press (Fig. 5.4b). A good sample tablet is thin and transparent. The samples were scanned by fourier-transform infrared spectra (FTIR) (Thermo Nicolet Model-6700) with spectral range from 4000 to 400 cm^{-1} and processed with the software program (Omnic 7.3). All the spectra were measured at a spectral resolution of 2 cm^{-1} and 64 scans were taken per sample. The FTIR instruments and its components are showed in Fig. 5.4.



Note. (a) FTIR (Thermo Nicolet Model-6700). (b) The hand press. (c)-(d) The die set.

Figure 5.4 The FTIR Instruments and Its Components

5.3 Results and Discussion

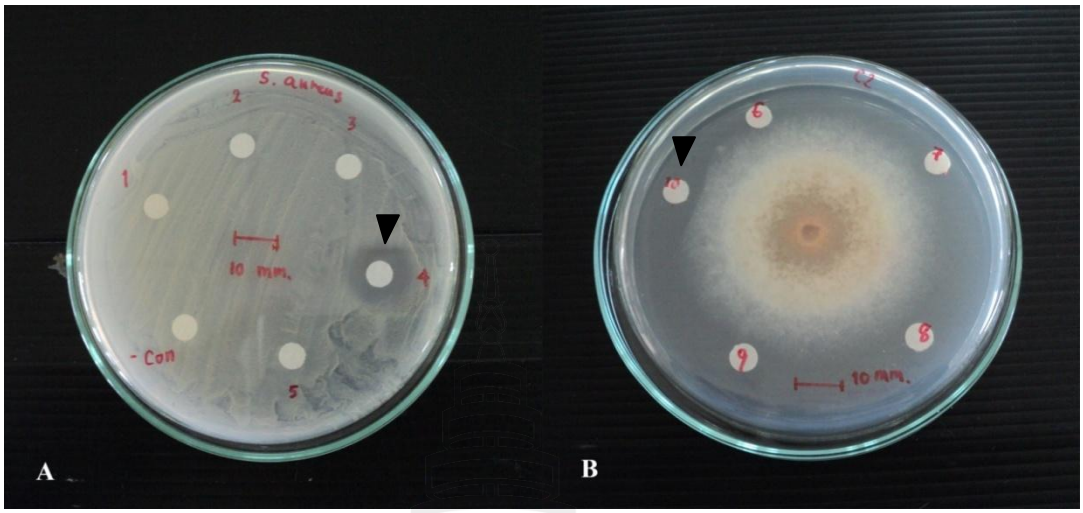
5.3.1 Antimicrobial Activity of Non-Fraction Extracts

The antimicrobial assay of ten fungal metabolites was processed *in vitro* by modified disc diffusion methods against nine pathogenic microorganisms. The result of microbial growth inhibition testing by fungal extracts was shown in Table 5.2. For antibacterial test, it was found that four fungal species inhibited the growth of at least one test bacteria. Among them, *Ophioceras* cf. *leptosporum* (JM18) showed the best antibacterial activity against *S. aureus* and followed by *B. cereus* (Fig. 5.5A). *Fusicoccum aesculi* (JM19-2) and *Chaetomium* sp. (CI18) also exhibited antimicrobial activity against *Ps. aeruginosa*. Another fungi, *Clonostachys rosea* (JM13) inhibited the growth of *B. cereus.* In antifungal test, only two fungal species *Chaetomium* sp. (CI18) and *Clonostachys rosea* (JM13) showed the inhibition of growth to *C. fructicola* (Fig. 5.5B), respectively.

Table 5.2 Antimicrobial Activities of Non-Fractioned Extracts against Some Pathogenic Microorganisms (Mean ± SD)

Fungal extracts	Zone of inhibition (mm)								
	BC	**EC**	**PA**	**ST**	**SA**	**CA**	**CF**	**F**	**A**
Stachybotrys parvispora JM09	0	0	0	0	0	0	0	0	0
Clonostachys rosea JM13	0	0	0	0	0	0	0	0	0
Beltrania rhombica JM14	0	0	8±0.6	0	0	0	11±1.3	0	0
Ophioceras cf. *leptosporum* JM18	8±0	0	0	0	0	0	8±1	0	0
Fusicoccum aesculi JM19-1	0	0	9±0.7	0	0	11±1.6	0	0	0
Fusicoccum aesculi JM19-2	0	0	0	0	0	10±1	0	0	0
Ascomycetes sp. 1 JM25	0	0	0	0	0	0	0	0	0
Hyphomycetes sp. 2 CI03	0	0	0	0	0	0	0	0	0
Hyphomycetes sp. 3 CI09	8±0.3	0	0	0	15±1	0	0	0	0
Chaetomium sp. CI18	0	0	0	0	0	0	0	0	0
Methanol	0	0	0	0	0	0	0	0	0
Streptomycin sulphate	20	20	15	20	14	0	0	0	0

Note. BC = *Bacillus cereus*, EC = *Escherichia coli*, PA = *Pseudomonas aeruginosa*, ST = *Salmonella typhimurium*, SA = *Staphylococcus aureus*, CA = *Candida albicans*, CF = *Colletotrichum fructicola*, F = *Fusarium* sp. and A = *Alternaria* sp.



Note. (A) Inhibition zone of *Ophioceras* cf. *leptosporum* (JM18) on *S. aureus* plate (arrow point). (B) Inhibition zone of *Chaetomium* sp. 1 (CI18) with *C. fructicola* (arrow point)

Figure 5.5 Disc Diffusion Assay Showing Antimicrobial Activity of Fungal Extracts

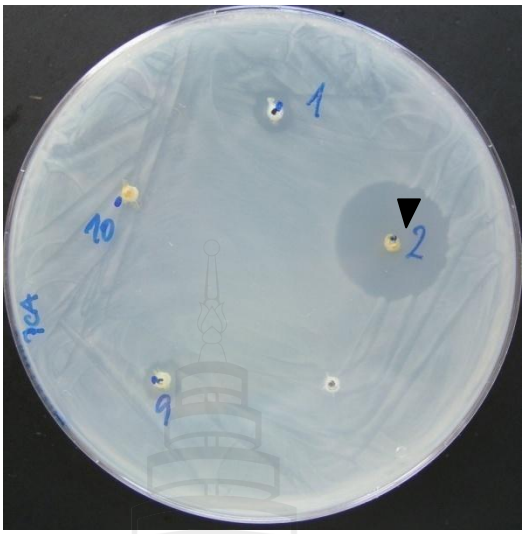
5.3.2 Antimicrobial Activity of Fractioned Extracts

For the antimicrobial assay of fractioned extracts, 9 selected saprobic fungi and 5 fungi obtained from Department of Biochemistry and Microbiology, Institute of chemical technology, Prague, Czech republic were extracted for metabolites and crude extracts were isolated into several fractions. The weight of fungal crude extracts in each fraction is shown in Appendix D (Table D1). The results of microbial growth inhibition testing by fungal extracts were shown in Tables 5.3. From the results, it can conclude that *Trichoderma* sp. (DBM4197), *Aspergillus oryzae* (DBM4336), *Botrytis cinerea* (DBM4208), *Stachybotrys chartarum* (DBM4297), *Fusicoccum aesculi* (JM019-1, JM019-2), *Chaetomium* sp. (CI18) Ascomycete sp. (JM25), *Clonostachys rosea* (JM13), *Beltrania rhombica* (JM14) and *Stachybotrys parvispora* (JM09) were effective inhibitors against some test microorganism (Fig. 5.6).

Table 5.3 Antimicrobial Activities of Fungal Extracts (Fraction A, C and D) against some Pathogenic Microorganisms

Fungal extracts	Fraction	Zone of inhibition (mm)						
		BC	**EC**	**EF**	**PA**	**SA**	**SC**	**SE**
Trichoderma sp. DBM4197	A	6	0	0	0	0	0	ND
Aspergillus oryzae DBM4336	A	20	0	3	0	13	0	ND
Aspergillus oryzae DBM4336	C	10	ND	ND	ND	11	ND	7
Botrytis cinerea DBM4208	A	0	0	0	0	0	0	ND
Botrytis cinerea DBM4208	C	8	ND	ND	ND	5	ND	6
Stachybotrys chartarum DBM4297	A	0	0	3	0	6	0	ND
Fusicoccum aesculi JM019-1	A	0	0	0	0	0	0	ND
Fusicoccum aesculi JM019-1	C	7	5	0	0	0	0	ND
Fusicoccum aesculi JM019-2	A	0	0	0	0	0	0	ND
Fusicoccum aesculi JM019-2	C	6	ND	ND	ND	0	ND	0
Chaetomium sp. CI18	A	7	0	0	0	0	0	ND
Chaetomium sp. CI18	C	6	0	0	0	0	0	ND
Chaetomium sp. CI18	D	0	ND	ND	ND	0	ND	9
Ascomycete sp. 1 JM25	A	0	0	0	0	7	0	0
Ascomycete sp. 1 JM25	C	0	0	0	0	0	0	ND
Clonostachys rosea JM13	A	5	0	0	0	0	0	ND
Clonostachys rosea JM13	C	10	6	5	0	4	4	9
Clonostachys rosea JM13	D	3	ND	ND	ND	4	ND	4
Beltrania rhombica JM14	A	0	0	0	0	0	0	ND
Beltrania rhombica JM14	C	7	ND	ND	ND	6	ND	5
Stachybotrys parvispora JM09	C	12	ND	ND	ND	5	ND	6
Hyphomycetes sp. 2 CI03	C	0	ND	ND	ND	0	ND	0

Note. SA = *Staphylococcus aureus*, BC = *Bacillus cereus*, EF = *Enterococcus faecalis*, PA = *Pseudomonas aeruginosa*, EC = *Enterobacter cloacae*, SC = *Saccharomyces cerevisiae*, SE = *Staphylococcus epidermidis*, ND = not determined



Note. no.1= *Trichoderma* sp., no.2 = *Aspergillus oryzae*, no.9 = *Clonostachys rosea*, no.10 = *Beltrania rhombica*

Figure 5.6 Antimicrobial Activity of Fungal Extracts in Fraction A

The screening of saprobic fungi for their antimicrobial activity showed some inhibition against at least one of test microorganisms. This represented a high potential of saprobic fungi for production of antimicrobial production. From all selected saprobic fungi, *Clonostachys rosea* (JM13) showed the highest antimicrobial activity in all tested fractions. The fungal extracts from this fungus have a broad spectrum of activity against several bacteria (gram negative and positive) and fungi including, *B. cereus*, *En. cloacae*, *En. faecalis*, *C. fructicola*, *S. cerevisiae*, *S. aureus* and *S. epidermidis*. *Clonostachys rosea* is known as mycoparasites of several pathogens including other fungi, bacteria, and insects and produces a wide range of volatile organic compounds (Toledo, Virla, Humber, Paradell & Lopez-Lastra, 2006; Yu & Sutton, 1997) *Clonostachys rosea* BAFC3874 was effective antagonist against the soil-borne pathogen *S. sclerotiorum* and an unusual family of compounds named peptaibols was isolated and identified from this fungal strain (Rodríguez et al., 2011). In addition two saprobic fungi; *Beltrania rhombica* (JM14) and *Stachybotry*

parvispora (JM09) have strong antimicrobial activity against bacteria especially in fraction C that contain various compounds. The fraction A extracts of *Beltrania rhombica* (JM14) had no inhibitory to all test microorganisms. This result showed that the active compounds might be not lipids compounds. Many antimicrobial compounds have been reported from these fungi. Rukachaisirikul et al. (2005) isolated two new eudesmane sesquiterpenes, named rhombidiol (1) and rhombitriol (2) from the aquatic fungus *Beltrania rhombica*. The screening of antimicrobial compounds from freshwater fungi found that *Beltrania rhombica* showed a broad activity spectrum, being able to affect Gram-positive, Gram-negative, yeast and some of the fungal phytopathogens (Gamboa-Angulo, De la Rosa-García, Heredia-Abarca & Medina-Baizabal, 2012). Moreover, Nozawa et al. (1997) founded that stachybotrin C and parvisporin isolated from *Stachybotry parvispora* showed cell survival activity.

There are also interesting saprobic fungi including *Chaetomium* (CI18), *Fusicoccum aesculi* (JM19-1, JM19-2) which showed effective antimicrobial activity against some bacterial and fungal pathogens. Species of *Chaetomium* are known to produce secondary metabolites including (-)-musanahol, 3-epi-aureonitol and chaetoviridins A, B which inhibited the growth of various pathogen (Marwah et al., 2007; Park et al, 2005a). Wherea, *Fusicoccum* species have been reported to produce Xanthofusin and Fusicoccin for antifungal and phytotoxic activity, respectively (Ballio et al., 1964; Breinholt et al., 1993).

Other than screen antimicrobial from saprobic fungi, fungal strains from Department of Biochemistry and Microbiology, Institute of chemical technology, Prague, Czech republic were also used to investigate for antimicrobial production. The results showed that these fungi had strong inhibitory activity against several bacteria. *Aspergillus oryzae* (DBM4336) represented the best antimicrobial activity when compare with all fungal strain including saprobic fungi. Although *Aspergillus oryzae* (DBM4336) showed the best inhibitory effect to test microorganism, but also *Clonostachys rosea* (JM13) showed a wide spectrum against more test organisms than *Aspergillus oryzae* (DBM4336). *Aspergillus oryzae* have been reported in production of mycotoxin to toxicity to animal (Blumenthal, 2004) and other active compounds such as indoloditerpenes for insecticidal and antimicrobial activities (Qiao et al., 2010). Besides, *Botrytis cinerea* (DBM4208) showed rather strong antimicrobial

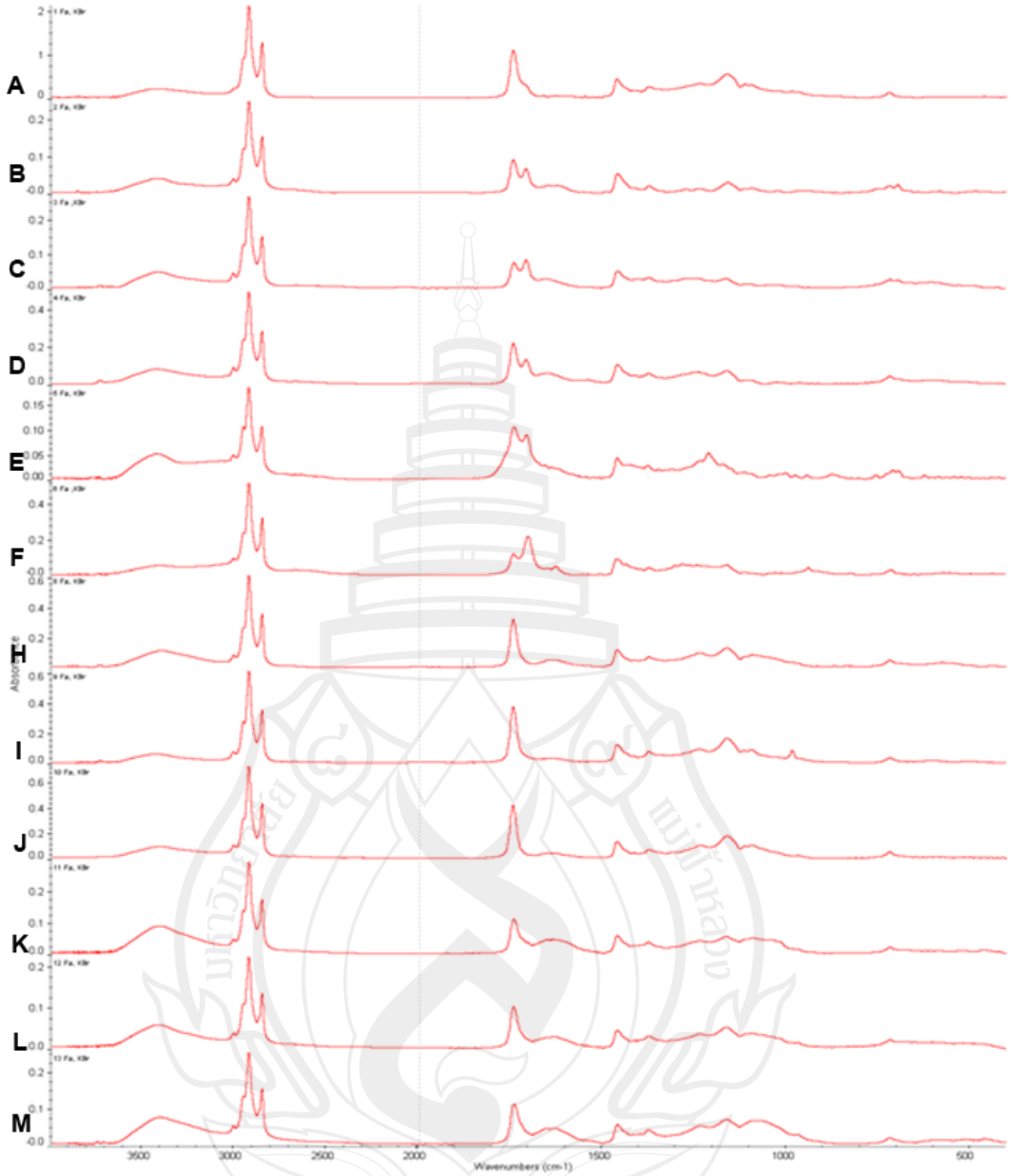
activity. In according to botrydial and dihydrobotrydial isolated from this fungus were antibiotic with activity against fungi and gram-positive bacteria (Fehlhaber et al, 1974; Kimura et al., 1986).

5.3.2 FTIR Analysis of Fractioned Extracts

All fractions of crude extracts were analyzed by FTIR. Obtained spectra are shown in Figs. 5.7-5.10. The spectra of fractions A contain characteristic of lipids (fats, fatty acids). According to FTIR, two obtained fractions B contain phenolics. Fractions C and D are mixture of various compounds (small molecules) including sugars, glycosides, organic acids, phenolics etc. The presence of proteins, or rather shorter peptides, is possible in some cases (*Trichoderma* sp. Fraction C). The fractions with some exceptions (mainly A) were highly colored that is an evidence of the presence of various pigments. From antimicrobial test, it was found that extracts in fraction C supported greater antimicrobial activity than extracts in other fractions in most of fungal strains. Active compounds from fraction C are shown to be more complex. Other techniques such as TLC, HPLC may be required for better isolation of compounds.

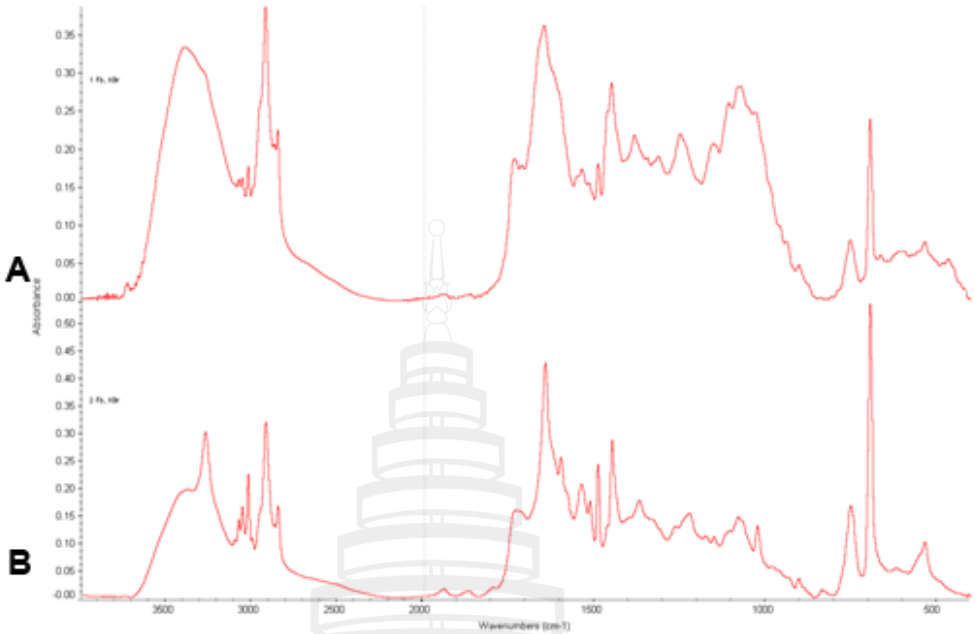
5.4 Conclusion

This study has shown that most of fungal isolates tested produce antimicrobial substances and some saprobic fungi have a promising prospect for production of useful bioactive compounds. The separation of extracts into several fractions caused more effective activity of antimicrobial production than non-fractioned extracts. However, in some case fungal extracts may be complex substances and each fraction is not well separated. Liouane et al. (2009) used different solvents of chloroform, ethyl acetate, butanol, and methanol to extract active compounds from *Gliocladium* sp. and these fraction was analyzed for antimicrobial activity. It was found that fungal chloroformic extract were effective against gram-negative bacteria. The mangrove endophytic fungi were screened for antimicrobial activities and the solvents composing ethyl acetate, diethyl ether and aqueous were used for metabolite



Note. A = *Trichoderma* sp. DBM4197, B = *Aspergillus oryzae* DBM4336, C = *Botrytis cinerea* DBM4208, D = *Stachybotrys chartarum* DBM4297, E = *Fusicoccum aesculi* JM019-2, F = *Fusicoccum aesculi* JM019-1, H = Ascomycete sp. 1 JM25, I = *Clonostachys rosea* JM13, J = *Beltrania rhombica* JM14, K = *Stachybotrys parvispora* JM09, L = Hyphomycetes sp. 2 CI03, M = Hyphomycetes sp. 3 CI09

Figure 5.7 FTIR Spectra of Fractions A: Petroleum Ether Soluble



Note. A = *Trichoderma* sp. DBM4197, B = *Aspergillus oryzae* DBM4336

Figure 5.8 FTIR Spectra of Fractions B: Acetone Soluble

extraction (Bharathidasan & Panneerselvam, 2012). From the results, ethyl acetates represented the maximum antibacterial activity. This study is just a primary study to screen for bioactive compounds from saprobic fungi. The potential fungi should be selected for isolation and characterization of chemical compounds. A large scale fermentation of secondary metabolites might be required and integrated with various analytical techniques such as TLC, HPLC, GC-MS and NMR to know exact active compounds. Their activities such as antioxidant, antitumor and cytotoxicity are also needed for further investigation. This may produce novel bioactive compounds for agricultural and pharmarceutical industry.

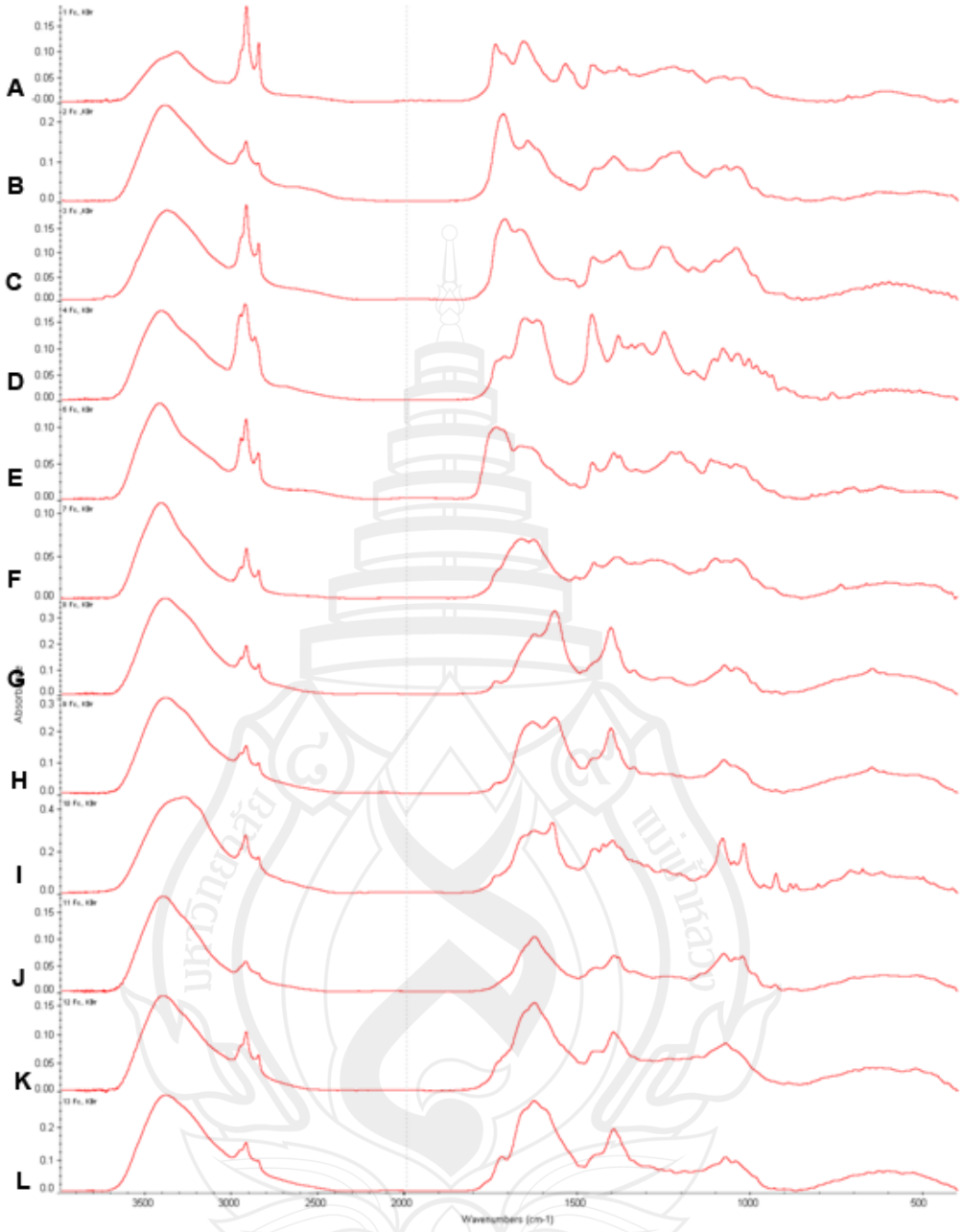
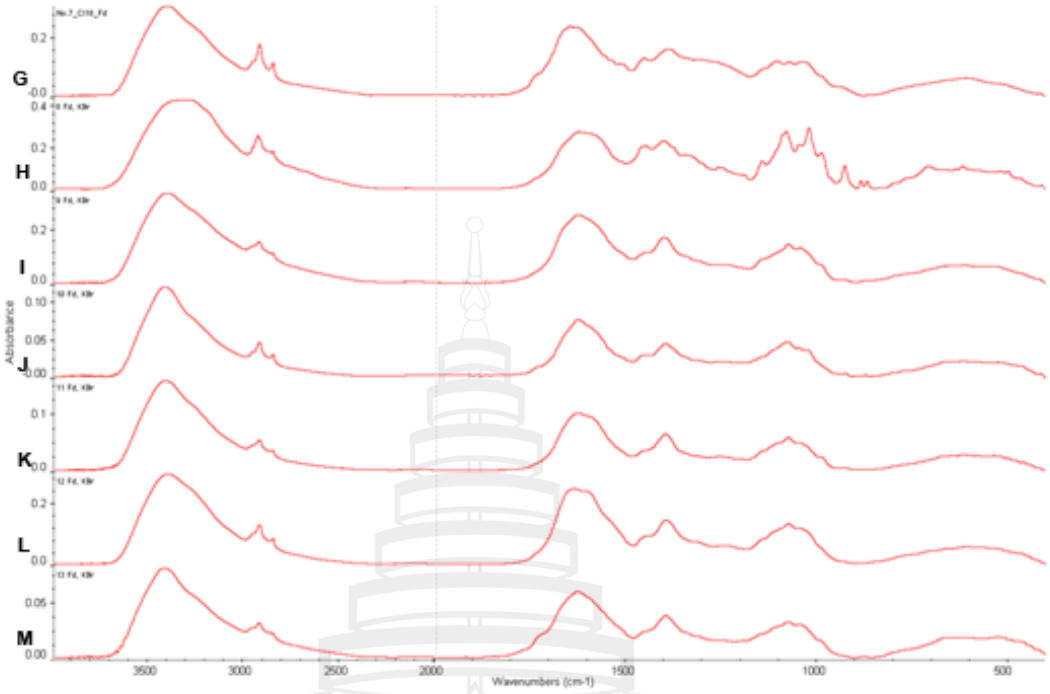


Figure 5.9 FTIR Spectra of Fractions C: Ethanol Soluble

Note. A = *Trichoderma* sp. DBM4197, B = *Aspergillus oryzae* DBM4336, C = *Botrytis cinerea* DBM4208, D = *Stachybotrys chartarum* DBM4297, E = *Fusicoccum aesculi* JM019-2, F = *Fusicoccum aesculi* JM019-1, G = *Chaetomium* sp. CI18, H = Ascomycete sp. 1 JM25, I = *Clonostachys rosea* JM13, J = *Beltrania rhombica* JM14, K = *Stachybotrys parvispora* JM09, L = Hyphomycetes sp. 2 CI03, M = Hyphomycetes sp. 3 CI09



Note. G = *Chaetomium* sp. CI18, H = Ascomycete sp. 1 JM25, I = *Clonostachys rosea* JM13, J = *Beltrania rhombica* JM14, K = *Stachybotrys parvispora* JM09, L = Hyphomycetes sp. 2 CI03, M = Hyphomycetes sp. 3 CI09

Figure 5.10 FTIR Spectra of Fractions D: Water Soluble

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CHAPTER 6

OVERALL CONCLUSIONS

Leaf litter is an excellent source of fungi to study biodiversity and their bioactive compounds. Previous studies have focused mainly on the diversity and taxonomy of saprobic fungi. The biotechnological application of these fungi is rather poor. This study was initiated in order to establish data for fungal saprobes on both of biodiversity and biotechnological applications. The fungal diversity on leaf litter of two selected hosts in northern Thailand was investigated and possible new taxa isolated from these hosts were studied. In addition, the morphological and molecular systematic of fungi in family *Planistromellaceae* were studied via type specimens. The potential of saprobic fungi in producing antimicrobial substances were tested and analyzed for the group of active compounds.

6.1 Diversity of saprobic fungi on *Magnolia liliifera* and *Cinnamomum iners*

In the current study, saprobic fungi on dead leaves of *Magnolia liliifera* and *Cinnamomum iners* collected in dry season at Doi Suthep-Pui forest, Chiang Mai, Thailand were studied and compared. Thirty-five fungi were found from *M. liliifer*a and 17 fungi were found from *C. iners*. Different fungal communities were obtained with low overlap of fungi between two hosts. *M. liliifera* had greater diversity of leaf litter fungi than *C. iners*. This result indicated the host-specificity of saprobic fungi. The stages of decomposition, incubation periods and parts of leaf are important factors that also influence fungal diversity on each host. On both hosts, leaves on early stage of decomposition supported higher fungal diversity than leaves on later

stage of decomposition. Saprobic fungi were specific to different tissues types (leaf lamina, midrib, petiole). This is the first study on fungal diversity of leaf liiter fungi from *C. iners* in Thailand. Furthermore, this work contributes more biodiversity data to global fungal estimates and new information of fungi in Thailand.

6.2 Possible new fungi discovered from this study

The study of diversity of fungi on leaf litter of *Magnolia liliifera* and *Cinnamomum iners* in Doi Suthep-Pui forest were obtained 52 taxa including three taxa are probably new to science. These taxa were described and illustrated. The nucleotide sequences of ITS and LSU region were blasted in GenBank to reveal the closest matches. From the results, three unidentified fungi show different morphological characters from other fungal genera and it is difficult to identify these fungi to the genus level. Molecular phylogenetic needs to be done to help identify these fungal taxa in the future.

6.3 Taxonomy and phylogeny studies of *Planistromellaceae* from type specimens

The taxonomic classification of *Planistromellaceae* has been confused with morphological characters and sequence data. It is important to reexamine the type specimens and study on molecular phylogeny. The type specimens of *Planistromellaceae* were examined, described and illustrated including *Comminutispora agavacearum, Eruptio acicola*, *Loratospora aestuarii*, *Microcyclus angolensis*, *Mycosphaerellopsis myricariae, Planistroma yuccigenum* and *Planistromella yuccifoliorum.* Phylogenetic analyses based on molecular data from LSU and ITS genes provide three different groups represented by the type species of *Piptarthron, Planistroma* and *Kellermania*. The *Planistromellaceae* is reduced to include *Kellermania* and *Planistroma* based on molecular data. *Mycosphaerellopsis* is

tentatively included, based on morphology, however there is no molecular data to support this, while four other genera are relocated to other family. Further studies are expecially needed with many more taxa being sequenced and carefully isolated from fresh materials to establish their correct classification of taxa identification.

6.4 Active compounds of saprobic fungi

Fractioned and non-fractioned extracts of nine saprobic fungi was assayed for the *in vitro* antimicrobial activity against nine pathogenic microorganisms. The fractioned extracts including A: petroleum ether, B: acetone, C: ethanol and D: water were analyzed by FTIR. The results showed that most of fungal isolates produced antimicrobial compounds. *Clonostachys rosea* (JM13) showed the highest antimicrobial activity with a broad spectrum of activity against several bacteria (gram negative and positive) and fungi. Among four fractions, ethyl acetates represented the maximum antibacterial activity. FTIR spectrums showed that these antimicrobial compounds are composed of various compounds. This study showed that saprobic fungi have a promising prospect for production of useful bioactive compounds.

6.5 Future Work

Possible new fungal taxa isolated in this study should be further explored especially on molecular phylogeny to reveal their identity. Further collections in wet season, on other hosts and in other locations, especially on different parts of Thailand and other tropical regions should be done. These future studies are important in understanding the effects of season and geographic distribution and to determine the host-specificity of saprobic fungi. The studies of endophytic and pathogenic fungi on *M. liliifera* and *C. iners* are required to investigate biodiversity and mode of life of saprobic fungi which are related between these hosts.

The screening of bioactive compounds from saprobic fungi should also be determined in other fungi. The potential fungi which produce antimicrobial compounds should be further studied for investigation of other activities and characterization of their chemical compounds. These novel compounds can be applied for agricultural and biotechnological uses.

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APPENDICES

APPENDIX A

CHEMICAL REAGENTS

1) Potassium Hydroxide (KOH) used in the rehydration of dried specimens. 3%, 5% and 10% KOH was used.

2) Lactoglycerol used for observing fungi and preparing semipermanent slide

Preparation of Lactoglycerol

Lactic acid 10 ml

Glycerol 10 ml

Distilled water 10 ml

Mix together 10 ml lactic acid, 10 ml glycerol, and 10 ml distilled water

3) Melzer's Reagent used for the examination of amyloid reaction in ascomycetes. Frequently colour-change reactions occur in Melzer’s reagent. The amyloid reaction (blue) of apical pore is often of taxonomic importance.

Preparation of Melzer's Reagent

Iodine 1.5 g

Potassium iodide 5 g, and

Chloral hydrate 100g to

Distilled Water 100 ml.

Dissolve 100 g of chloral hydrate, 5 g of potassium iodide and 1.5 g iodine in 100 ml distilled water.

4) India ink used for observing mucilaginous appendages and sheaths of spores in some fungi. India ink should be diluted at a ratio of 1:10 with distilled water before use.

APPENDIX B

LIST OF SAPROBIC FUNGI FOUND IN THIS STUDY AND THEIR INFORMATION

Table B1 List of Saprobic Fungi Found in This Study and Their Information

No.	Code	Fungal species	Number of cultures	Deposit Code		Host	Remark
				MFLUCC	BCC		
1	JM01	*Phaeosphaeria* sp.	1	10-0278	-	*Magnolia liliifera*	Might be new species
2	JM02	*Colletotrichum fructicola*	2	10-0262, 10-0263	-	*Magnolia liliifera*	
3	JM03	*Lasiosphaeria-like* sp.	1	10-0279	-	*Magnolia liliifera*	
4	JM04	*Dicyma pulvinata*	0	-	-	*Magnolia liliifera*	
5	JM05	*Pestalotiopsis* sp.	0	-	-	*Magnolia liliifera*	
6	JM06	*Cladosporium* sp. 1	1	10-0280	-	*Magnolia liliifera*	
7	JM07	*Phoma* sp. 1	0	-	-	*Magnolia liliifera*	
8	JM09	*Stachybotrys parvispora*	1	10-0292	-	*Magnolia liliifera*	
9	JM10	*Zygosporium* sp.	0	-	-	*Magnolia liliifera*	

Table B1 (continued)

No.	Code	Fungal species	Number of cultures	Deposit Code		Host	Remark
				MFLUCC	BCC		
10	JM11	*Sporidesmium* sp.	1	10-0264	-	*Magnolia liliifera*	
11	JM12	Anamorph of *Eutypa* sp. 1	0	-	-	*Magnolia liliifera*	
12	JM13	*Clonostachys rosea*	1	10-0261	-	*Magnolia liliifera*	
13	JM14	*Beltrania rhombica*	1	10-0293	-	*Magnolia liliifera*	
14	JM15	*Clonostachys rogersoniana*	1	10-0265	-	*Magnolia liliifera*	
15	JM16	Ceolomycetes sp. 1	0	-	-	*Magnolia liliifera*	
16	JM18	*Ophioceras* cf. *leptosporum*	1	10-0281	-	*Magnolia liliifera*	
17	JM19	*Fusicoccum aesculi*	2	10-0260, 10-0266	*	*Magnolia liliifera*	
18	JM20	*Volutella consors*	1	10-0267	-	*Magnolia liliifera*	
19	JM21	*Lasiodiplodia theobromae*	1	10-0268	-	*Magnolia liliifera*	
20	JM23	*Botryosphaeria* sp.	0	-	-	*Magnolia liliifera*	
21	JM25	Ascomycetes sp. 1	1	10-0282	-	*Magnolia liliifera*	Might be new species
22	JM26	*Nidulisporium* sp.	0	-	-	*Magnolia liliifera*	
23	JM27	*Stachylidium bicolor*	0	-	-	*Magnolia liliifera*	
24	JM28	Hyphomycetes sp. 1	0	-	-	*Magnolia liliifera*	
25	JM29	*Clanostachy compactiuscula*	0	-	-	*Magnolia liliifera*	
26	JM31	*Phialophora sp.*	1	10-0294	-	*Magnolia liliifera*	
27	JM32	*Montagnula* sp.	0	-	-	*Magnolia liliifera*	

Table B1 (continued)

No.	Code	Fungal species	Number of cultures	Deposit Code		Host	Remark
				MFLUCC	BCC		
28	JM33	*Cladosporium* sp. 2	1	10-0295	-	*Magnolia liliifera*	
29	JM34	*Canalisporium caribense*	0	-	-	*Magnolia liliifera*	
30	JM35	Ceolomycetes sp. 2	1	10-0283	-	*Magnolia liliifera*	
31	JM36	Ascomycetes sp. 2	0	-	-	*Magnolia liliifera*	
32	JM37	*Phoma* sp. 2	1	10-0296	-	*Magnolia liliifera*	
33	JM38	*Phomopsis* sp.	0	-	-	*Magnolia liliifera*	
34	JM39	*Phomatospora* sp.	0	-	-	*Magnolia liliifera*	
35	JM40	Ceolomycetes sp. 3	0	-	-	*Magnolia liliifera*	
36	CI01	Coelomycete sp. 4	1	10-0748	-	*Cinnamomum iners*	
37	CI02	*Pyricularia costina*	1	10-0749	-	*Cinnamomum iners*	
38	CI03	Hyphomycetes sp. 2	1	10-0750	-	*Cinnamomum iners*	Might be new species
39	CI04	*Pleurophragmium* sp.	1	10-0751	-	*Cinnamomum iners*	
40	CI05	*Ophioceras* cf. *commune*	0	-	-	*Cinnamomum iners*	
41	CI06	Anamorph of *Eutypa* sp. 2	1	10-0752	-	*Cinnamomum iners*	
42	CI07	*Colletotrichum* sp.	1	10-0753	-	*Cinnamomum iners*	
43	CI08	*Lasiodiplodia theobromae*	1	10-0754	-	*Cinnamomum iners*	
44	CI09	*Hyphomycetes* sp. 3	1	10-0755	-	*Cinnamomum iners*	Might be new species
45	CI10	*Ellisiopsis occulta*	0	-	-	*Cinnamomum iners*	

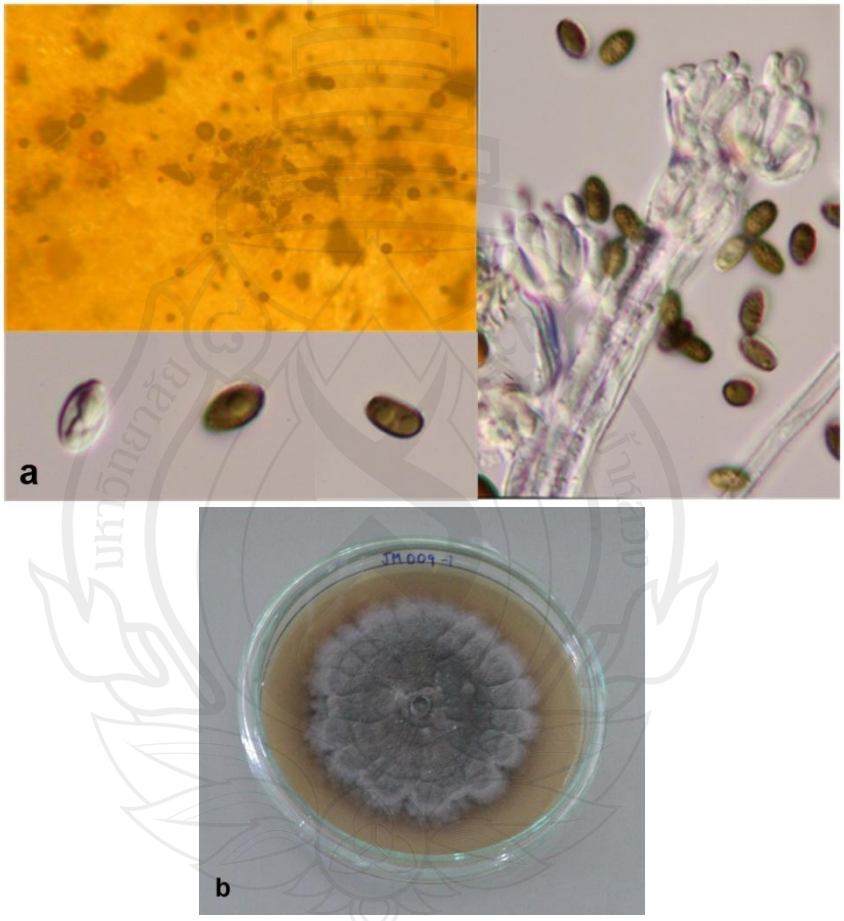
Table B1 (continued)

No.	Code	Fungal species	Number of cultures	Deposit Code		Host	Remark
				MFLUCC	BCC		
46	CI11	*Pyrenochaeta* sp.	1	10-0756	-	*Cinnamomum iners*	
47	CI12	*Gliocladium* sp.	0	-	-	*Cinnamomum iners*	
48	CI13	Coelomycetes sp. 5	0	-	-	*Cinnamomum iners*	
49	CI14	*Acremonium* sp.	1	10-0757	-	*Cinnamomum iners*	
50	CI15	*Graphium penicillioides*	1	10-0758	-	*Cinnamomum iners*	
51	CI17	*Graphium* sp.	1	10-0760	-	*Cinnamomum iners*	
52	CI18	*Chaetomium* sp.	1	10-0761	-	*Cinnamomum iners*	

Note. - = not deposited, * = deposited but waiting for code

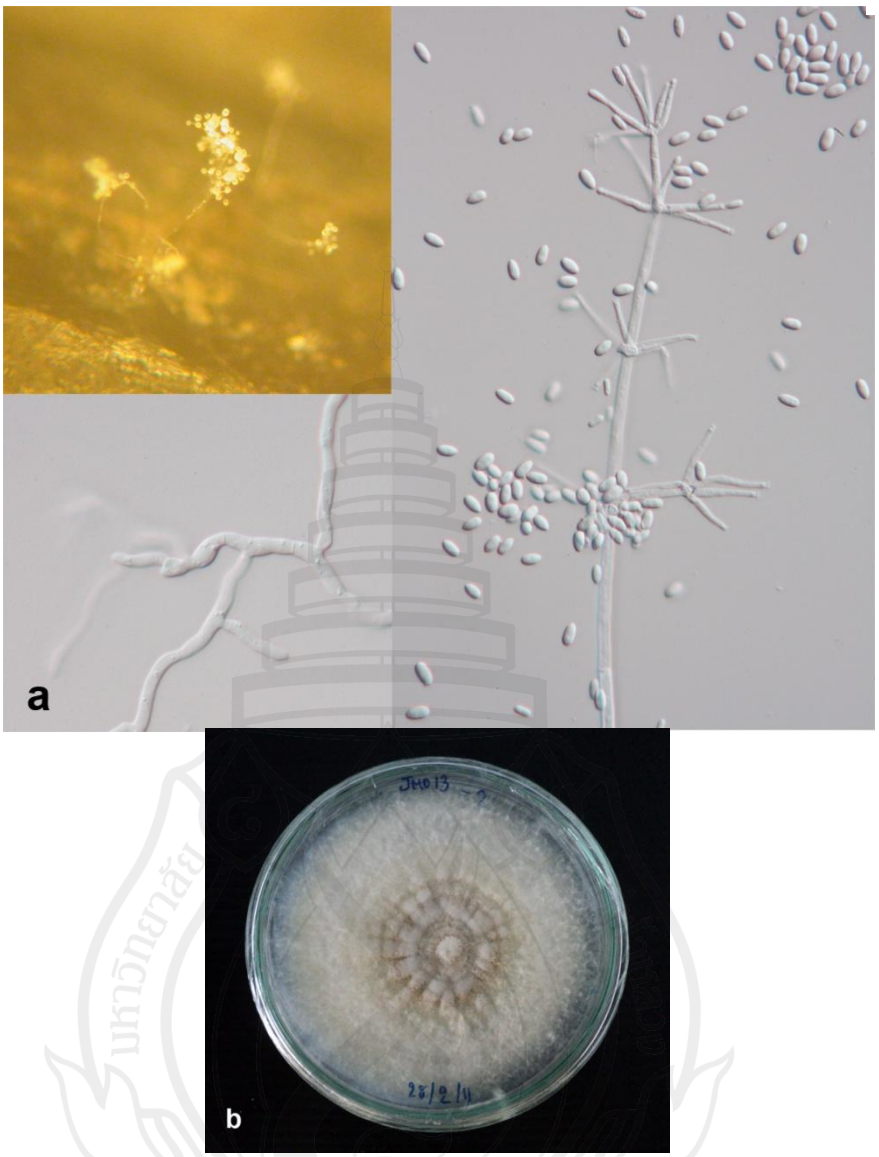
APPENDIX C

THE CHARACTERISTICS OF SELECTED FUNGAL ISOLATES



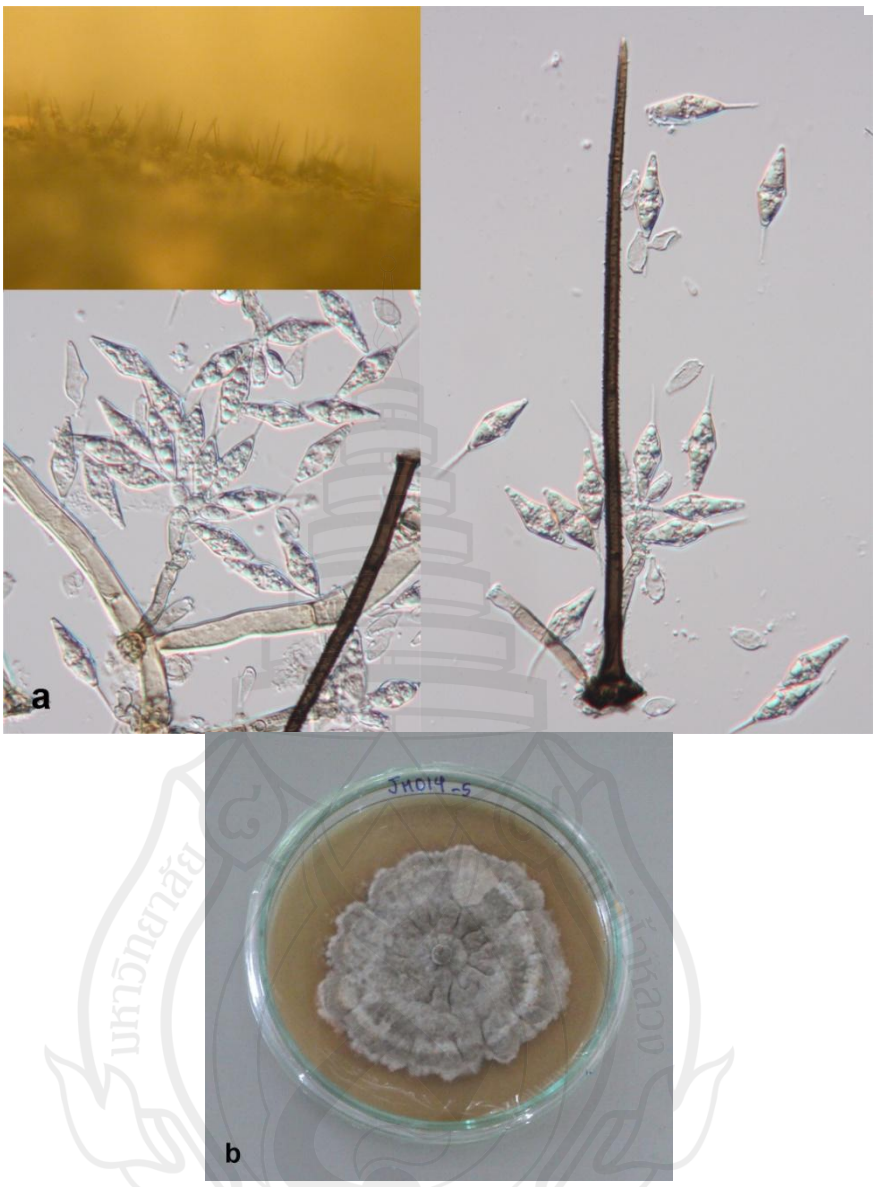
Note. (a) Morphological character on *Magnolia liliifera* leaf. (b) Colony characteristic on SDA. at 28 °C, 30 days.

Figure C1 *Stachybotrys parvispora* (JM09)



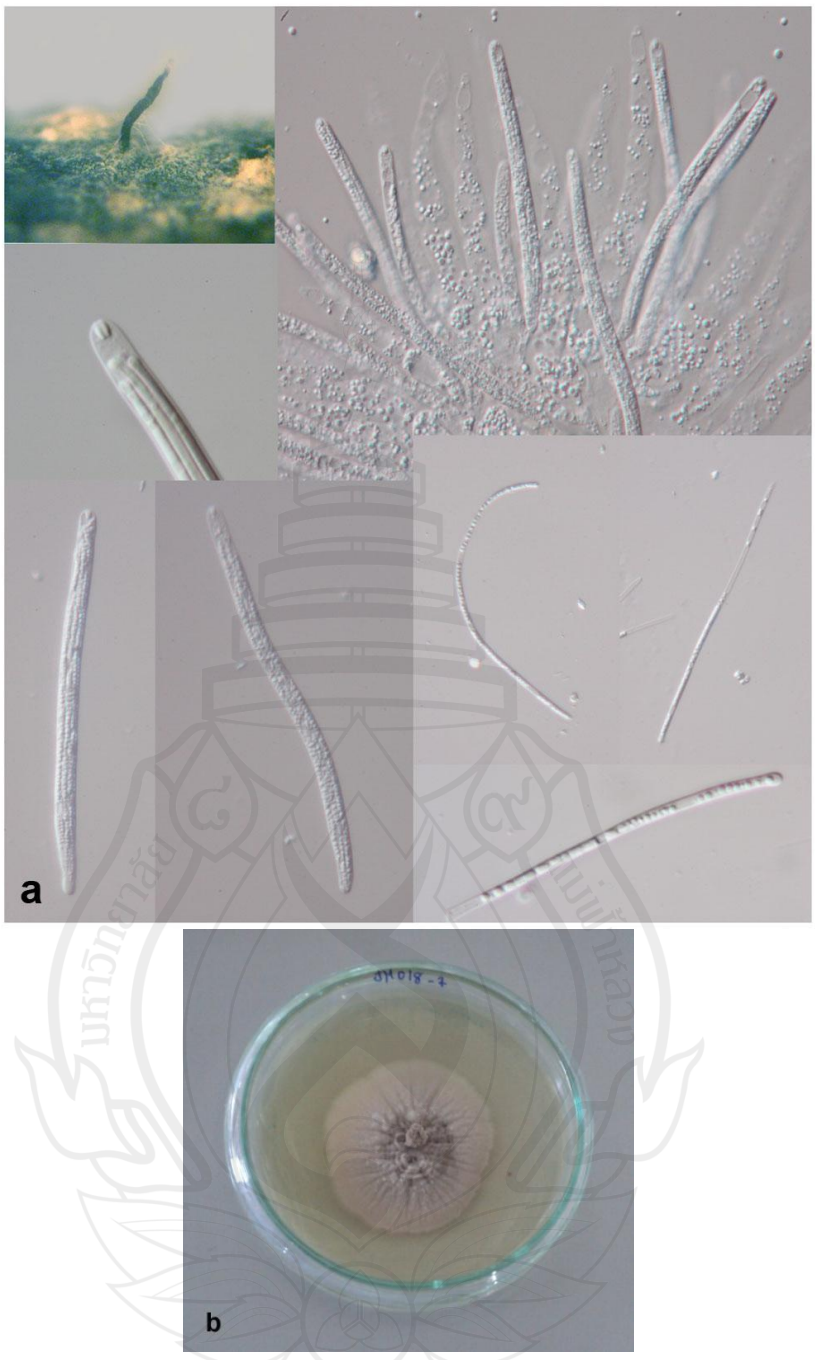
Note. (a) Morphological character on *Magnolia liliifera* leaf. (b) Colony characteristic on SDA. at 28 °C, 30 days.

Figure C2 *Clonostachys rosea* (JM13)



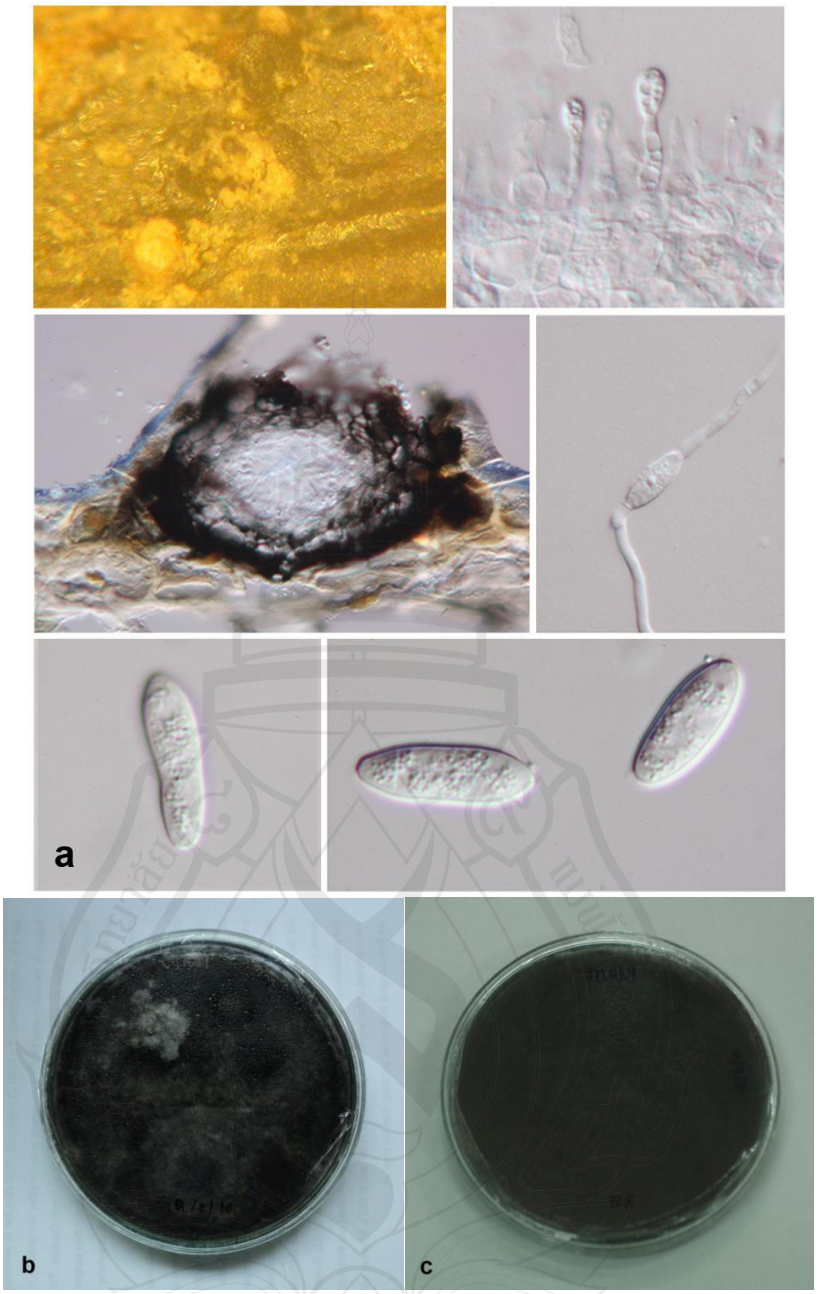
Note. (a) Morphological character on *Magnolia liliifera* leaf. (b) Colony characteristic on SDA. at 28 °C, 30 days.

Figure C3 *Beltrania rhombica* (JM14)



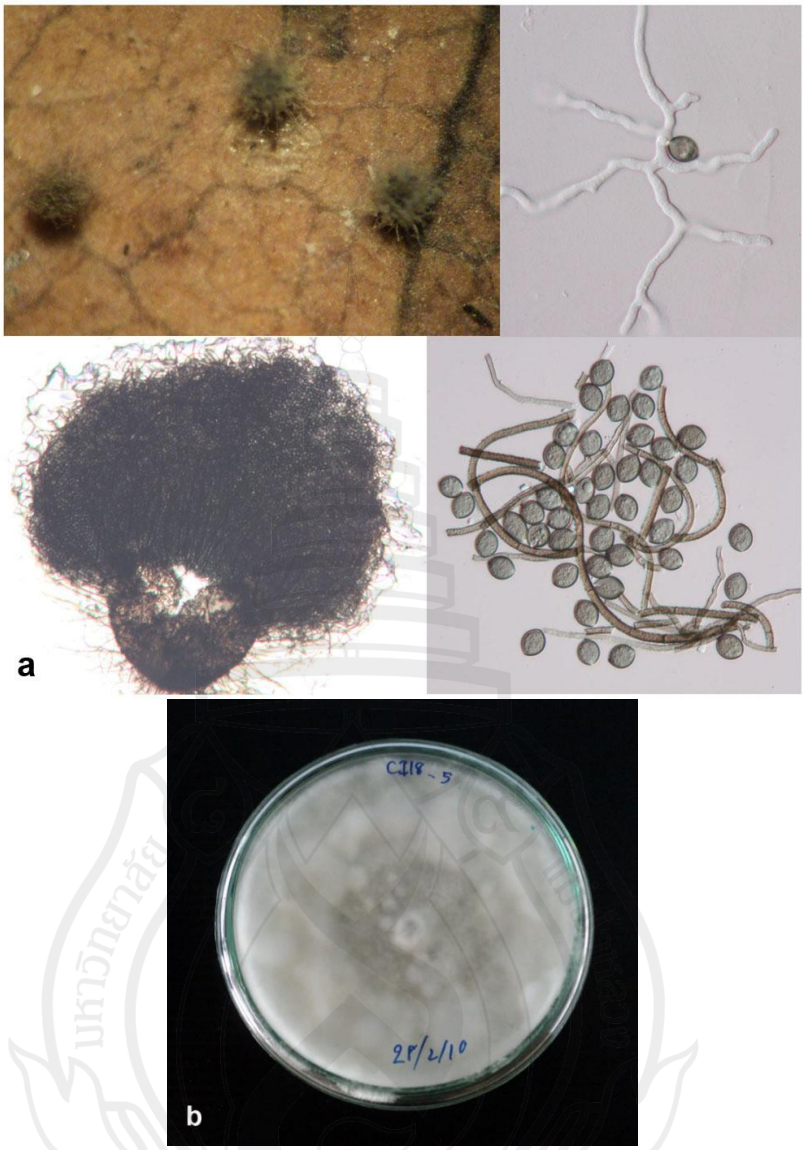
Note. (a) Morphological character on *Magnolia liliifera* leaf. (b) Colony characteristic on SDA. at 28 °C, 30 days

Figure C4 *Ophioceras cf. leptosporum* (JM18)



Note. (a) Morphological characters of strains JM19-1 and JM19-2 on *Magnolia liliifera* leaf. (b) Colony characteristic of strain JM19-1 on SDA. at 28 °C, 30 days. (c) Colony characteristic of strain JM19-2 on SDA. at 28 °C, 30 days.

Figure C5 *Fusicoccum aesculi* (JM19-1, JM19-2)



Note. (a) Morphological character on *Cinnamomum iners* leaf. (b) Colony characteristic on SDA. at 28 °C, 30 days

Figure C6 *Chaetomium* sp. (CI18)

APPENDIX D

THE WEIGHT OF FUNGAL CRUDE EXTRACTS IN EACH FRACTION

Table D1 The Weight of Fungal Crude Extracts in Each Fraction

No	Code	Fungi	Weigh (g)				Remark
			A:Petroleum ether	B:Acetone	C:Ethanol	D:Water	
1	DBM 4197	*Trichoderma* sp.	0.090	0.015	0.200	-	From Czech Republic
2	DBM 4336	*Aspergillus oryzae*	0.050	0.015	0.200	-	From Czech Republic
3	DBM 4208	*Botrytis cinerea*	0.020	0.010	0.040	-	From Czech Republic
4	DBM 4297	*Stachybotrys chartarum*	0.030	0.002	0.130	-	From Czech Republic
5	JM19-1	*Fusicoccum aesculi*	0.010	0.009	0.130	-	From Thailand
6	JM19-2	*Fusicoccum aesculi*	0.030	0.005	1.774	0.321	From Thailand
7	CI18	*Chaetomium* sp.	0.120	-	0.850	0.320	From Thailand
8	JM25	Ascomycete sp. 1	0.210	-	0.070	0.333	From Thailand

Table D1 (continued)

No	Code	Fungi	Weigh (g)				Remark
			A:Petroleum ether	B:Acetone	C:Ethanol	D:Water	
9	JM13	*Clonostachys rosea*	0.210	-	0.460	0.320	From Thailand
10	JM014	*Beltrania rhombica*	0.290	-	0.180	0.280	From Thailand
11	JM09	*Stachybotrys parvispora*	0.070	-	0.146	0.075	From Thailand
12	CI03	Hyphomycetes sp. 1	0.100	-	0.237	0.076	From Thailand
13	CI09	Hyphomycetes sp. 1	0.100	-	0.285	0.155	From Thailand
14	DS77	*Cladosporium* sp.	0.053	-	0.642	0.005	From Czech Republic

APPENDIX E

ABSTRACT PRESENTED AT CONFERENCE AND PUBLICATIONS

Diversity of Fungi on Leaf and Woody Litter from Some Selected Trees in Northern Thailand and Screening for Insecticide Production

Monkai, J.[1], Yacharone, S.[1], Chukeatirote, E.[1], Promputtha, I.[2], Kodsueb, R.[3], McKenzie, E.[4], Hyde, K. D.[1*]

[1]School of Science, Mae Fah Luang University, Chiang Rai, Thailand
[2]School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, Thailand
[3]Department of biology, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, Thailand
[4]Herbarium PDD, Landcare Research, Private Bag 92170, Auckland, New Zealand

Abstract
Leaf and woody litter fungi are important decomposers in terrestrial ecosystems and these fungi can produce secondary metabolites that may inhibit the growth of insects. This research aims to study the diversity of saprobic fungi on leaves and woody litter in northern Thailand and screen the fungi for insecticide production. Decaying leaves and woody litter of various tree species were selected from Doi Suthep-Pui National Park and Doi Tung National Park in the dry and wet seasons of 2010. A moist chamber method was used for incubation. A total of 94 taxa were identified by morphological character and comprises 18 ascomycetes, 70 anamorphic taxa (26 coelomycetes and 44 hyphomycetes) and 2 myxomycetes. Five taxa are probably new to science and will be published following molecular analysis. Seventy isolates have been obtained and these will be selected for molecular phylogenetic analysis and all isolates will be assayed for insecticidal activities.

Keywords: leaf litter, woody litter, saprobic fungi, screening insecticide

*Corresponding author.
Tel.: 0-5391-6248; Fax: 0-5391-6034, 0-5391-7049
E-mail: kdhyde3@gmail.com

O-BD010

Diversity of fungi on leaf litter of *Magnolia lillifera* and *Cinnamomum iners* from Doi Suthep-Pui National Park, Thailand

Monkai, J.[1], Promputtha, I.[2], Chukeatirote, E.[1], McKenzie, E.H.C.[3] and Hyde, K.D.[1,4]

[1]School of Science, Mae Fah Luang University, Chiang Rai, 57100 Thailand
[2]School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, Thailand
[3]Landcare Research, Auckland, New Zealand
[4]International Fungal Research & Development Centre, The Research Institute of Resource Insects, Chinese Academy of Forestry Beilongsi, Kunming, Yunnan, China

Leaf litter fungi play an important role as decomposers in natural ecosystems. One major aim to study these saprobic fungi is to establish their interaction with plant hosts whether they are generalists, host specific or host recurrent. In this study, two tree species namely *Magnolia liliifera* and *Cinnamomum iners* in the forest of Doi Suthep-Pui National Park, Chiang Mai Province were selected. Decaying leaves of each host were collected during dry season and examined for fungal presence. Fungal communities (numbers and species) from different hosts were then recorded and these data were used to assess fungal diversity using statistical analyses. For *M. liliifera*, 36 taxa were identified comprising 9 ascomycetes and 27 anamorphic fungi (11 coelomycetes and 16 hyphomycetes). Dominant species were *Ellisembia* sp.1 (46.7%), *Stachybotrys* sp.1 (40.0%), *Colletotrichum* sp.1 (26.7%) and *Ophioceras* sp.1 (26.7%). Eighteen taxa were identified from *C. iners* including 2 ascomycetes and 16 anamorphic fungi (6 coelomycetes and 10 hyphomycetes). Dominant species were anamorph of *Eutypa* sp.1 (26.7%), *Pleurophragmium* sp.1 (20.0%) and *Colletotrichum* sp.1 (16.7%). The overlapping fungi of both tree species are anamorph of *Eutypa* sp., *Colletotrichum* sp., *Diplodia* sp. and *Ophioceras* sp.

Keywords: fungal diversity, leaf litter fungi, Thailand

Publications in International Journals

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Planistromellaceae (Botryosphaeriales)

Jutamart MONKAI[a,b,c], Jian-Kui LIU[b,c], Saranyaphat BOONMEE[b,c], Putarak CHOMNUNTI[b,c], Ekachai CHUKEATIROTE[b,c], E. B. Gareth JONES[d], Yong WANG[a] & Kevin D. HYDE[b,c]*

[a]*Department of Plant Pathology, Agriculture College, Guizhou University, Guiyang 550025, China*

[b]*School of Science, Mae Fah Luang University, Chiang Rai, 57100, Thailand*

[c] *Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai, 57100, Thailand*

[d]*Institute of Ocean and Earth Sciences (IOES), C308, Institute of Postgraduate Studies Building, University of Malaya, Kuala Lumpur 50603, Malaysia*

Abstract – In this paper, we re-examine, re-describe and illustrate all sexual generic type specimens of *Planistromellaceae* including *Comminutispora agavacearum, Eruptio acicola*, *Loratospora aestuarii*, *Microcyclus angolensis*, *Mycosphaerellopsis myricariae, Planistroma yuccigenum* and *Planistromella yuccifoliorum.* We also use molecular data from GenBank to show the taxonomic placement of some of these genera. Members of family *Planistromellaceae* (*Botryosphaeriales*) are saprobes or pathogens on various plants and characterized by multi or uniloculate ascostromata which are erumpent through cracking or splitting of host tissues and have periphysate ostioles. The ascostromata comprise several layers of brown to black thick-walled cells, pseudoparaphyses are not obvious in mature specimens, and asci are bitunicate. The asexual morphs were previously reported to be found in the genera *Aposphaeria*-like, *Fusicladium, Hyphospora*, *Kellermania, Lecanosticta, Pazschkeella* and *Piptarthron*. Following this study, phylogenetic analyses based on molecular data from LSU and ITS genes provide strong support for the monophyly of the *Planistromellaceae* in the *Botryosphaeriales*, while the *Planistromellaceae* clade separates into three different groups represented by the type species of *Piptarthron*, *Planistroma* and *Kellermania*, respectively. We accept *Kellermania* (= *Planistromella* and possibly *Piptarthron*), *Planistroma* and *Mycosphaerellopsis* (the latter with no molecular support) in *Planistromellaceae*, while four other genera are redisposed of as follows: *Comminutispora* clusters in *Capnodiales*, *Eruptio* and *Microcyclus* have been shown to be members of *Mycosphaerellaceae*, and *Loratospora* has been shown to belong in *Phaeosphaeriaceae.*

***Aposphaeria*-like / *Comminutispora* / *Eruptio* / *Fusicladium* / *Hyphospora* / *Kellermania* / *Lecanosticta* / *Loratospora* / *Microcyclus* / molecular phylogeny / *Mycosphaerellopsis* / *Pazschkeella* / *Piptarthron* / *Planistroma* / *Planistromella* / taxonomy / type specimens**

INTRODUCTION

The class *Dothideomycetes* contains the largest species numbers and is the most phylogenetically diverse group in the phylum *Ascomycota*. Development in this group is ascolocular and asci are bitunicate (Kirk *et al.*, 2008). Previously, the classification of *Dothideomycetes* was determined using morphological characters

* Corresponding author: Yong Wang, email address:yongwangbis@yahoo.cn

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Fungi on decaying leaves of *Magnolia liliifera* and *Cinnamomum iners* show litter fungi to be hyperdiverse

Monkai J[1,2*], Promputtha I[3], Kodsueb R[4], Chukeatirote E[1,2], McKenzie EHC[5] and Hyde KD[1,2]

[1]School of Science, Mae Fah Luang University, Thasud, Chiang Rai 57100, Thailand
[2]Institute of Excellence in Fungal Research, Mae Fah Luang University, 57100 Chiang Rai, Thailand
[3]School of Cosmetic Science, Mae Fah Luang University, Thasud, Chiang Rai 57100, Thailand
[4]Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok 65000, Thailand
[5]Landcare Research, Private Bag 92170, Auckland, New Zealand

Monkai J, Promputtha I, Kodsueb R, Chukeatirote E, McKenzie EHC, Hyde KD 2013 – Fungi on decaying leaves of *Magnolia liliifera* and *Cinnamomum iners* show litter fungi to be hyperdiverse. Mycosphere 4(2), 292–301, Doi 10.5943/mycosphere/4/2/12

Diversity of fungi on decaying leaves of *Magnolia liliifera* and *Cinnamomum iners* collected during the dry season at Doi Suthep-Pui forest, Chiang Mai, Thailand were studied and compared. Thirty-five taxa were identified from *Magnolia liliifera* comprising 8 sexual (ascomycetes) and 27 asexual taxa. The most abundant species found were *Sporidesmium* sp., *Colletotrichum fructicola* and *Stachybotrys parvispora*. Seventeen taxa were identified from *Cinnamomum iners* comprising 2 ascomycetes and 15 asexual taxa. Anamorph of *Eutypa* sp. 2 and *Pleurophragmium* sp. were the most abundant species on *Cinnamomum iners*. There is very little overlap between the fungi occurring on the two host species. Distinct fungal communities were found between the two hosts at each stage of decomposition. Decaying leaves of both hosts collected in the early stage of decomposition supported a greater fungal diversity than those collected at the later stage of decomposition. Saprobic fungi on the two plants are shown to be hyperdiverse.

Key words – biodiversity estimates – fungal ecology – saprobic fungi

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*Corresponding author: Jutamart Monkai – e-mail – mjutamart@gmail.com

Introduction

Saprobic fungi function as decomposers of organic materials and recycle nutrients to other organisms (Cooke & Rayner 1984). The number of fungi worldwide was estimated at 1.5 million species by Hawksworth (1991, 2001b). However, only 70,000 species are presently described leaving the remaining 1.43 million (or 95%) as being undescribed. It is therefore important to search for these undescribed fungi which occur in unexplored habitats, hosts or poorly studied countries especially in tropical regions (Hawksworth & Rossman 1997). Forests of the northern part of Thailand have great plant diversity (Gardner et al. 2000) and several studies on their fungal diversity have been carried out in recent years (Duong et al. 2008, Thongkantha et al. 2008,

CURRICULUM VITAE

CURRICULUM VITAE

NAME Miss Jutamart Monkai

DATE OF BIRTH 6 December 1983

ADDRESS 31 Sripingmuang road
Changklan District, Muang,
Chiang Mai Thailand
57100

EDUCATIONAL BACKGROUND

2006 Bachelor of Science
Microbiology, Chiang Mai University

WORK EXPERIENCE

2006-Present Scientist
The Scientific and Technological
Instruments Center, Mae Fah Luang
University

PUBLICATIONS

Liu, J. K., Phookamsak, R., Doilom, M., Wikee, S., Li, Y. M., Ariyawansha, H., Boonmee, S., Chomnunti, P., Dai, D. Q., Bhat, D. J., Romero, A. I., Zhuang, W. Y., Monkai, J., Jones, E. B. G., Chukeatirote, E., Ko Ko, T. W., Zhao, Y. C., Wang, Y. & Hyde K. D. (2012). Towards a natural classification of *Botryosphaeriales.* **Fungal. Diver., 57,** 149–210.

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