FUNGI ASSOCIATED WITH COFFEE BERRIES IN CHIANG MAI, THAILAND

HARYUDIAN PRIHASTUTI

MASTER OF SCIENCE
IN BIOTECHNOLOGY

MAE FAH LUANG UNIVERSITY

2008

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IN CHIANG MAI, THAILAND

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A THESIS SUBMITTED TO MAE FAH LUANG UNIVERSITY
IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
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EXAMINING COMMITTEE

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ACKNOWLEDGEMENT

I would like to express my sincere appreciation to all the support and help given from my teachers, friends and family during this study.

I am grateful to my advisor, Prof. Dr. Kevin D. Hyde for introducing me to the world of mycology and plant pathology, and providing stimulus and direction for this project. I would like to appreciate my co-advisor, Assist. Prof. Dr. Ekachai Chukeatirote for his valuable advices, suggestion and encouragement whenever I request his help throughout my project.

I am also grateful to Dr Lei Cai (Novozymes, China) for his invaluable guide, teaching and motivation for molecular practices, phylogenetic analysis and scientific writing. I would always be grateful to Dr. Po Po Than for introducing and teaching me to the world of *Colletotrichum*.

I am greatly indebted to Dr. Hyde for financial support to enable me to do molecular work at the International Fungal Research and Development Centre, The Research Institute of Resource Insects, the Chinese Academy of Forestry, China from July 2008 to January 2009, sending me to attend at International Symposium on Fungal Diversity which was held on October 2008 in Hangzhou, China. Dr. Chen Hang for providing materials for my molecular work at the International Fungal Research and Development Centre, The Research Institute of Resource Insects, the Chinese Academy of Forestry, China from July 2008 to January 2009.

The Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS), Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia (BRIP), CABI Europe-UK, Bakeham Lane, Egham, Surrey, UK are highly appreciated for providing fungal cultures used in this study.

I am also grateful to Biro Perencanaan dan Kerjasama Luar Negeri-Departemen Pendidikan Nasional, Indonesia for the award of a master scholarship. Thanks should also be extended to Prof. Liliek Sulistyowati for sending me to study in Thailand.

Thanks go to all of my colleagues in our mycological lab. P'Sack, P'Bo, P'Dear, P'Mongkol, P'Pho, P'Nook, P' Kaegh, Paul, Yang Youlian, Samantha, Mama, Thida, Elvi and Melati are thanked for their friendship. Mei, Xia, Hu, Jack and RIRI's friends are sincerely thanked for their various kind help during my visit to the International Fungal Research and Development Centre in Kunming, China. All Indonesian students in Thailand are thanked for their friendship.

I am deeply thankful to my beloved parents, my grandpa and grandma, my young brother and sister, my families for their eternally love, support, and encouragement. Finally, my fiancé, it has been a long ride, thank you for being there, I could not have done it without you.

Haryudian Prihastuti

Thesis Title	Fungi Associated with Coffee Berries in Chiang Mai, Thailand
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Degree	Master of Science (Biotechnology)
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ABSTRACT

In this project, fungi associated with coffee berries were studied. Coffee berries samples were collected from Chiang Mai, Thailand and used to analyze the epiphytic and endophytic fungal communities. A total of 352 fungal strains were isolated and grouped into 12 genera. The most common taxon was *Fusarium* with 24 % frequency of occurrence. In this study, *Moniliopthora* is the first record in coffee plantation. The results showed that variation of the fungal diversity between epiphytic and endophytic communities was insignificant ($0.05<P<0.10$). It was also found that fungal diversity in Pha Daeng village was significantly higher than that in Mae Lod village ($P < 0.001$).

This study was further carried out emphasizing on the genus of *Colletotrichum*. The combination of morphological and cultural characters, biochemical, pathogenicity testing and DNA barcoding clustered three morphogroups of *Colletotrichum* associated with coffee berries from Chiang Mai, Thailand and introduced them as new species. *Colletotrichum asianum*, *C. coffeae* and *C. coffeigenum* are introduced as new species in the present study. Furthermore, the combined datasets of actin, partial β-tubulin-2 (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region revealed *Colletotrichum* relationships congruent with their morphological characters. The biochemical and

DNA barcoding data from multi-genes used here also showed clear differences between *C. kahawae*, *C. gloeosporioides* and the new *Colletotrichum* species.

Keywords: Coffee berries/ *Colletotrichum*/ endophytes/ epiphytes.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Coffee (*Coffea arabica*) is one of the most important beverage crops in the world and a valuable agricultural export commodity. The plant originated in Ethiopia and is now cultivated in over 80 countries (Coste, 1992; Orozco-Castillo, *et al*., 1994; Pearl, *et al*., 2004). Coffee is one of the most economically important incomes for Thailand, being the third largest coffee producer in Southeast Asia (Angkasith, 2001). However, the production of coffee has been limited due to damage by disease and insect pests, and injury by successive cropping. Production losses due to several destructive disease such as Coffee Berry Disease (CBD) are especially important (Van der Vossen, *et al*., 1976; Firman & Waller, 1977).

Coffee berry disease is caused by *Colletotrichum kahawae*, which has reported as a serious disease in the major production area of Arabica coffee in Eastern Africa (Van der Vossen, *et al*., 1976; Firman & Waller, 1977). *Colletotrichum acutatum* and *C. gloeosporioides* has been reported as members of coffee mycobiota (Masaba & Waller, 1992; Waller, *et al*., 1993; Santamaría & Bayman, 2005). *Colletotrichum kahawae* is related to *C. gloeosporiodes*, but they were classified as distinct species (Waller, *et al*., 1993). Coffee Berry Disease (CBD) poses a major threat to the Arabica coffee industries of other tropical countries, beside in African continent (Bridge, *et al*., 2008). The fungal diversity on coffee berries should therefore be understood, then accurate identification could be used for developing and implementing effective disease control strategies.

1.2 Coffee

1.2.1 Classification of coffee

Coffee belongs to the genus *Coffea*, in the family Rubiaceae. According to Cramer (1957), there are at least 100 species of coffee, all in the genus *Coffea*. Two main species are used in coffee production, *Coffea arabica* L. (Arabica coffee) and *C. canephora* Pierre (Robusta coffee). *Coffea arabica* is indigenous to Ethiopia and about 73% of the world coffee production is now derived from Arabica coffee (Orozco-Castillo, *et al*., 1994).

1.2.2 Importance of coffee to agriculture

Coffee is a popular beverage and hence a considerable value agricultural export for its major producers. It is an important source of annual income and employment, significantly contributing to the economics of developing countries within Asia, Africa and Latin America (Orozco-Castillo, *et al*., 1994; Anthony, *et al*., 2001). Four million tons of green beans are produced annually from 6.75 million hectares of coffee plantations worldwide, with sales between 6 to 12 US billion dollars (Perfecto & Armbrecht, 2003; González, 2000; Sera, *et al*., 2000). The top ten coffee producing and exporting countries are led by Brazil and Vietnam, followed by Columbia, Indonesia, Mexico, Ivory Coast, Ethiopia, Uganda, Guatemala and India (Sera, *et al*., 2000).

1.2.3 Coffee in Thailand

Robusta coffee is the majority of the coffee grown in Thailand; it is grown in the southern part of the country and commonly doused with chemicals for disease control. At the end of the 1970s, planting of Arabica coffee in the northern highlands was encouraged in order to replace the cultivation of opium poppies, as well as to counter deforestation from shifting agriculture practiced by many of the local ethnic groups known as “hill tribes” (Anonymous, 2008).

Thailand is the third largest coffee producer in Southeast Asia - after Vietnam and Indonesia (Angkasith, 2001). While there are many species of coffee trees in the world, only Arabica and Robusta have major economic significance, both of which are grown in Thailand (Songset, *et al*., 2005). Consumption demands for both instant and roasted coffee are growing in

Thailand. Exports of coffee to all destination countries between April 2006 and March 2007 by Thailand were 317, 318 ton (ICO, 2008).

1.3 Fungal Diversity

1.3.1 Epiphytic fungi

Epiphytes are organisms which grow on the surface of trees or other plants without parasitizing the host (Butin, *et al*., 1995). In the recent years much research has been published concerning epiphytes with an adverse effect on plant pathogens (Jayaswal, *et al*., 1990; Janisiewicz, 1992; Sholberg, *et al*., 1995; Sinigaglia, *et al*., 1998).

An important role in plant's defense responses can be played by interactions among epiphytes. The nature of the interactions depends, among other things, on the qualitative-quantitative composition of epiphytic communities (Kaczmarkowa & Pedziwilk, 2001).

1.3.2 Endophytic fungi

Petrini (1991) provided a working definition for endophytes that has since been widely accepted: endophytes comprise "all organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host". This includes symptomless latent pathogens (Brown, *et al*., 1998) and those fungi which also have an epiphytic phase of their life cycle. Latent infection of pathogens associated with the plants has been long recognized (Gäumann, 1951). It is a situation in which a pathogen infects a host but the plant does not show symptoms.

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi.

Plants	Endophytic Fungi	References
Apocynaceae	*Alternaria* spp., Ascomycete spp., *Aspergillus* spp., *Aureobasidium pullulans*, *Chaetomella* sp., *Chaetomium* sp., *Cladosporium* sp., Coelomycetes spp., *Colletotrichum* spp., *Fusarium* spp., *Gliocladium* sp., *Helmithosporium* sp., Hyphomycete spp., Mycelia Sterilia, *Pestalotiopsis* sp., *Phoma* spp., *Phomopsis* spp., *Phyllosticta* sp., *Pyrenochaeta* sp., *Torula* sp. and *Xylariales* sp.	Huang, *et al*. (2008).
Asclepiadaceae	*Alternaria* spp., Ascomycete spp., *Aspergillus* spp., *Aureobasidium pullulans*, *Chaetomella* sp., *Cladosporium* sp., *Colletotrichum* spp., *Ellisembia* sp., *Fusarium* spp., *Gliocladium* sp., Hyphomycete spp., Mycelia Sterilia, *Pestalotiopsis* sp., *Phoma* spp., *Phomopsis* spp., *Phyllosticta* sp., *Pyrenochaeta* sp., *Pyriculariopsis* sp., *Torula* sp., *Verticillium* sp. and *Xylariales* sp.	Huang, *et al*. (2008).

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi (Cont.)

Plants	Endophytic Fungi	References
Asteraceae	*Alternaria* spp., Ascomycete spp., *Aspergillus* spp., *Aureobasidium pullulans*, Coelomycetes sp., *Colletotrichum* spp., *Drechslera*-like sp., *Ephelis* sp., *Fusarium* spp., *Gliocladium* sp., Hyphomycete spp., Mycelia Sterilia, *Pestalotiopsis* sp., *Phoma* spp., *Phomopsis* spp., *Phyllosticta* sp., *Pyrenochaeta* sp., *Torula* sp. and *Xylariales* sp.	Huang, *et al*. (2008).
Banana	*Alternaria alternata,* Basidiomycetes*, Cladosporium cladosporioides, Cladosporium musae, Colletotrichum gloeosporioides, Colletotrichum musae, Cordona musae, Cryptosporiopsis* sp.*, Curvularia* sp.*, Deightoniella torulosa, Epicoccum nigrum, Fusarium lateritium, Fusarium solani, Glomerella cingulata, Guignardia cocoicola, Lasiodiplodia theobromae, Microsphaeriopsis* sp.*, Nigrospora musae, Nigrospora*	Brown, *et al*. (1998); Photita, *et al*. (2004).

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi (Cont.)

Plants	Endophytic Fungi	References
	oryzae, Periconiella musae, Pestalotiopsis palmarum, Pestalotiopsis sp.*, Phoma* sp.*, Phomopsis* sp., *Phyllasticta musicola, Xylaria* sp. and Xylariaceae sp.	
Cupressus arizonica	Dothideomycetes, Eurotiomycetes, Pezizomycetes and Sordariomycetes.	Hoffman & Arnold (2008).
Drimys winteri	Anamorphic taxa, Basidiomycete and *Bjerkandera adusta*.	Oses, *et al*. (2008).
Dryas integrifolia	Botryosphaeriaceae, Mycosphaerellaceae, Pleosporales and Rhytismatales.	Higgins, *et al*. (2007).
Grasses	*Acremonium*, *Atkinsonella*, *Balansia*, *Balansiopsis*, *Epichloë*, and *Myriogenospora*.	Bacon & White (1994); Groppe, *et al*. (1999); Saikkonen, *et al*. (2000); Rudgers & Clay (2007).
Huperzia selago	Botryosphaeriaceae, Mycosphaerellaceae, Pleosporales and Rhytismatales.	Higgins, *et al*. (2007).
Juniperus virginiana	Dothideomycetes, Eurotiomycetes, Pezizomycetes and Sordariomycetes.	Hoffman & Arnold (2008).

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi (Cont.)

Plants	Endophytic Fungi	References
Lamiaceae	*Aureobasidium pullulans*, *Colletotrichum* spp., Mycelia sterilia, *Pestalotiopsis* sp., *Phoma* spp., *Pyrenochaeta* sp. and *Spiropes* sp.	Huang, *et al*. (2008).
Magnolia liliifera	*Bionectria* (*Bionectriaceae*), *Diaporthe*, *Glomerella* (*Phyllachoraceae*), *Hypoxylon* (*Xylariaceae*), *Massarina* (*Lophiostomataceae*), *Phomopsis* and *Xylaria* (*Xylariaceae*).	Promputtha, *et al*. (2005).
Mangroves	Ascomycetes, Mitosporic fungi and Sterile mycelia.	Suryanarayanan, *et al*. (1998); Kumaresan & Suryanarayanan (2001); Kumaresan, *et al*. (2002).
Nothofagus oblique	Basidiomycete and *Xylaria* sp.	Oses, *et al*. (2008).
Palms	Ascomycetes, Mitosporic Fungi, *Glomerella cingulata*, *Phomopsis* spp., *Xylariaceae* sp., *Oxydothis* sp., *Stagonospora* spp. and *Phoma multirostrata*.	Rodrigues (1994); Fröhlich & Hyde (1999); Taylor, *et al*. (1999); Fröhlich, *et al*. (2000).
Picea mariana	Botryosphaeriaceae, Mycosphaerellaceae, Pleosporales and Rhytismatales.	Higgins *et al*. (2007).

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi (Cont.)

Plants	Endophytic Fungi	References
Pinus taeda	Leotiomycetes and Sordariomycetes.	Arnold, *et al*. (2007).
Platycladus orientalis	Dothideomycetes, Eurotiomycetes, Pezizomycetes and Sordariomycetes.	Hoffman & Arnold (2008).
Podocarpus saligna	*Inonotus* sp.	Oses, *et al*. (2008).
Polygonaceae	*Alternaria* spp., Ascomycete spp., *Aspergillus* spp., *Aureobasidium pullulans*, *Botryosphaeria* sp., *Colletotrichum* spp., *Flagellospora* sp., *Fusarium* spp., *Gliocladium* sp., Hyphomycete spp., Mycelia sterilia, *Pestalotiopsis* sp., *Phoma* spp., *Phomopsis* spp., *Pyrenochaeta* sp., *Torula* sp. and *Xylariales* sp.	Huang, *et al*. (2008)
Prumnopitys andina	Basidiomycete and *Bipolaris* sp.	Oses, *et al*. (2008)
Rubiaceae	*Alternaria* spp., *Colletotrichum* spp., Mycelia sterilia and *Phomopsis* spp.	Huang, *et al*. (2008)
Solanaceae	*Alternaria* spp., *Aureobasidium pullulans*, *Colletotrichum* spp., Mycelia sterilia, *Pestalotiopsis* sp., *Phomopsis* spp., *Physalospora* sp., *Pyrenochaeta* sp. and *Rhizosphaera* sp.	Huang, *et al*. (2008)

Table 1.1 Selected list of terrestrial plant colonized by endophytic fungi (Cont.)

Plants	Endophytic Fungi	References
Theobroma cacao	*Botryosphaeria* sp., *Botryosphaeria ribis, Clonostachys rosea, Colletotrichum gloeosporioides, Colletotrichum* sp., *Fusarium* (Teleomorph: *Nectria* spp.), *Guignardia* sp., *Phomopsis* sp., *Trichoderma* sp. and *Xylaria* sp.	Arnold *et al*. (2003); Arnold and Lutzoni (2007); Mejía *et al*. (2008).

Among terrestrial plants, endophytic fungi appear to be ubiquitous. Every plant species which has been examined to date harbors at least one fungal endophyte species (Petrini, 1991; Stone, *et al*., 2000; Arnold, 2001) and many plant species may be associated with tens to hundreds of endophytes species (Saikkonen, *et al*., 1998; Stone, *et al*., 2000). Endophytic fungi may inhabit all parts of plants, including leaves, petioles, stems, twigs, bark, xylem, roots, fruit, flowers and seeds (Boddy, *et al*., 1987; Chapela & Boddy, 1988; Sieber, 1989; Clay, 1990; Bills & Polishook, 1991; Bohn, 1993; Fisher, *et al*., 1993; Lodge, *et al*., 1996; Lupo, *et al*., 2001; Meyer, *et al*., 2001, Oses, *et al*., 2008).

The attributes endophytes being in common have been considered by Stone, *et al*. (1994) and include 1) infected host tissues remain symptomless; 2) they establish at least a transitory biotrophic nutritional relationship with their host; 3) they are internal, at least subticular and have contact with and derive nutrition from the living host tissue.

1.4 *Colletotrichum* species

The genus of *Colletotrichum* was first reported by Tode in 1790 as the genus *Vermicularia*, but was later redescribed as *Colletotrichum* by Corda in 1837 (order Melanconiales; class Coelomycetes; subdivision Deuteromycotina) comprises imperfect fungal species which exist as *Glomerella* in teleomorph stage (Sutton, 1992; Latunde-Dada, 2001). The genus *Colletotrichum* comprises a number of endophytic, saprophytic and plant pathogenic species of worldwide importance on a wide range of economic crops and ornamentals (Sutton, 1992; TeBeest, *et al*., 1997; Kumar & Hyde, 2004; Photita, *et al*., 2004).

Colletotrichum is one of the most important plant pathogens worldwide which causes anthracnose disease in a wide range of hosts including cereals and grasses, legumes, vegetables, perennial crops and tree fruits (Bailey & Jeger, 1992; Lenné, 1992; Latunde-Dada, 2001). *Colletotrichum* species have been found on wild fruits in Hong Kong (Tang, *et al*., 2003). *Colletotrichum acutatum, C. capsici* and *C. gloeosporioides* have also been reported causing anthracnose disease on chilli fruits in Thailand (Than, *et al*., 2008a). *Colletotrichum* species that cause serious plant disease are also commonly isolated as endophytes from healthy plants and have been identified as saprobes on dead plant material (Photita, *et al*., 2001; Promputtha, *et al*., 2002; Toofanee & Dulymamode, 2002; Kumar & Hyde, 2004). In addition to being plant pathogens, *Colletotrichum* strains have also been reported as biological control agents against weeds (Templeton, 1992).

The pathogenesis of *Colletotrichum* is diverse, arising from nutritional and ecological diversity within the genus, which vary from intracellular hemibiotrophy to subcuticular intramural necrotrophy (Bailey & Jeger, 1992; Pring, *et al*., 1995). Specialized infection structures are produced by *Colletotrichum* species such as germ tubes, appressoria, intracellular hyphae and secondary necrotrophic hyphae (Perfect, *et al*., 1999). *Colletotrichum* infect hosts by either colonizing subcuticular tissues intramurally or being established intracellularly. The preinfection stages of the both in *Colletotrichum* are very similar, in which deposition of conidia of susceptible hosts include adhesion, germination, appressoria formation and penetration (Bailey & Jeger, 1992; TeBeest, *et al*., 1997; Prusky, *et al*., 2000). The events following penetration, the pathogens that colonize the intramural region beneath the cuticle invade in a necrotrophic manner and spread rapidly throughout the tissues (O′Connell, *et al*., 1985). There is no detectable biotrophic stage in this form of parasitism. In contrast, most anthracnose pathogens exhibit a biotrophic infection strategy initially by colonizing the plasmalemma and cell wall intracelularly. The biotrophic stage generally is short-lived includes all events in which infection develops without visible disruption of host systems. Subsequently, intracellular hyphae colonize one or two cells and produce secondary necrotrophic hyphae (Bailey & Jeger, 1992; TeBeest, *et al*., 1997). These pathogens are regarded as hemiobiotrophs or facultative biotrophs (Kim, *et al*., 2004). An example of hemiobiotrophs is *C. gloeosporioides* on avocado, chilli and citrus which produce both intracellular biotrophy at an early stage and later intramural necrotrophy (O′Connell, *et al*., 1985). Though, the infection process in *Colletotrichum* species is apparently similar in prepenetration process, there are differences between species in the later process such as conidia adhesion, melanization and cutinization in penetration of the plant cuticle by the appressoria.

1.5 Coffee Berry Disease

Coffee berry disease (CBD) is the major factor limiting the economic production of Arabica coffee in Africa (Masaba & Waller, 1992). CBD is an anthracnose of green and ripe coffee berries caused by *Colletotrichum coffeanum* Noack *sensu* Hindorf, renamed *C. kahawae* (Waller, *et al*., 1993). The name of *C. coffeanum* was applied to a *Colletotrichum* species found on coffee in Brazil by Noack at the end of the 19 century, where CBD is not present and coffee leaves were the source of Delacroix's material. Thus, it is clear that the name *C. coffeanum* was not based on type material associated with coffee berry disease and the name *C. coffeanum* is no longer valid as it is presumed to be a synonym of *C. gloeosporioides*. *Colletotrichum* causing CBD was differentiated by Waller, *et al*. (1993) biochemically and culturally as shown on Table 1.2.

Coffee berry disease was first reported in 1922 in Kenya, near to the border with Uganda and spread to many estates west of the Rift Valley in Kenya (McDonald, 1926). Other reports came from Zaire in 1938 (Hendrickx, 1939), Cameroon (Muller, 1964), Tanzania (Tapley, 1964), Rwanda and Uganda (Butt & Butters, 1966). More recently CBD was reported in Malawi, Zimbabwe and Zambia in 1985 (Waller, 1987). This disease remains restricted to the African continent, but it poses a major threat to the Arabica coffee industries of other tropical countries (Bridge, *et al*., 2008).

Table 1.2 Differentiation of *Colleetotrichum kahawae* and *C. gloeosporioides* isolates from coffee

	C. gloeosporioides	***C. kahawae***
Colony characteristics (fresh single-conidial isolates on 2% MEA)	Faster growing (3-6 mm day-1 at 25), white to pale grey mycelium, sporulation from acervuli or simple hyphae	Slow growing (2-4 mm day-1 at 25), profuse olivaceous to greenish dark grey mycelium, no acervular conidiomata produced, sporulation occurs from simple hyphae
Metabolism	Utilize citrate, tartrate or both as sole carbon source	Not utilize citrate or tartrate as sole carbon sources
Pathogenicity	Not pathogenic to young berries or seedling hypocotyls	Pathogenic to young berries or seedling hypocotyls, causing dark sunken anthracnose lesions

The fungus affects green and ripe berries and the coffee flower at any stage in its development. The symptom of CBD on berries has two forms. Active lesions are initially small dark sunken areas that spread and coalesce to cover the berry surface, when the berry ripens and anthracnose fully develops the bean can also become infected (Figure 1.1). In some instances, scab lesions can also occur, which in appear only slightly sunken and normally pale tan in colour (Masaba & Waller, 1992; Waller, *et al*., 1993).

Figure 1.1 Dark sunken lesion with sporulation

Source: (Silva & Várzea, 2006)

The colonies of *C. kahawae* from sporulating lesions are densely floccose, grey to dark olivaceous-grey, dark greenish in reverse, attaining 14-28 mm diameter on 2% malt extract agar in 7 days (Waller, *et al*., 1993). Colonies become variable with succesive transfer cultures, often paler or brownish. Acervuli are absent and conidia are produced from simple hyphae. Conidia are straight, cylindrical, aseptate, invariably guttule, obtuse at the apex, and 12.5–19 x 4 µm. Appressoria are moderately abundant, pale to medium brown, circular or slightly irregular, 8-9.5 x 5.5-6.5 µm, often becoming complex. Biochemically, *C. kahawae* are unable to metabolize citrate and tartrate as a sole carbon sources in culture. *Colletotrichum kahawae* is pathogenic to young green berries and seedling hypocotyls of Arabica coffee causing dark sunken anthracnose lesions.

1.6 Current Status of *Colletotrichum*

Colletotrichum is the genus that has continued to rank highly as one of most studied genera of phytopathogenic fungi and Oomycetes. Latunde-Dada (2001) have concluded that *Colletotrichum's* rating as judged by the number of hits recorded in the *Web of Science* (Institute for Scientific Information of the United Kingdom) over the period 1981 to March 2001 compares with *Puccinia, Botrytis* and *Verticillium*. Although, *Colletotrichum* is surpassed by *Fusarium, Phytophthora* and *Rhizoctonia* in listed publications.

Identification within the genus of *Colletotrichum* has long been in uncertain due to the lack of good morphological characters, the existence of both anamorphic and teleomorphic forms in some taxa, their extensive host range, uncertain host relationships and pathological variations relative to environmental influences (Simmonds, 1965; Bailey & Jeger, 1992; TeBeest, *et al*., 1997; Freeman, *et al*., 2000; Latunde-Dada, 2001; Du, *et al*., 2005; Thaung, 2008). TeBeest, *et al*. (1997) have concluded that taxonomic uncertainty has made accurate identification difficult and complicated efforts to understand host relationships, diagnose disease accurately, develop effective control strategies and establish cost effective quarantine programs.

Traditionally, *Colletotrichum* species are identified and delimited mainly on the morphological characters; several features have been utilized by taxonomists including size and shape of conidia and appressoria; presence or absence of setae, sclerotia, acervuli and teleomorph state and cultural characters such as colony colour, growth rate and texture (Simmonds, 1965; Smith & Black, 1990; Sutton, 1992; TeBeest, *et al*., 1997; Photita, *et al*., 2005; Than, *et al*., 2008a; Than, *et al*., 2008b., Than, *et al*., 2008c; Thaung, 2008). These criteria alone are not always adequate for reliable differentiation among *Colletotrichum* species due to variation in morphology and phenotype among species under environmental influences. To overcome the inadequacies of these traditional schemes, molecular techniques have been used to characterize and identify taxa within *Colletotrichum* (Sreenivasaprasad, *et al*., 1996; Abang, *et al*., 2002; Moriwaki, *et al*., 2002; Peres, *et al*., 2002; Guerber, *et al*., 2003; Photita, *et al*., 2005; Du, *et al*., 2005; Peres, *et al*., 2008; Shenoy, *et al*., 2007; Whitelaw-Weckert, *et al*., 2007; Than, *et al*., 2008a; Than, *et al*., 2008b., Than, *et al*., 2008c). Cannon, *et al.* (2000) stated that nucleic acid analyses should provide the most reliable framework to classify *Colletotrichum*, as DNA characters did not directly influenced by environmental factors.

A combined technique of molecular diagnostic tools along with traditional morphological techniques is at present an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon, *et al*., 2000). Photita, *et al*. (2005) separated 34 isolates of *Colletotrichum* spp. were isolated from banana, ginger, *Euphatorium thymifolia*, soybean, longan, mango and *Draceana sanderiana* from Thailand into four morpho-groups viz:

C. musae, *C. gloeosporioides* group 1, *C. gloeosporioides* group 2, *C. gloeosporioides* group 3 and *C. truncatum*. Whitelaw-Weckert, *et al.* (2007) proposed a new *C. acutatum* group based on cultural, morphological, RAPD-PCR and sequencing of parts of the 5.8S-ITS regions and the ß-tubulin-2 gene. Than, *et al*. (2008a) differentiated the isolates of chilli anthracnose from Thailand into three species viz: *C. acutatum*, *C. capsici* and *C. gloeosporioides* based on morphological characterization, sequencing based on rDNA- ITS region and beta tubulin gene and pathogenicity testing.

1.7 Aims of This Study

The main objectives of this study were as follows:

1. To investigate the fungal diversity of the coffee berry by comparing the community of epiphytic and endophytic fungi from coffee berry.

2. To characterize the species of *Colletotrichum* isolates associated with coffee berry disease in Chiang Mai, Thailand by their morphological and cultural characters, biochemical, pathogenicity testing and DNA barcoding.

CHAPTER 2

FUNGAL DIVERSITY ASSOCIATED WITH COFFEE BERRIES IN CHIANG MAI, THAILAND

2.1 Abstract

In this study the epiphytic and endophytic fungal communities associated with coffee berries of Arabica coffee in Chiang Mai, Thailand were compared. Epiphytic and endophytic fungi were isolated by placing berry pieces on potato dextrose agar with and without surface sterilization, respectively in order to establish whether the fungal communities on the surface of a berry differs from the community within the berry. A total of 352 fungal strains were isolated and grouped into 12 genera. Surprisingly, two genera were found only in Pha Daeng village, and *Moniliopthora* is the first record from a coffee plantation. The results show that variation of the fungal diversity between epiphytic and endophytic communities is insignificant ($0.05 < P < 0.10$). Two sites were investigated and it was found that fungal diversity in Pha Daeng village was significantly higher than that in Mae Lod village ($P < 0.001$).

2.2 Introduction

Almost every plant species and even plant part is inhabited by different fungi (Hyde, 1995). They may be present as epiphytes, endophytes, pathogens, parasites or commensals. Fungi have shown potential as biocontrol agents, are chemical producers of bioactive compounds and are used in the pharmaceutical and many other industries, and are decomposers in natural ecosystems (Yuen, *et al*., 1999; Bucher, *et al*., 2004; Duarte, *et al*., 2006; Gadd, 2007). Hawksworth (2001) was described the estimated numbers of fungi to be 1.5 million. The importance of the fungal species pool however, is likely to be related to the economic impact of fungi in the environment (Hyde, 2001).

Fungi that live on the surface of their host have been defined as epiphytes (De Barry, 1866). In epiphytic communities the saprobic mycota can include organisms pathogenic to plants (Clark & Paul, 1970). In recent years much research has been published concerning epiphytes having an adverse effect on plant pathogens (Jayaswal, *et al*., 1990; Janisiewicz, 1992; Sholberg, *et al*., 1995; Sinigaglia, *et al*., 1998).

Endophytes are microorganisms that colonize and cause unapparent (symptomless) infections in healthy plant tissues (Petrini, 1991; Wilson, 1995). Fungal endophytes have been studied from many plant species (See Table 1.1).

Relationships between epiphytes and endophytes have important implications for fungal biodiversity and plant health. Therefore, the goals of this study were 1) to establish whether the fungal community on the surface of a berry differs from the community within the berry and 2) to establish whether the fungal community differs among sites.

2.3 Materials and methods

2.3.1 Collection of berries and isolation of epiphytic and endophytic fungi

Healthy red berries were collected from coffee plants at Pha Daeng and Mae Lod village in Chiang Mai, Thailand. Five plants per site were chosen and five healthy red berries were sampled per plant. From each berry, three pieces were isolated and cultured on water agar (WA).

In order to isolate epiphytes, 5 x 5 mm pieces of tissue berry were placed on WA and incubated at room temperature (28-30°C) without surface sterilizing the pieces. In order to isolate endophytes, 5 x 5 mm pieces of tissue berry were used, the pieces of berry were surface sterilized in 1% $NaClO_2$ (1 min), 70% EtOH (1 min) based on the modified procedure described by Bayman, *et al*. (1998), Bayman, *et al*. (2002) and Gamboa & Bayman (2001) and rinsed three times with sterilized water and drying in sterilized tissue paper. They were then placed on WA and incubated at room temperature (28-30°C). The growing edges of any fungal hyphae developing from the tissues were then transferred aseptically to potato dextrose agar (PDA, Criterion®, Santa Maria, USA).

The fungi were identified following sporulation while fungi that did not sporulate or could not be readily identified was then cultured on malt extract agar (MEA, Difco™) (Guo, *et al*., 1998). Cultures were examined periodically and identified when isolates sporulated. The remaining isolates which failed to produce spores were treated as mycelia sterilia. All mycelia sterilia were separated into morphospecies (*sensu* Lacap, *et al*., 2003) and differentiated from other sporulating isolates.

2.3.2 Data Analyses

The frequency of occurrence was calculated in order to compare the dominance of fungi among different communities. The data acquired from each site is presented as frequency of occurrence which is the number of samples that a particular genus occurred on, divided by the number of samples examined at one site (Tsui, *et al*., 2001; Yanna, *et al*., 2002). The total number of genera and frequency of occurrence of each genus were recorded and calculated. Shannon-Weiner diversity index (H'), species richness and species evenness were applied to evaluate the diversity of fungal communities (Begon, *et al*., 1992). A t test was performed to compare the Shannon-Weiner indices between different fungal communities and sites (Hutcheson, 1970; Zar, 1999; Cai, 2006).

2.4 Results

A total of 352 fungal strains were isolated from 50 coffee berries in two different sites which include endophytes and epiphytes in Chiang Mai, Thailand. In this study, taxa were only identified to generic level as all genera isolated were complicated speciose genera and require the use of molecular sequencing for identification. The remaining isolates which did not sporulate in culture and could not be identified were named mycelia sterilia.

In total of 160 isolates of fungal endophytes were isolated from two sites. The number of isolates of endophytic fungi among sites showed that Pha Daeng had higher than Mae Lod village. Composition of the endophytic fungi between two sites was comprised similar genera, with the exception of *Gliocladium* (Table 2.1). This genus was found only at Pha Daeng village. The predominant of endophytic fungi among different sites was *Fusarium*, ninety-nine isolates at Pha Daeng and sixty-one isolates at Mae Lod village.

In total of 192 isolates of fungal epiphytes were isolated from two sites. The number of isolates of epiphytic fungi among different sites showed that Pha Daeng had higher than Mae Lod village. Composition of the epiphytic fungi between two sites was varied (Table 2.1). It showed that three genera were not presented at Mae Lod village. *Moniliopthora* was presented only at Pha Daeng and is a first record from a coffee plantation. The predominant of epiphytic fungi among different sites was *Fusarium*, one-hundred-eighteen isolates at Pha Daeng and seventy four isolates.

The distribution of taxa was varied in both among different communities and sites (Figure 2.1). *Fusarium* was predominant either on different communities or different sites. It was followed by *Pestalotiopsis*, *Cladosporium*, *Penicillium*, *Trichoderma* and *Colletotrichum* which varied of the distribution either on different communities or different sites.

In order to compare the fungal diversity among different communities and/or different sites, the total frequency of occurrence of each genus, species richness, species evenness and Shannon-Weiner diversity index (H') of each community and site were calculated and are also shown in Table 2.2. Higher species richness was found from the epiphytic community compared with endophytic community (192 isolates of epiphytes versus 160 isolates of endophytes) and also from Pha Daeng village compared with Mae Lod village (217 isolates at Pha Daeng versus 135 isolates at Mae Lod village).

The *t* test was performed to compare the fungal diversity between epiphytes and endophytes; and Pha Daeng and Mae Lod village. It indicates that the fungal diversity between epiphytes and endophytes insignificant ($0.05 < P < 0.10$), while that between Pha Daeng and Mae Lod village is significant ($P < 0.001$) (Table 2.2).

Table 2.1 List of endophytic and epiphytic fungi isolated from coffee berries in Chiang Mai, Thailand.

	Pha Daeng village	Mae Lod village
Endophytic:		
Aspergillus	7	6
Cladosporium	17	9
Colletotrichum	7	4
Fusarium	21	20
Gliocladium	4	-
Mycelia sterilia	2	3
Penicillium	8	3
Pestalotiopsis	7	8
Phoma	7	1
Phomopsis	8	3
Trichoderma	11	4
Total endophytic	**99**	**61**
Epiphytic:		
Aspergillus	5	4
Cladosporium	18	13
Colletotrichum	10	6
Fusarium	23	21
Moniliopthora	2	-
Mycelia sterilia	4	2
Penicillium	14	3
Pestalotiopsis	22	20
Phoma	7	-
Phomopsis	3	-
Trichoderma	10	5
Total epiphytic	**118**	**74**
Total	**217**	**135**

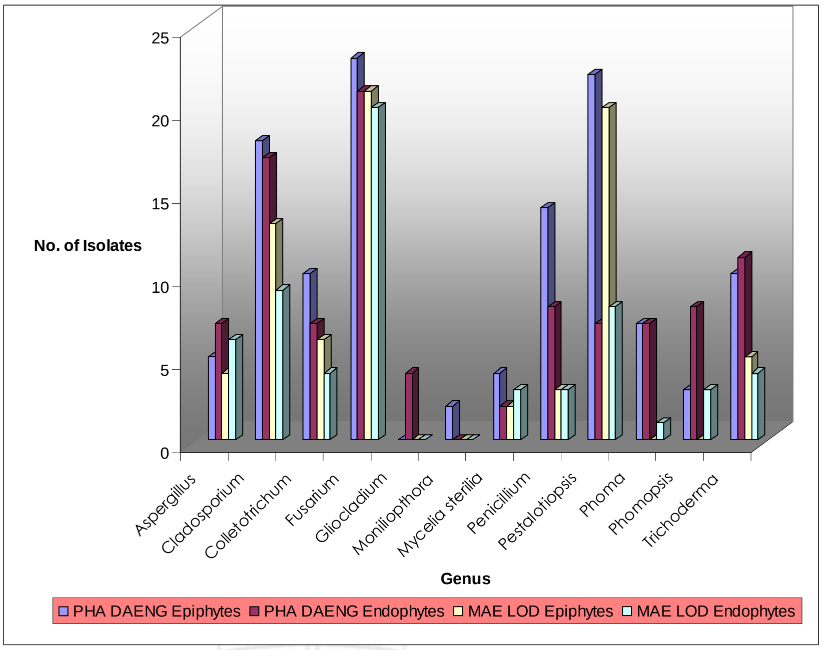

Figure 2.1 Fungal distribution on coffee berry (*Coffea arabica*)

Table 2.2 Frequency of occurrence of fungi on coffee berry (*Coffea arabica*).

Genus	**Frequency occurrence (%)**			
	Epiphytic	**Endophytic**	**1st site (Pha Daeng)**	**2nd site (Mae Lod)**
Aspergillus	4.7	**8.1**	5.5	**7.4**
Cladosporium	**16**	**16**	**16**	**16**
Colletotrichum	**8.3**	**6.9**	7.8	**7.4**
Fusarium	**23**	**26**	**20**	**30**
Gliocladium	-	2.5	1.8	-
Moniliopthora	1	-	0.9	-
Mycelia sterilia	3.1	3.1	2.8	3.7
Penicillium	**8.9**	**6.9**	**10**	4.4
Pestalotiopsis	**22**	**9.4**	**13**	**21**
Phoma	3.6	5	6.5	0.7

Table 2.2 Frequency of occurrence of fungi on coffee berry (*Coffea arabica*) (Cont.)

Genus	Frequency occurrence (%)			
	Epiphytic	Endophytic	1st site (Pha Daeng)	2nd site (Mae Lod)
Phomopsis	1.6	**6.9**	5.1	2.2
Trichoderma	7.8	**9.4**	**9.7**	**6.7**
Species richness	192	160	217	135
Species evenness (J')	0.863	0.915	0.905	0.839
Shannon-Weiner indices (H')	0.899	0.953	0.976	0.839

Note. Frequency of occurrence of the five most common genera occurring in each community and site are in bold.

Table 2.3 Comparison of Shannon-Wiener diversity indices between different community and site.

H_0: epiphytic and endophytic community support same fungal diversity. **H_A: null hypotheses is not true**	**H_0: Pha Daeng and Mae Lod village support same fungal diversity.** **H_A: null hypotheses is not true**
Community:	**Site:**
H' (epiphytic) = 0.899	H' (Pha Daeng) = 0.976
H' (endophyticl) = 0.953	H' (Mae Lod) = 0.839
Calculated $t = -1.738$	Calculated $t = 4.123$
Calculated freedom $v = 350$	Calculated freedom $v = 237$
t 0.05(2), 350 = 1.967 > t	t 0.05(2), 237 = 1.969 < t
Thus, null hypothesis is accepted,	Thus, null hypothesis is rejected,
($0.05 < P < 0.10$).	($P < 0.001$).

2.5 Discussion

The fungal distribution was varied either on different communities or different sites (Table 2.1 and Figure 2.1). Differences among sites might be due to differences in environmental conditions or presence of inoculum (Rodrigues, 1994; Andrews & Harris, 2000). The composition of fungal communities is affected by a number of factors and correlated to the size and quantity of available resources (Strong, 1992; Lodge & Cantrell, 1995; Hyde, *et al*., 2005). A few studies of endophytic mycota suggest that studies should include plants from different populations, because single populations may not be representative (Lodge, *et al*., 1996; Lodge, 1997; Gamboa & Bayman, 2001).

Results show that each of the five most common genera both in epiphytic and endophytic communities comprises similar genera. Generally, a large number of surface contaminants (e.g. *Aspergillus*, *Cladosporium*, *Penicillum*) were presented on the fungal endophytes isolates. Similar results have been reported by Hyde & Soytong (2008).

In this study, *Fusarium* was predominated both the epiphyte and endophyte communities; this genus has been reported as frequently collected in tropical and temperate areas (Tang, *et al*., 2003; Hyde, *et al*., 2005; Kvas, *et al*., 2009). Ramos-Mariano (1997) have compared endophytic and epiphytic fungi in coconut leaves in Brazil; 45 species were exclusively endophytic and 44 species were exclusively epiphytic, with 29 species found in both communities. The most common epiphyte was *Cladosporium* spp. and the most common endophyte was *Pestalotiopsis palmarum*. In a study of epiphytes and endophytes in coffee leaves in Brazil, *Botryosphaeria* and *Pestalotia* were common as endophytes and *Xylaria*, *Collletotrichum*, and *Guignardia* were common as epiphytes (Santamaria & Bayman, 2005).

The fungal diversity of epiphytes was higher than endophytes, although endophyte communities were not noticeably less than epiphyte communities. Surprisingly, differences in epiphyte and endophyte communities were insignificant when using the *t* test (Table 2.3). The diversity indices of epiphytes and endophytes could not be compared as they are usually under-sampled (Santamaría & Bayman, 2005; Hyde & Soytong, 2008). Furthermore, the techniques to study endophytes severely influence the fungal endophytes isolated (Guo, *et al*., 2001; Hyde & Soytong, 2008). A method for studying endophytes was described by Schulz, *et al*. (1998), which was efficient for eliminating epiphytes. Therefore, Sánchez Márquez, *et al*. (2007) recommended that this method should be used in all endophytes studies in the future.

Epiphytic and endophytic fungi probably interact in ways that affect the host plant. Interactions within each community are poorly understood and interactions between them are completely unexplored. To understand such interactions, bacteria and their interactions with fungi should be explored, because this is also poorly understood. Recent studies have shown that bacteria may influence development of human digestive systems and other aspects of animal development (Hamilton, 1999). It is possible that endophyte and epiphyte communities influence not only plant function and health, but also plant development. Endophytes has been implicated in mutualism, decreased herbivory, increased drought resistance, increased disease resistance and enhancement of plant growth (Fröhlich, *et al*., 2000; MacMillan, 2002; Sieber, 2007), although the mutualism has been inconclusive in the field (Sieber, 2007). Understanding these communities and interactions and manipulating them to improve plant health, represents one of the most promising and poorly understood areas of agricultural bicotechnology (Cotty, *et al*., 1994; Sturz, *et al*., 2000; Santamaria & Bayman, 2005). Also, their potential to produce bioactive compounds has become one of the main areas in endophyte study in recent years (Kumar & Hyde, 2004; Tejesvi, *et al*., 2007; Huang, *et al*., 2008). While comprehensive understanding of microbial communities has theoretical interest, it is also provides valuable knowledge of causes and consequences of microbial mediated interactions in agricultural ecosystems which may have economical value (Saikkonen, 2007).

CHAPTER 3

CHARACTERIZATION OF *Colletotrichum* SPECIES ASSOCIATED WITH COFFEE BERRIES IN CHIANG MAI, THAILAND

3.1 Abstract

Colletotrichum species are known to occur on and cause diseases of coffee (*Coffea arabica*), but their identities are not yet clearly understood. There is also little known about the characterization of *Colletotrichum* species associated with coffee berry disease in Thailand. In this paper we report the *Colletotrichum* species associated with coffee berries in northern Thailand and compare these isolates with previous species reported to cause coffee berry disease elsewhere. The combination of morphological and cultural characters, biochemical, pathogenicity testing and DNA barcoding resulted in the isolates clustering into three unknown *Colletotrichum* species. *Colletotrichum asianum*, *C. coffeae* and *C. coffeigenum* are therefore introduced as new species in the present study. Furthermore, the combined datasets of actin, partial β-tubulin-2 (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region revealed *Colletotrichum* relationships congruent with their morphological characters. The biochemical and DNA barcoding data from multi-genes used here also showed clear differences between *C. kahawae*, *C. gloeosporioides* and the new *Colletotrichum* isolates.

3.2 Introduction

Coffee is a tropical crop and according to FAO statistics production of green beans is around 4 million tons annually with sales of between 6 to 12 billion dollars (González, 2000). Coffee is a major source of income for Thailand being the third largest producer in Southeast Asia - after Vietnam and Indonesia (Angkasith, 2001). While there are many species of coffee trees in the world, only Arabica and Robusta have major economic significance, and both species are grown in Thailand (Songset, *et al*., 2005). Arabica coffee is produced in the cooler highland areas of the northern part of Thailand while robusta coffee is produced in Southern part of Thailand (Angkasith, 2001). Consumption demands for both instant and roasted coffee are growing in Thailand.

Despite of its importance, coffee production has often been limited by a disease known as coffee berry disease. Coffee berry disease is the major factor limiting Arabica coffee production in Kenya and other countries in Eastern Africa (Van der Vossen, *et al*., 1976; Firman, *et al*., 1977). Coffee berry disease is caused by the fungus *Colletotrichum coffeanum*, renamed *C. kahawae* (Waller, *et al*., 1993). It can cause a devastating anthracnose on developing berries in Arabica coffee in Africa and is particularly serious at high altitudes (Van der Graaff, 1992; Masaba & Waller, 1992). Coffee berry disease may cause crop losses of between 50 to 80% (Van der Vossen, *et al*., 1976) in years favorable to a severe disease epidemic - prolonged wet and cool weather) (Van der Vossen, 2001).

Several species or strains of *Colletotrichum* have been reported from coffee including saprobic or pathogenic strains, although saprobic strains of *Colletotrichum* occur most commonly. *Colletotrichum, Xylaria and Guignardia* are the most common genera of endophytes of coffee in Puerto Rico (Masaba & Waller, 1992; Santamaría & Bayman, 2005). However, there is little known about the characterization of *Colletotrichum* species associated with coffee berry disease in Thailand and it is not clear whether all the species that can be isolated from berries are equally pathogenic. Thus, this study has characterized the species of *Colletotrichum* isolates associated with apparently healthy coffee berries and infected berries in Chiang Mai, Thailand using morphological characters, biochemical tests, phylogenetic relationships and pathogenicity testing.

3.3 Materials and Methods

3.3.1 Selected genus of epiphytic and endophytic community

Epiphytic and endophytic *Colletotrichum* strains were isolated from coffee berry as described in Chapter two. They were initially characterized to species level using morphological characters, and then subjected to biochemical analysis, phylogenetic relationships and pathogenicity testing as described below.

3.3.2 Isolation of *Colletotrichum* species from coffee berry disease infected berries

Colletotrichum isolates were collected from anthracnose lesions on infected coffee berries (*Coffea arabica* L.) at two sites in Chiang Mai, Thailand. Isolation was carried out by two methods depending on fungal sporulation. 1) Isolates were obtained from fruit without visible sporulation using the modified procedure described by Photita, *et al*. (2005) and Than, *et al*. (2008a). Three 5 x 5 mm pieces of tissue were taken from the margin of infected tissues, surface sterilized by dipping 1% sodium hyphochlorite for 1 minute, immersed in 70% ethanol for 1 minute and rinsed three times with sterilized water and finally dried in sterilized tissue paper. Samples were placed on water agar and incubated at room temperature (28-30°C). The growing edges of any fungal hyphae developing from the tissues were then transferred aseptically to potato dextrose agar (PDA, Criterion®, Santa Maria, USA). The fungi were identified following sporulation. Single spore subcultures were obtained for each *Colletotrichum* isolate by using the modified procedure described by Goh (1999). 2) Alternatively, direct examination and single spore isolation from the spore masses on infected berries was also carried out. Spore masses were pick off with a sterilized wire loop and streaked on the surface of water agar followed by incubation overnight. A single germinated spore was picked up with a sterilized needle and transferred onto PDA. Pure cultures were stored in sterilized water and agar slants in Eppendorf tubes at 6°C. Cultures are deposited in the culture collection of BIOTEC Culture Collection (BCC) Thailand.

3.3.3 Morphological studies of *Colletotrichum* species

Mycelial discs (4 mm) were transferred onto potato dextrose agar (PDA, Criterion®, Santa Maria, USA) taken from actively sporulating areas near the growing edge of a 7 day old culture of each isolate. Cultures were incubated at 28°C. Each plug was placed onto PDA plates and incubated under the same condition as starter cultures for 7 days. Three replicate cultures of each isolate was investigated. After 7 days, conidial size and shape from 20 conidia harvested from the culture of each isolate were recorded (Than, *et al*., 2008a).

Colony diameter of every culture was measured daily for 7 days. Growth rate was calculated as the 7 day average of mean daily growth (mm per day). After 7 days, colony size and color of the conidial masses and zonation were recorded.

Appresoria were produced using a slide culture technique, in which 10 mm^2 squares of PDA were placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip was placed over the inoculated agar (Johnston & Jones, 1997). The slide cultures were incubated at room temperature (28-30°C). After 3-7 days, the shape and size of the appresoria formed across the underside of the cover slip were then studied.

Morphological data were analyzed using analysis of variance ($P<0.05$) with Duncan's Multiple Range Test (DMRT) by using SPSS software version 16.0 (SPSS Inc., Chicago, USA) (Kirkpatrick & Feeney, 2006).

3.3.4 Biochemical testing

The biochemical test based on substrate utilization was assessed in agar plates according to the method of Bridge, *et al*. (2008). Utilization of citrate and tartrate as a carbon source were assayed on agar plates. Medium B with 1.2 % (w/v) agar was supplemented with 1 % (w/v) citric acid or ammonium tartrate and 0.005 % (w/v) bromocresol purple. Positive and negative controls containing, respectively, glucose or no additional carbon source were included for each isolate.

All media was adjusted to pH 4.5 with NaOH prior to sterilization by autoclaving at 105°C for 20 minutes. Test media were inoculated with agar plugs (4 mm diameter) taken from 7 day old single conidium derived cultures. Utilization was assessed by visual comparison of growth and a rise in the pH of the medium adequate to produce a dark blue to purple colour of bromocresol purple (Waller, *et al*., 1993; Bridge, *et al*., 2008).

3.3.5 Pathogenicity testing

Three representative isolates of each morpho-group were used for pathogenicity testing. Isolates were *Colletotrichum* Group 1 (BML-I6, BML-I15, BPD-I2), *Colletotrichum*

Group 2 (BML-I3, BML-I14, BPD-I4), *Colletotrichum* Group 3 (BPD-I12, BPD-I16, BPD-I18), and two isolates of *C. kahawae* were also included.

Single spore cultures of three representative isolates were grown on potato dextrose agar for 7 days at room temperature. The spores were harvested by adding 10 ml of sterilized distilled water onto the culture, which was then gently swirled to dislodge the conidia with the concentration adjusted to 10^6conidia/ml using a haemocytometer; and used as standard inoculum for carrying out all experiments. The conidial suspension was filtered through two layers of muslin cloth (Than, *et al*., 2008a).

The coffee berries (*Coffea arabica*) were supplied by Royal Agricultural Project Coffee from Chiang Mai, Thailand. Non-infected berries were disinfected with 1% sodium hypochlorite for 5 minutes, and washed three times with distilled water. The berries were blotted dry with a sterilized tissue paper and inoculated by using wound/drop and non-wound/drop inoculation method (Lin, *et al*., 2002; Than, *et al*., 2008a). The wound/drop inoculation method involved pin pricking the coffee berry wall to a 1 mm depth and then placing 6 µl of conidia suspension (10^6conidia/ml) over the wound. Control fruits were inoculated with 6 µl of sterilized distilled water onto the wound. The inoculated fruits were incubated at room temperature in a closed sterile container. This experiment was arranged by using Completely Randomized Design (CRD), with three replicate per isolate. Three berries of green and red berries were tested per isolate. Infected berries were disposed of following sterilization. The anthracnose lesion area was evaluated by measuring length, width and area of the typical anthracnose lesion developed on the berries, from 1 to 15 days after inoculation (DAI). Percentage of infected area in fruits was obtained from the lesion area divided by fruit area and multiplied by one hundred (Than, *et al*., 2008a). After 15 days following inoculation, conidial from diseased berries were aseptically transferred onto potato dextrose agar (PDA, Criterion®, Santa Maria, USA) and incubated at room temperature. The resultant cultures were checked for morphological characters to confirm Koch's postulates. All infected berries were disposed of following sterilization.

Data of the anthracnose lesion area were analyzed with Duncan's Multiple Range Test (DMRT) by using SPSS software version 16.0 (SPSS Inc., Chicago, USA) (Kirkpatrick & Feeney, 2006).

3.3.6 Molecular examination

1. DNA extraction

Three isolates from each suspected morpho-species of *Colletotrichum* isolated from coffee berries and *Colletotrichum* epitypes were included in phylogenetic study. For production of mycelium for DNA extractions, isolates were grown on PDA and incubated for 7 days. Mycelium was obtained from the surface by scrapping. Genomic DNA was extracted by using a Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the instructions of the manufacturer. DNA concentrations were estimated visually in agarose gel by comparing band intensity with a DNA ladder 100 bp (Transgen Biotech®).

2. PCR amplification and DNA sequencing

Nucleotide sequence analysis of the actin, partial of β-tubulin-2 gene (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region for 15 representative *Colletotrichum* strains including the epitypes and *C. falcatum* as a outgroup were performed by PCR amplification.

The PCR amplification for actin, partial of β-tubulin-2 gene (tub2) and the complete rDNA-ITS region were amplified with primers-pair ACT512F (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT783R (5'-TACGAGTCCTTCTGGCCCAT-3') (P. Johnston, *personal communication*), Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Than, *et al*., 2008a), ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990), respectively. The cycling parameters consisted of a 3 minutes denaturing step at 95°C followed by 34 cycles at 95°C for 1 minutes, 52°C for 30 seconds, 72°C for 1 minutes and a final cycle of 10 minutes at 72°C.

Calmoudulin was amplified using primers-pair CL1 (5'-GARTWCAAGGAGGCCTTCTC-3') and CL2 (5'-TTTTTGCATCATGAGTTGGAC-3') (P. Johnston, *personal communication*). The cycling parameters was initiated at 94°C for 2.5 minutes followed by 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds and a final step at 72°C for 15 minutes.

The glutamine synthetase and glyceraldehyde 3-phosphate dehydrogenase were amplified with GSF1 (5'-ATGGCCGAGTACATCTGG-3') and GSR1 (5'-GAACCGTCGAAGTTCCAC-3') (Guerber, *et al*., 2003); GDF1 (5'-GCCGTCAACGACCCCTTCATTGA-3') and GDR1 (5'-GGGTGGAGTCGTACTTGAGCATGT-3') (Peres, *et al*., 2008) respectively. The cycling parameters consisted of a denaturation step at 94°C for 4 minutes, followed by 34 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 minutes and a final cycle at 72°C for 10 minutes.

The PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis gels. DNA sequencing for actin, partial of β-tubulin-2 gene (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region were performed by sequencing at the International Fungal Research and Development Centre, The Research Institute of Resource Insects, the Chinese Academy of Forestry, China.

3. Phylogenetic analysis

Separate or combined datasets of actin, partial of β-tubulin-2 gene (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region sequences were used for analyses by using the PAUP program All sequences were aligned by BioEdit and Clustal X (Thompson, *et al*., 1997; Santamaría and Bayman, 2005) and optimized manually, gaps were excluded. Branch supports of the trees resulting from the parsimony were assessed by bootstrapping based on 1000 replicates (Felsenstein, 1985).

3.4 Results

3.4.1 Collection of *Colletotrichum* species

A summary of *Colletotrichum* isolates used in this study are listed in Table 3.1. Thirty-four isolates of *Colletotrichum* were collected from Pha Daeng and Mae Lod village which included epiphytes, endophytes and pathogens. Eighteen isolates were collected from Pha Daeng village of which ten are epiphytes, seven endophytes and one a pathogen. Mae Lod village yielded sixteen isolates of which are four epiphytes, six endophytes and six pathogens. In addition, isolates of *C. acutatum* from *Carica papaya* was provided from the Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia "BRIP". One isolate of *C. acutatum* and one isolate of *C. gloeosporioides* were provided from Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Two isolates of *C. kahawae* from *Coffea arabica* were provided from CABI Bioscience UK Centre, Egham, Surrey, UK "IMI". One isolate of *C. falcatum* isolated from *Saccharum officinarum* was collected from Gedheg village, Comal District, Central Java Province, Indonesia and will designated as the epitype.

3.4.2 Morphological and cultural characterization

Distinctness in conidial morphology, colony characters and growth rates among *Colletotrichum* isolates resulted in morphological groups. Three distinct groups within *Colletotrichum* were recognized in this study. Group 1 comprised 5 isolates; 2 epiphytes and 3 endophytes, group 2 comprised 20 isolates; 12 epiphytes, 3 endophytes and 5 pathogens and group 3 comprised 9 isolates; 2 epiphytes, 5 endophytes and 2 pathogens (Table 3.2).

1. Colony characters: Distinct morphological types on PDA were observed in each morphological group after 7 days. Colonies produced by isolates from Group 1 varied from pale yellowish to pinkish colonies with dense whitish-grey aerial mycelium and a few bright-orange conidial masses near the inoculum point (Figure 3.1). Isolates from Group 2 produced colonies with greyish-green colonies in the centre, green in reverse with sparse white aerial mycelium and orange conidial masses near the inoculum point (Figure 3.1). Colonies produced by isolates from Group 3 varied from grey to dark grey colonies with dense pale grey aerial mycelium (Figure 3.1).

2. Growth rate: An important comparative character was the growth rate of the colony in culture. There was a statistically significant difference in growth rates among the 34 isolates. Isolates of *Colletotrichum* Group 3 (10.72 ± 0.53 mm day^{-1}) grew significantly faster, followed by Group 1 (9.12 ± 1.95 mm/day) and Group 2 (5.09 ± 0.38 mm/day) ($P = 0.05$) (Table 3.3).

3. Conidia morphology: Conidia of all the isolates had varied shapes. Four types of cylindrical conidia were recorded, viz, cylindrical conidia with obtuse ends (oblong), cylindrical conidia with obtuse and slightly tapered ends, cylindrical conidia narrowing at the center, and cylindrical conidia with slightly rounded ends. Conidia produced by isolates of

Colletotrichum Group 1 varied from fusiform with obtuse to slightly rounded ends, sometimes oblong (Figure 3.1). *Colletotrichum* Group 2 produced cylindrical conidia with obtuse ends (oblong) with narrowing at the center (Figure 3.1). *Colletotrichum* Group 3 produced cylindrical conidia with obtuse to slightly rounded ends (Figure 3.1). There were statistically significant differences in length and width of conidia among the Groups in this genus (Table 3.3).

4. Appressoria morphology: There was little distinction among the groups in size and shape of appressoria. Appressoria-shape produced by slide cultures varied from ovoid, clavate and slightly irregular to irregular in shape (Table 3.3, Figure 3.1).

Table 3.1 Synopsis of characters of *Colletotrichum* isolates.

***Colletotrichum* species**	**Number of isolates**	**Substrate and host**	**Location**
Colletotrichum Group 1	2	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 1	3	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
Colletotrichum Group 2	9	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 2	11	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
Colletotrichum Group 3	7	Coffee berries (*Coffea arabica*)	Pha Daeng Village, Chiang Mai Thailand
Colletotrichum Group 3	2	Coffee berries (*Coffea arabica*)	Mae Lod Village, Chiang Mai Thailand
*C. acutatum**	1	Papaya (*Carica papaya*)	Type of *C. acutatum* Queensland, Australia
*C. acutatum***	1	Papaya (*Carica papaya*)	Ex-epitype : Queensland, Australia

Table 3.1 Synopsis of characters of *Colletotrichum* isolates (Cont.).

Colletotrichum species	Number of isolates	Substrate and host	Location
*C. falcatum****	1	Sugarcane leaves (*Saccharum officinarum*)	Epitype : Indonesia
*C. gloeosporioides**	1	Citrus leaves (*Citrus sinensis*)	Epitype of *Vermicularia gloeosporioides* Italy, Calabria, Lamezia terme
*C. kahawae*****	2	Coffee (*Coffea arabica*)	Holotype (IMI 319418), Kenya Paratype (IMI 363578), Kenya

Note. *Isolate supplied from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **Isolates supplied from Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; ***Isolate will be designated as the epitype (Gedheg, Comal, Central Java, Indonesia); ****Isolates supplied from CABI Europe – UK, Bakeham Lane, Egham, Surrey TW209TY, UK.

Table 3.2 Summary of the life mode of *Colletotrichum* isolates.

Morpho-group	Isolates	Life Mode
Colletotrichum Group 1	BPD-I2	endophyte
(5 isolates)	BPD-I13	epiphyte
	BML-I6	endophyte
	BML-I13	epiphyte
	BML-I15	endophyte
Colletotrichum Group 2	BPD-I3	epiphyte
(20 isolates)	BPD-I4	endophyte
	BPD-I6	epiphyte

Table 3.2 Summary of the life mode of *Colletotrichum* isolates (Cont.)

Morpho-group	Isolates	Life Mode
Colletotrichum Group 2	BPD-I7	epiphyte
(20 isolates)	BPD-I8	epiphyte
	BPD-I9	epiphyte
	BPD-I10	epiphyte
	BPD-I11	epiphyte
	BPD-I14	endophyte
	BML-I1	epiphyte
	BML-I2	epiphyte
	BML-I3	pathogen
	BML-I4	epiphyte
	BML-I5	epiphyte
	BML-I8	epiphyte
	BML-I9	pathogen
	BML-I10	pathogen
	BML-I11	pathogen
	BML-I14	endophyte
	BML-I16	pathogen
Colletotrichum Group 3	BPD-I1	epiphyte
(9 isolates)	BPD-I5	epiphyte
	BPD-I12	endophyte
	BPD-I15	endophyte
	BPD-I16	endophyte
	BPD-I17	endophyte
	BPD-I18	pathogen
	BML-I7	endophyte
	BML-I12	pathogen

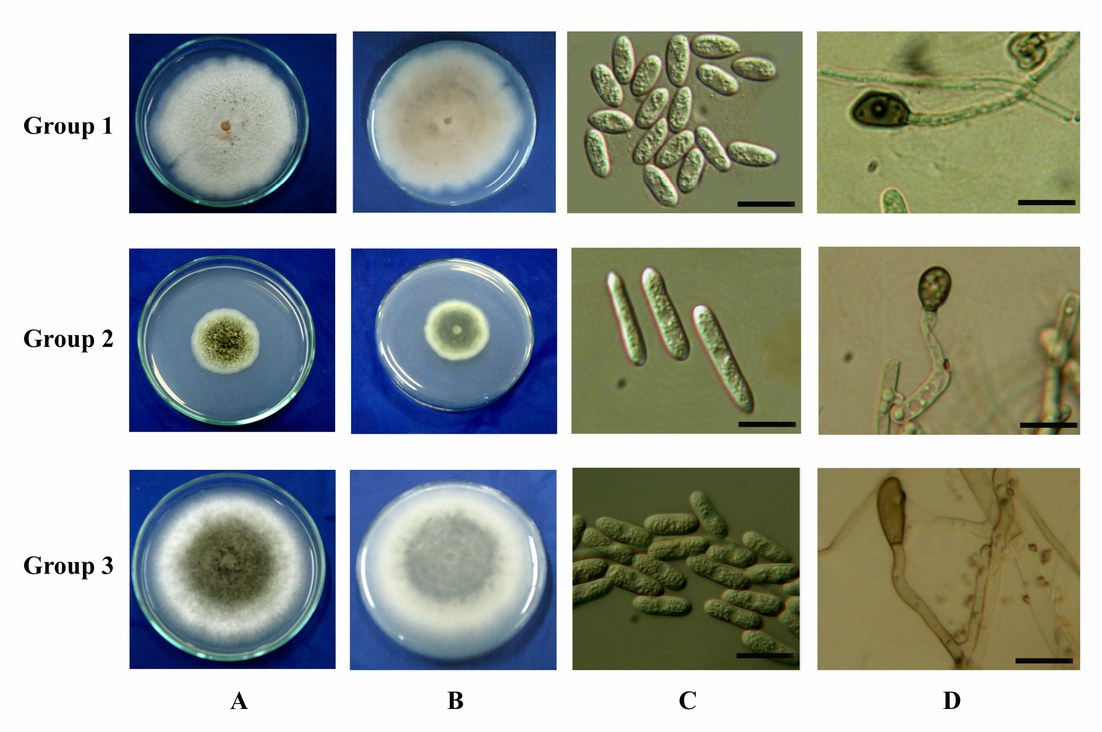

Figure 3.1 Morphology characters of *Colletotrichum* isolates. Upper (A) and Reverse (B) sides of cultures on PDA seven days after inoculation; (C) Conidia; (D) Appressoria (Bars = 10 µm).

Table 3.3 Summary of the morphological data of *Colletotrichum* isolates.

Species	**Colony characters**	**Conidia**			**Appresoria**		**Growth rate (mm/day)**
		Length (µm)	Width (µm)	Shape	Length (µm)	Width (µm)	
Colletotrichum Group 1	Cottony, dense white-greyish aerial mycelium, pale yellowish to pinkish colony	10.17±1.49 a (7 – 18.3)	3.60±0.48 c (3 - 6)	Fusiform	6.67±1.89 a (4 – 15.3)	4.26±0.45 a (3.5 – 5.3)	9.12±1.95 b (6.5 – 11.5)
Colletotrichum Group 2	Tufts, sparse white aerial mycelium, greyish-green colony and slow growing	12.26±1.66 c (7 - 20.3)	3.38±0.36 a (3 – 5.7)	Cylindrical	6.72±1.34 a (4.3 – 10.7)	4.72±0.85 b (3.3 – 8)	5.09±0.38 a (4.08 – 5.67)
Colletotrichum Group 3	Cottony, dense pale grey aerial mycelium, grey to dark grey colony and fast growing	11.37±0.96 b (9.7 – 14)	3.54±0.35 b (3 – 4.3)	Cylindrical	7.54±1.55 b (4.3 – 11.7)	4.35±0.85 a (3 – 7.3)	10.72±0.53 c (9.67 – 11.5)

*The mean difference is significant at the 0.05 level; values with same letter based on Duncan's multiple range tests in a column do not differ significantly

3.4.3 Biochemical testing

All *Colletotrichum* species isolated from coffee berries were tested for the ability to utilize citrate or tartrate as a sole of carbon source (Figure 3.2). Biochemical testing was used to distinguish *Colletotrichum* isolates on coffee. The two isolates of *C. kahawae* were unable to grow on either citrate or tartrate sufficiently to produce any colour change. Thirty-four isolates from Chiang Mai, Thailand had the ability to grow and change the colour to a dark blue to purple of the indicator on one or both substrates (Table 3.4). In the citrate test, four isolates of *Colletotrichum* Group 1, seventeen isolates of *Colletotrichum* Group 2 and six isolates of *Colletotrichum* Group 3 were unable to grow which sufficiently to produce any colour change.

Table 3.4 Summary of the substrate utilization of *Colletotrichum* isolates.

Species	Citrate		Tartrate	
	Positive	**Negative**	**Positive**	**Negative**
Colletotrichum Group 1 (5 isolates)	1	4	5	0
Colletotrichum Group 2 (20 isolates)	3	17	20	0
Colletotrichum Group 3 (9 isolates)	3	6	9	0
C. kahawae (2 isolates)	0	2	0	2

<u>**Note.**</u> Number of tests showing positive/negative utilization of organic acids as a sole carbon source. (Positive: able to grow either on citrate or tartrate and sufficiently gave a dark blue or purple colour; negative: unable to grow either on citrate or tartrate and sufficiently produce any colour change).

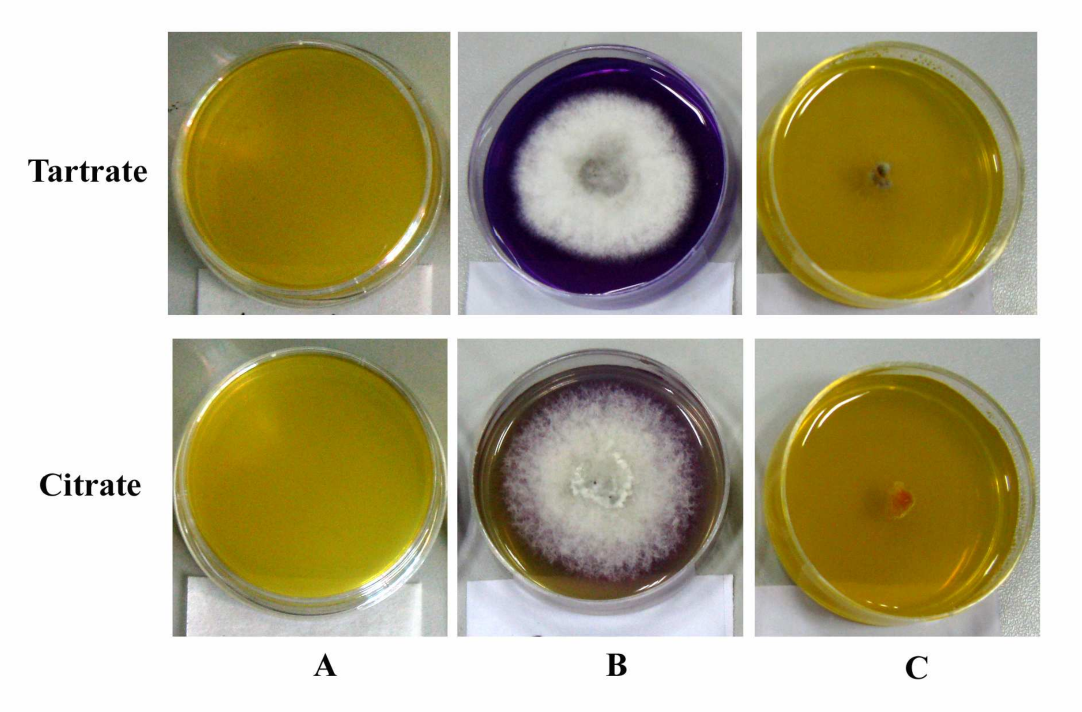

Figure 3.2 Biochemical reaction of *Colletotrichum* isolates. (A) Before inoculation; (B) Postitive reaction; (C) Negative reaction.

3.4.4 Pathogenicity testing

Both non-wound/drop and wound/drop inoculation methods using green and red berries, resulted in fruit infection (Figure 3.3, Table 3.5). Fruit symptoms from all morpho-groups were similar to each other. The wound/drop inoculation method produced a higher incidence of disease than the non-wound/drop method. Statistically, the non-wound/drop inoculation method which was applied to green berries did not show any difference among *Colletotrichum* groups in contrast to red berries. In the wound/drop inoculation method, using both green and red berries, *Colletotrichum* Group 3 had a higher incidence of disease among the groups. *Colletotrichum kahawae* produced symptoms more rapidly than the other isolates both in non-wound/drop and wound/drop methods and resulted in sunken lesions on the berry 6-10 days after inoculation (data not shown).

Table 3.5 Summary of the disease incidence of *Colletotrichum* isolates.

Species	Isolate	Non-Wound/drop		Wound/drop	
		Green (%)	Red (%)	Green (%)	Red (%)
Colletotrichum	BML-I6	0	25.57	0	42.33
Group 1	BML-I15	19.10	60.27	54.57	54.43
	BPD-I2	11.13	51.23	46.37	53.80
	Mean	**10.08 ± 15.50 a**	**45.69 ± 19.52 b**	**33.64 ± 25.52 a**	**50.19 ± 8.10 a**
Colletotrichum	BML-I3	26.30	8.93	17.37	42.57
Group 2	BML-I14	0	25.47	43.30	46.60
	BPD-I14	0	41.10	0	100
	Mean	**8.77 ± 17.45 a**	**25.17 ± 16.52 a**	**20.22 ± 24.41 a**	**63.06 ± 29.28 a**
Colletotrichum	BPD-I12	16.03	51.27	51.10	100
Group 3	BPD-I16	0	100	22.97	100
	BPD-I18	0	85.47	52.13	69.50
	Mean	**5.34 ± 16.03 a**	**78.91 ± 25.06 c**	**42.07 ± 17.50 a**	**89.93 ± 20.17 b**

Table 3.5 Summary of the disease incidence of *Colletotrichum* isolates (Cont.).

Species	Isolate	Non-Wound/drop		Wound/drop	
		Green (%)	Red (%)	Green (%)	Red (%)
	IMI 319418	100	100	100	100
C. kahawae	IMI 363578	100	100	100	100
	Mean	**100 ± 0 b**	**100 ± 0 d**	**100 ± 0 b**	**100 ± 0 b**

*The mean difference is significant at the 0.05 level; values with same letter based on Duncan's multiple range tests in a column do not differ significantly.

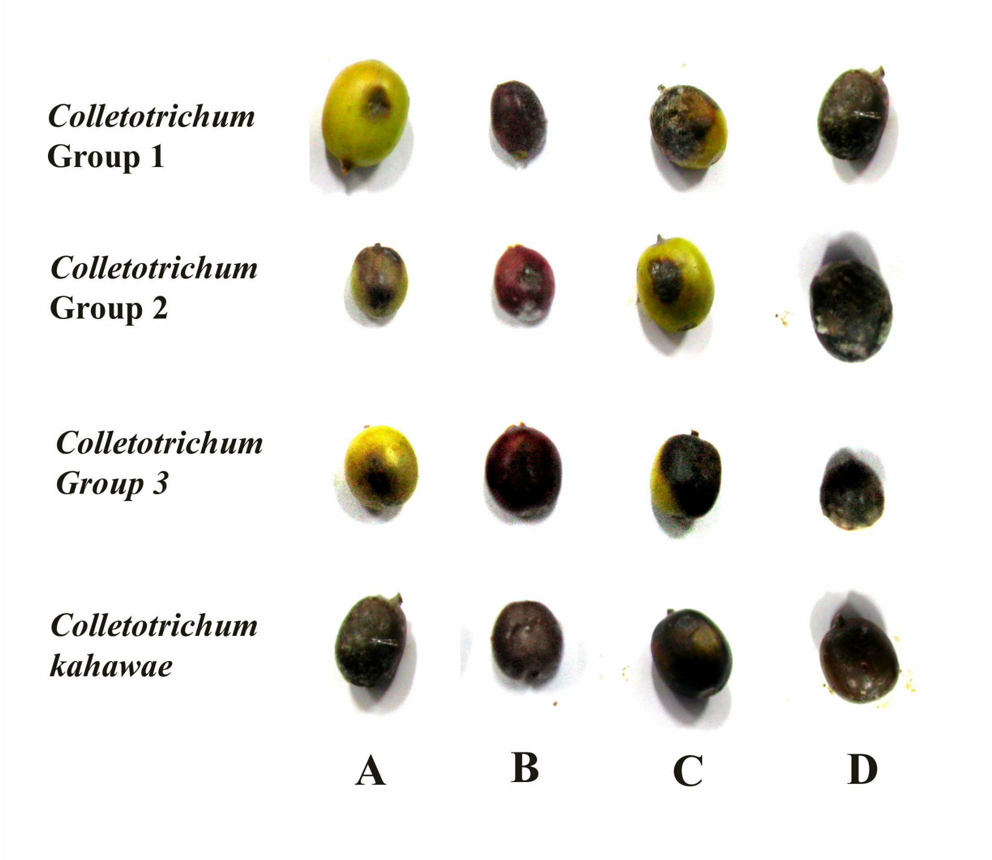

Figure 3.3 Pathogenicity testing of *Colletotrichum* isolates. (A) Non-wound/drop method on green berries; (B) Non-wound/drop method on red berries; (C) Wound/drop method on green berries; (D) Wound/drop method on red berries.

3.4.5 Phylogenetic study

The phylogeny was estimated using sequence data from the Actin, partial β-tubulin-2 (tub2), Calmoudulin, Glutamine synthetase, Glyceraldehyde-3-Phosphate Dehydrogenase, the complete rDNA-ITS regions and combined datasets of those genes. Datasets were obtained for the representative 9 taxa isolated from coffee berries from Chiang Mai, Thailand and the 6 epitype isolates. One thousand maximum parsimony bootstrap replicates were performed on the tree's internal nodes and the most parsimonious tree was generated from weighted parsimony, treating gaps as missing data (Figure 3.4 - 3.10).

The actin gene comprised 494 characters after alignment by Clustal X, of which 58 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that eight trees were not significantly different. One of the most eight parsimonius trees (TL = 71, CI = 0.972, RI = 0.976, RC = 0.949, HI = 0.028) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.4. The coffee berry isolates represented in Group 1, 2 and 3 revealed a monophyletic clade with 52% bootstrap. *Colletotrichum* Group 1 showed ambiguity in placement which is shown in node 51% bootstrap.

The partial β-tubulin-2 (tub2) gene comprised 506 characters after alignment by Clustal X, of which 95 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that 140 trees were not significantly different. One of the most 140 parsimonius trees (TL = 117, CI = 0.957, RI = 0.964, RC = 0.923, HI = 0.043) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.5. All representative *Colletotrichum* isolates from Chiang Mai, Thailand formed high bootstrap supported monophyletic clade (88%). On the other hand, BPD-I4 isolate clustered with each other as sister group of other members of the Group 2 (Figure 3.5).

The calmoudulin gene comprised 838 characters after alignment by Clustal X, of which 193 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that three trees were not significantly different. One of the most three parsimonius trees (TL = 262, CI = 0.916, RI = 0.937, RC = 0.858, HI = 0.084) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.6. The phylogeny generated from this gene revealed associations of *Colletotrichum* groups isolated from coffee berry. All subclades had high bootstrap support (>98%) which reflected homology within group. On the other hand, BML-I6 isolate clustered with each other as sister group of other members of the Group 1 (Figure 3.6).

The glutamine synthetase gene comprised 1123 characters after alignment by Clustal X, of which 360 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that two trees were not significantly different. One of the most two parsimonius trees (TL = 554, CI = 0.865, RI = 0.881, RC = 0.761, HI = 0.135) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.7. The phylogenetic placement of *Colletotrichum* groups, *C. kahawae* and *C. gloeosporioides* from this single tree revealed monophyletic group with high bootstrap support (100%).

The glyceraldehyde 3-phosphate dehydrogenase gene comprised 185 characters after alignment by Clustal X, of which 94 characters parsimony informative were included.

Kishino-Hasegawa (KH) test showed that four trees were not significantly different. One of the most four parsimonius trees (TL = 139, CI = 0.921, RI = 0.933, RC = 0.859, HI = 0.079) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.8. The coffee berry isolates represented in Group 1, 2 and 3 revealed a monophyletic clade with high bootstrap support (92%).

The complete rDNA-ITS region comprised 630 characters after alignment by Clustal X, of which 40 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that two hundreds and twenty five trees were not significantly different. One of the most 225 parsimonius trees (TL = 51 CI = 0.882, RI = 0.918, RC = 0.810, HI = 0.118) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.9. This analysis revealed monophyletic groups among *Colletotrichum* species from coffee berries, *C. gloeosporioides* and *C. kahawae* (100% bootstrap support).

The combined datasets of actin, partial β-tubulin-2 (tub2), calmoudulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase and the complete rDNA-ITS region comprised 2949 characters after alignment by Clustal X, of which 351 characters parsimony informative were included. Kishino-Hasegawa (KH) test showed that two trees were not significantly different. One of the most two parsimonius trees (TL = 518, CI = 0.809, RI = 0.845, RC = 0.684, HI = 0.191) generated from weighted parsimony and treating gaps as missing data is shown in Figure 3.10. The phylogram constructed using combined dataset showed that coffee berries isolates represented in Groups 1, 2 and 3 clustered into 3 distinct clades with high bootstrap support (>98%). Groups 1, 2 and 3 were distinct clade from the type species of *C. acutatum*, *C. gloeosporiodes* and *C. kahawae* which indicate that they are new species and recorded in the present study. Furthermore, the combination of these genes revealed that *C. gloeosporioides* and *C. kahawae* are distinct species which is supported by high bootstrap confidence (100%).

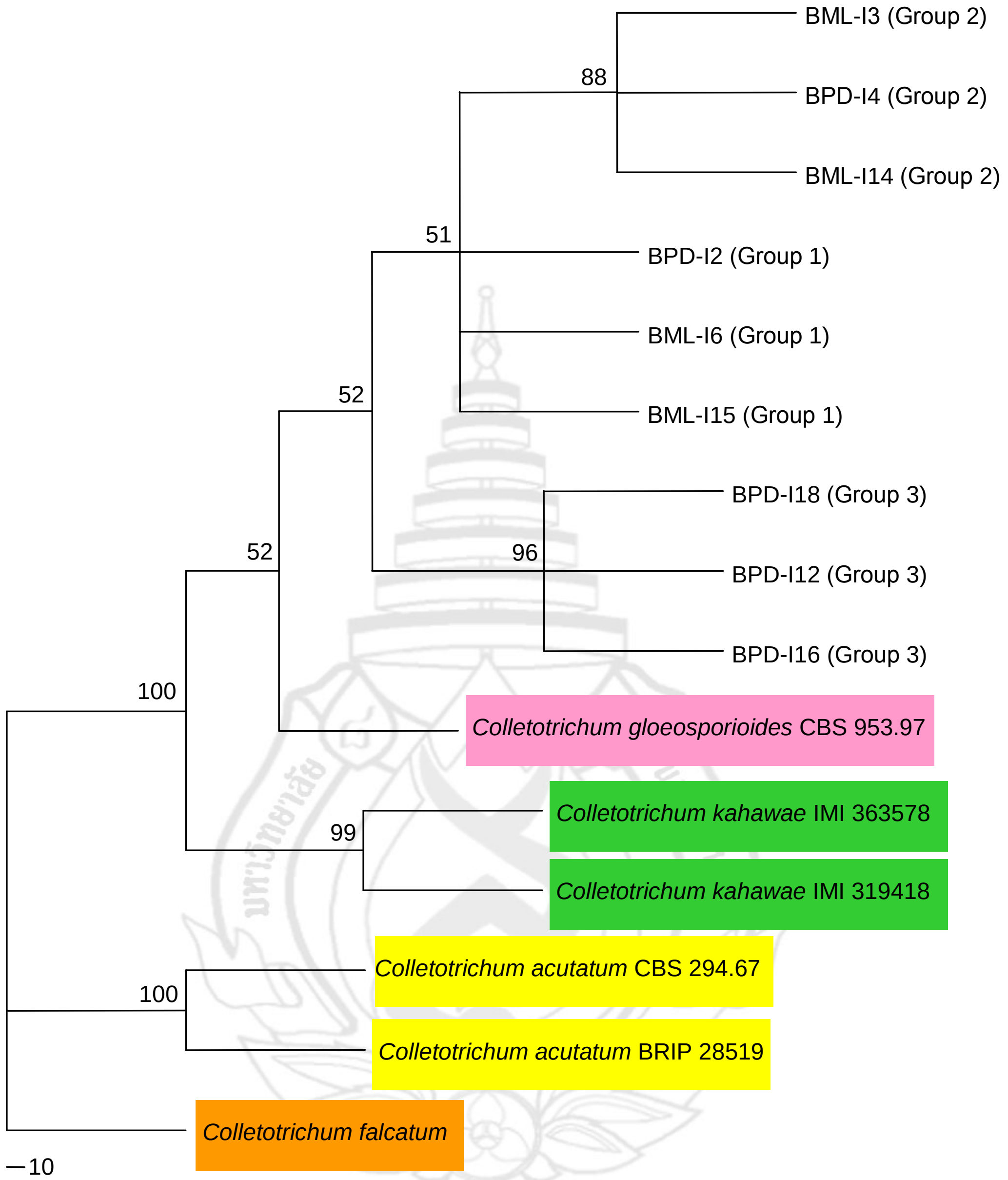

Figure 3.4 Phylogenetic tree generated from parsimony analysis based on actin sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from 1000 replicates (equal or above 50%). Bar = 10 % sequence divergence.

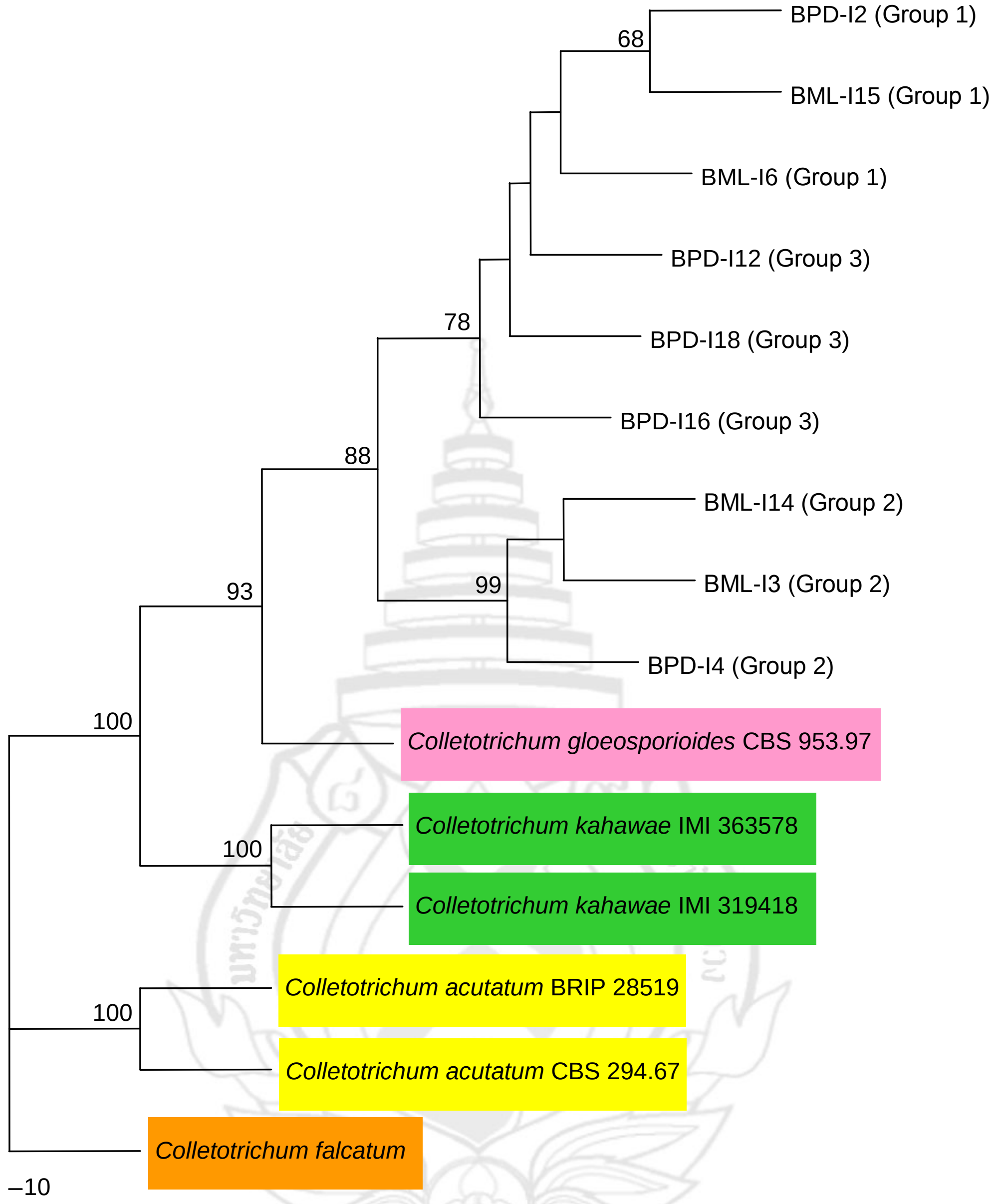

Figure 3.5 Phylogenetic tree generated from parsimony analysis based on partial β-tubulin-2 (tub2) sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from 1000 replicates (equal or above 50%). Bar = 10 % sequence divergence.

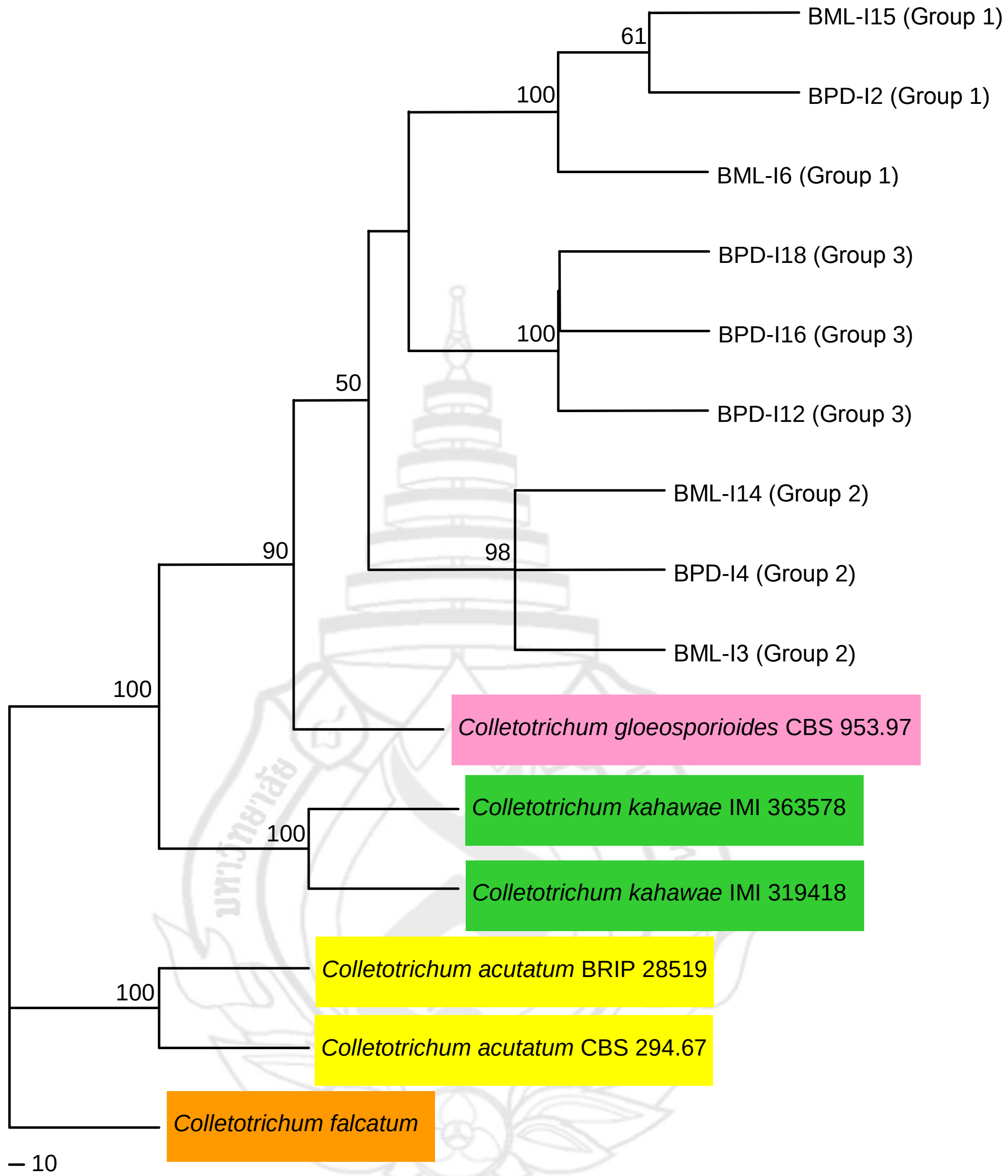

Figure 3.6 Phylogenetic tree generated from parsimony analysis based on calmoudulin sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from 1000 replicates (equal or above 50%). Bar = 10 % sequence divergence.

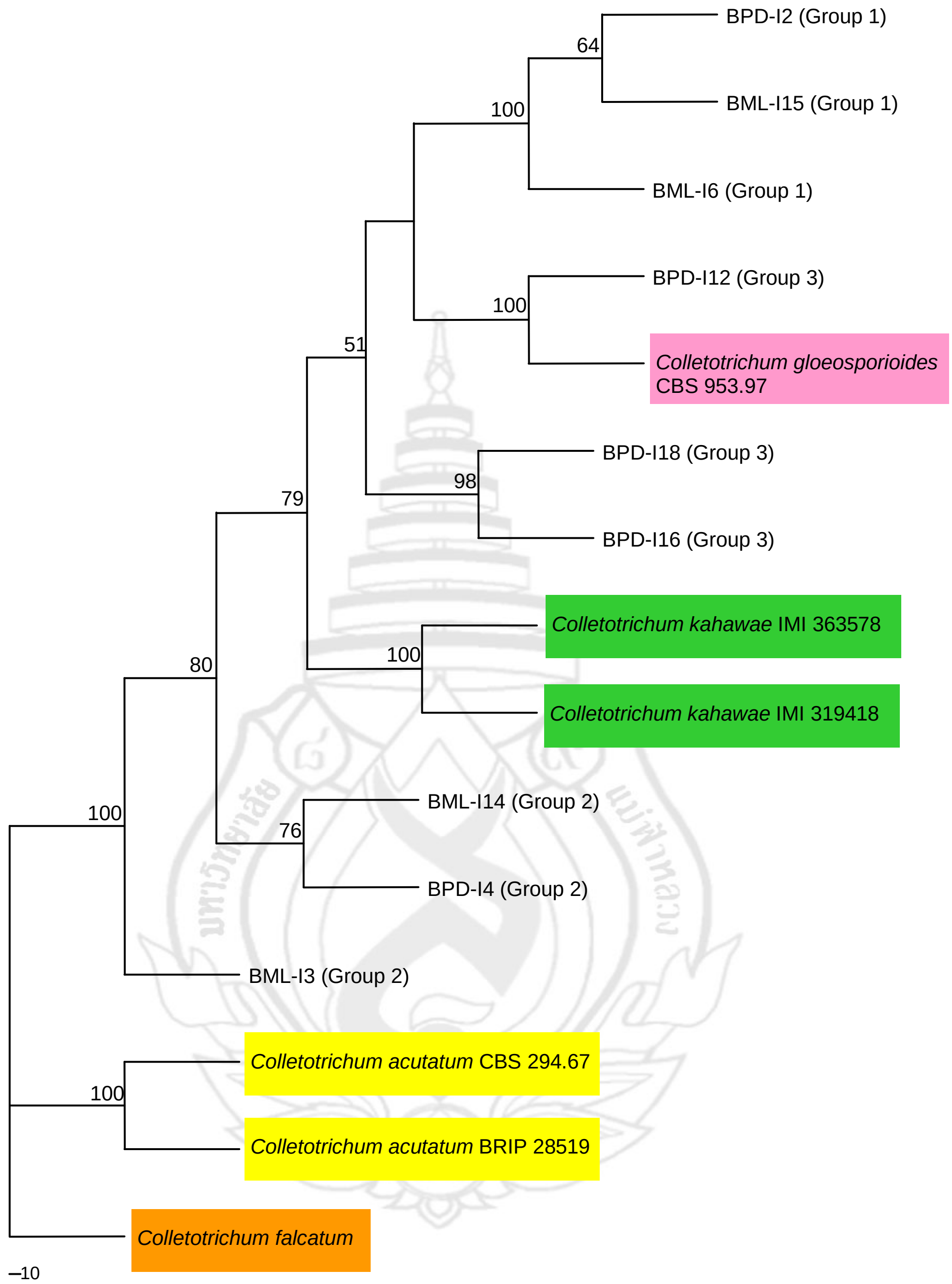

Figure 3.7 Phylogenetic tree generated from parsimony analysis based on glutamine synthetase sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from 1000 replicates (equal or above 50%). Bar = 10 % sequence divergence.

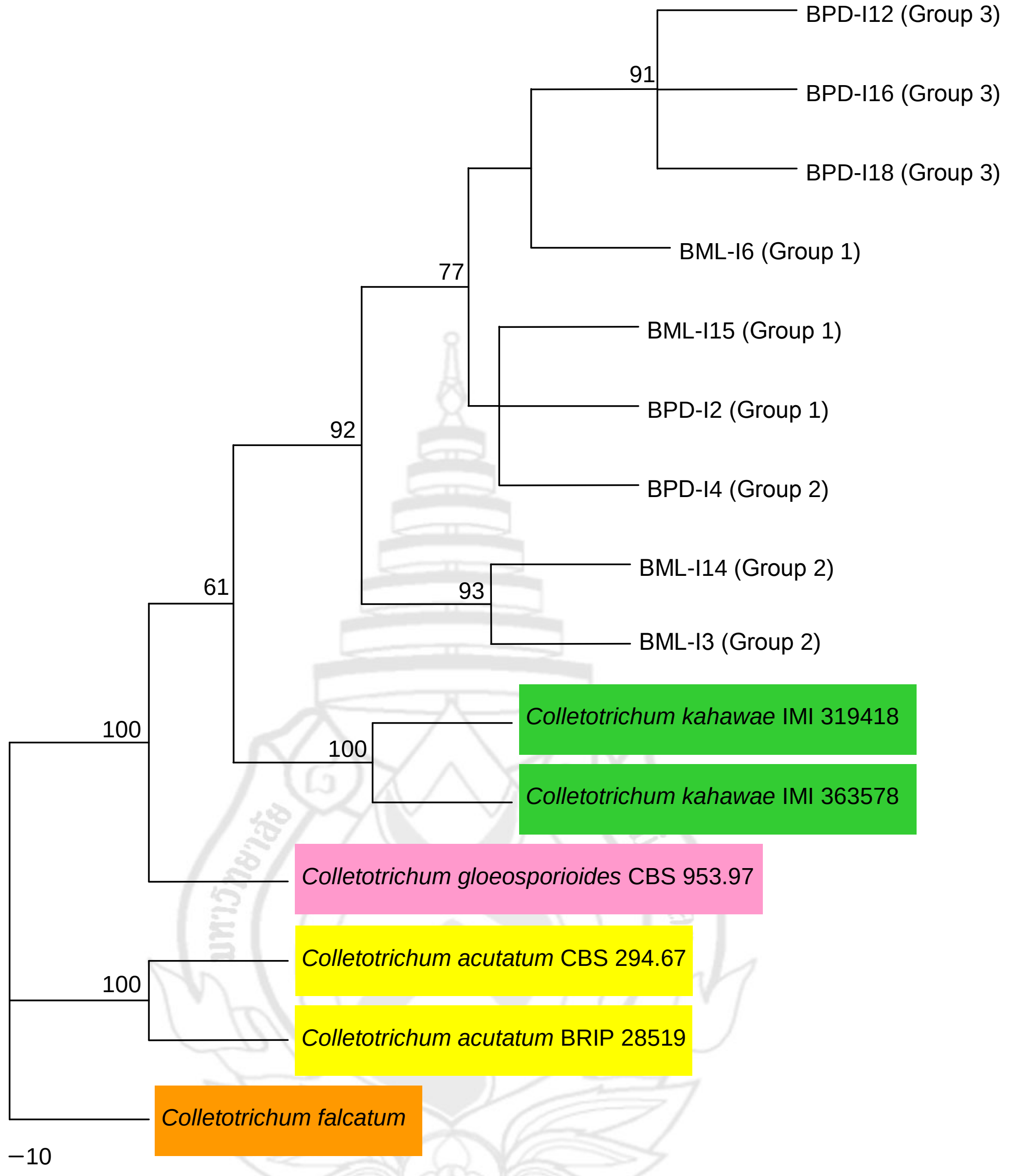

Figure 3.8 Phylogenetic tree generated from parsimony analysis based on glyceraldehyde 3-phosphate dehydrogenase sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from (equal or above 50%). Bar = 10 % sequence divergence.

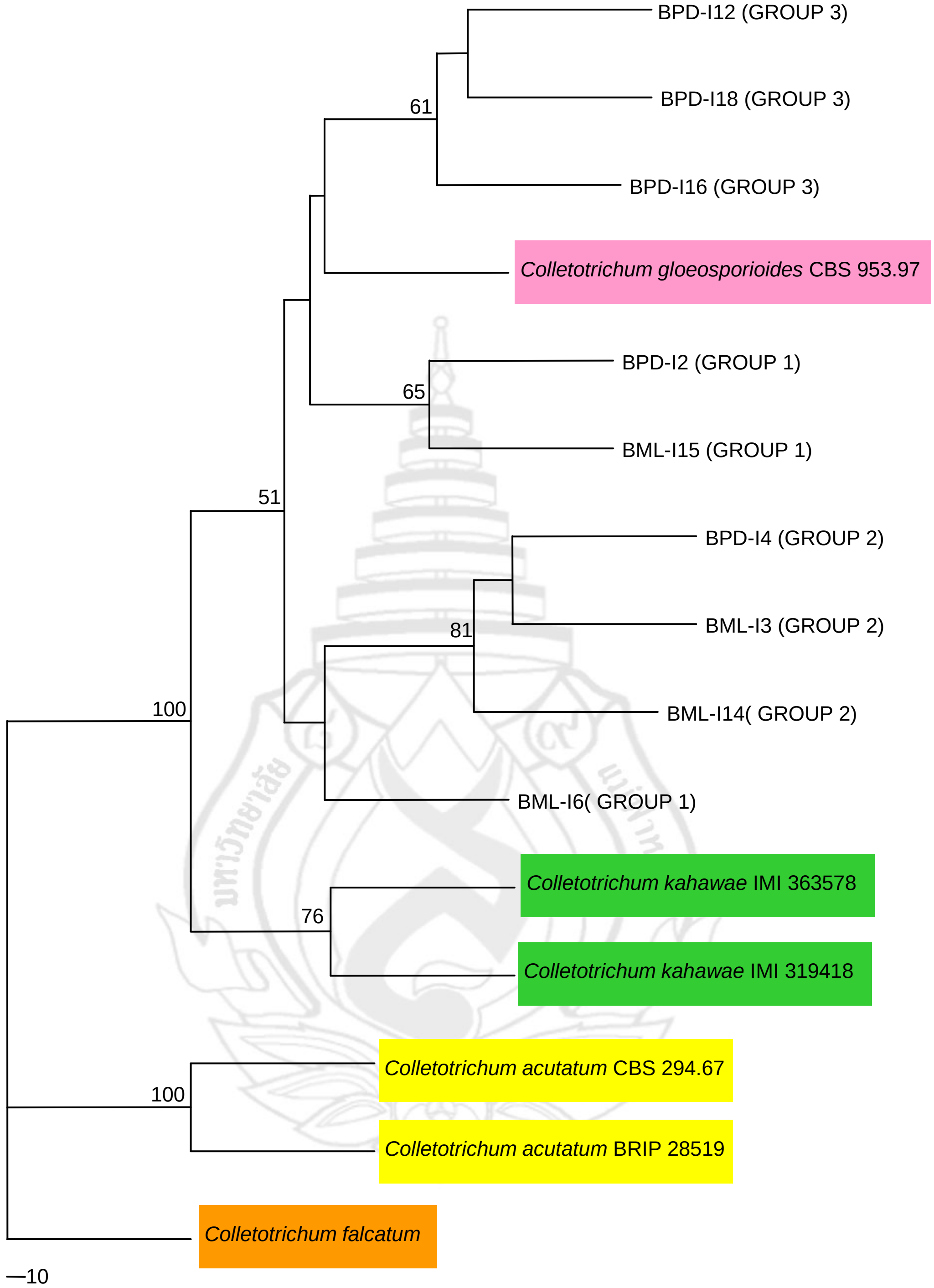

Figure 3.9 Phylogenetic tree generated from parsimony analysis based on the complete rDNA-ITS region sequences, with *C. falcatum* as the outgroup. Values above the branching nodes indicate percentage bootstrap support calculated from 1000 replicates (equal or above 50%). Bar = 10 % sequence divergence.

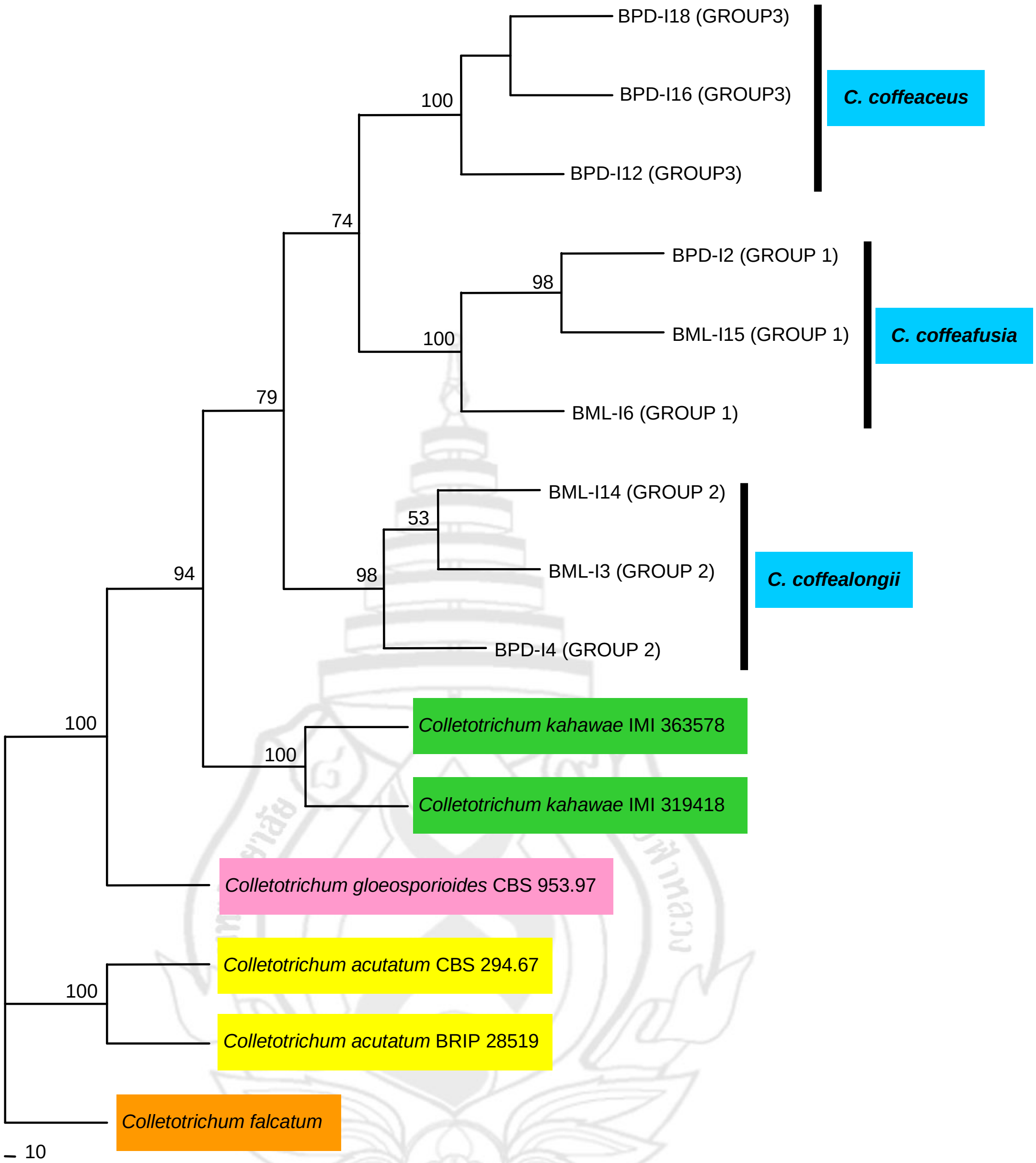

Figure 3.10 Phylogenetic tree generated from parsimony analysis based on the combined of Actin, partial β-tubulin-2 (tub2), Calmoudulin, Glutamine synthetase, Glyceraldehyde-3-Phosphate Dehydrogenase and the complete rDNA-ITS region, with *C. falcatum* as the outgroup. Values above branching node indicate percentage bootstrap support calculated from (equal or above 50%). Bar = 10 % sequence divergence.

3.4.6 Taxonomy

Three new species of *Colletotrichum* were recorded in the present study, of which *Colletotrichum asianum*, *C. coffeae* and *C. coffeigenum* are introduced. The type species of *C. gloeosporioides* and *C. kahawae* were included for comparison.

Colletotrichum asianum Prihastuti, L. Cai and K.D. Hyde, sp. nov. (Figure 3.11)

Etymology: *asianum*, in reference to the habitat where the type was found.

Description: Colonies on PDA at first greenish-white and becoming grayish-green to dark green at the centre with age, reverse dark green at the centre, max. attaining 42 mm radial diam. in 7 days at 28°C, growth rate 4.67-5.5 mm/day ($\overline{x}$ = 5.20 ± 0.31, n = 9) (Figure 3.11). Aerial mycelium in small tufts, white, sparse, with orange to dark orange visible conidial masses. *Sclerotia* absent. *Acervuli* absent in culture. *Setae* absent. *Conidia* 8.7-20.3 x 3–4.7 µm ($\overline{x}$ = 12.87 ± 2.52 x 3.40 ± 0.46, n = 180), common both in mycelium and conidial masses, one-unicellular celled, smooth-walled, guttulate, hyaline, cylindrical with obtuse ends (oblong) with narrowing to slight narrowing at the center. *Appressoria* 4.7-10.7 x 3.3-6.7 µm ($\overline{x}$ = 7.67 ± 1.72 x 5.07 ± 0.96, n = 30) in slide cultures, mostly formed from conidia, brown to dark brown, ovoid, clavate and slightly irregular to irregular in shape and often becoming complex with age.

Teleomorph: not produced in culture (during this study).

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti. (MFU 090232).

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (paratype in MFU 090233); THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (paratype in MFU 090234).

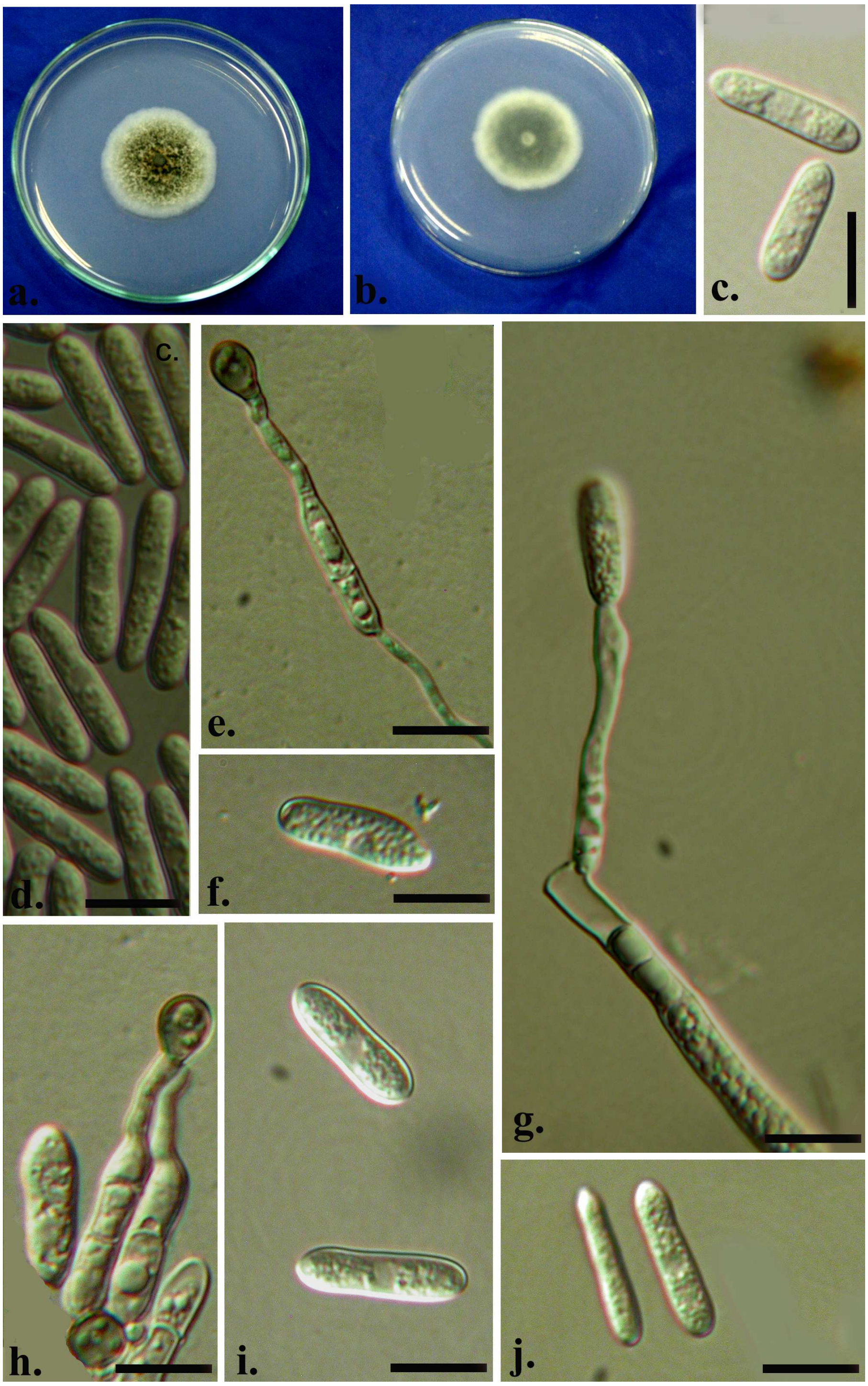

Figure 3.11 *Colletotrichum asianum* (from holotype). Upper (A) and reverse (B) sides of cultures on PDA seven days after inoculation; (c, d, f, i, j) conidia; (e and h) appressoria; (g) conidiogenous cell (Bars = 10 µm).

Colletotrichum coffeae Prihastuti, L. Cai and K.D. Hyde, sp. nov. (Figure 3.12)

Etymology: *coffeae*, in reference to the habitat where the type was found.

Description: Colonies on PDA at first white and becoming pale brownish to pinkish, reverse pale yellowish to pinkish colonies, max. attaining 82 mm radial diam. in 7 days at 28°C, growth rate 6.58–11.5 mm/day ($\bar{x}$ = 9.30 ± 1.93, n = 9) (Figure 3.13). Aerial mycelia grayish-white, dense, cottony, with visible conidial masses at the inoculum point. *Sclerotia* present in some culture. *Acervuli* brown to dark brown, conspicuous for their brown setae. *Conidia* 7-18.3 x 3–4.3 μm ($\bar{x}$ = 10.18 ± 1.74 x 3.46 ± 0.36, n = 180), common in mycelium, one-celled, smooth-walled, guttulate, hyaline, fusiform with obtuse to slightly rounded ends, sometimes oblong. *Appressoria* 4.7-8.3 x 3.5-5 μm ($\bar{x}$ = 6.67 ± 1.05 x 4.13 ± 0.44, n = 30) in slide cultures, mostly formed from mycelia, brown, ovoid, sometimes clavate and often becoming complex with age.

Teleomorph: not produced in culture (during this study).

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti. (MFU 090229).

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (paratype in MFU 090230); THAILAND, Chiang Mai Province, Mae Taeng District, Mae Lod Village, Royal Agricultural Project Coffee, on berry of *Coffea arabica*, 16 January 2008, H. Prihastuti (paratype in MFU 090231).

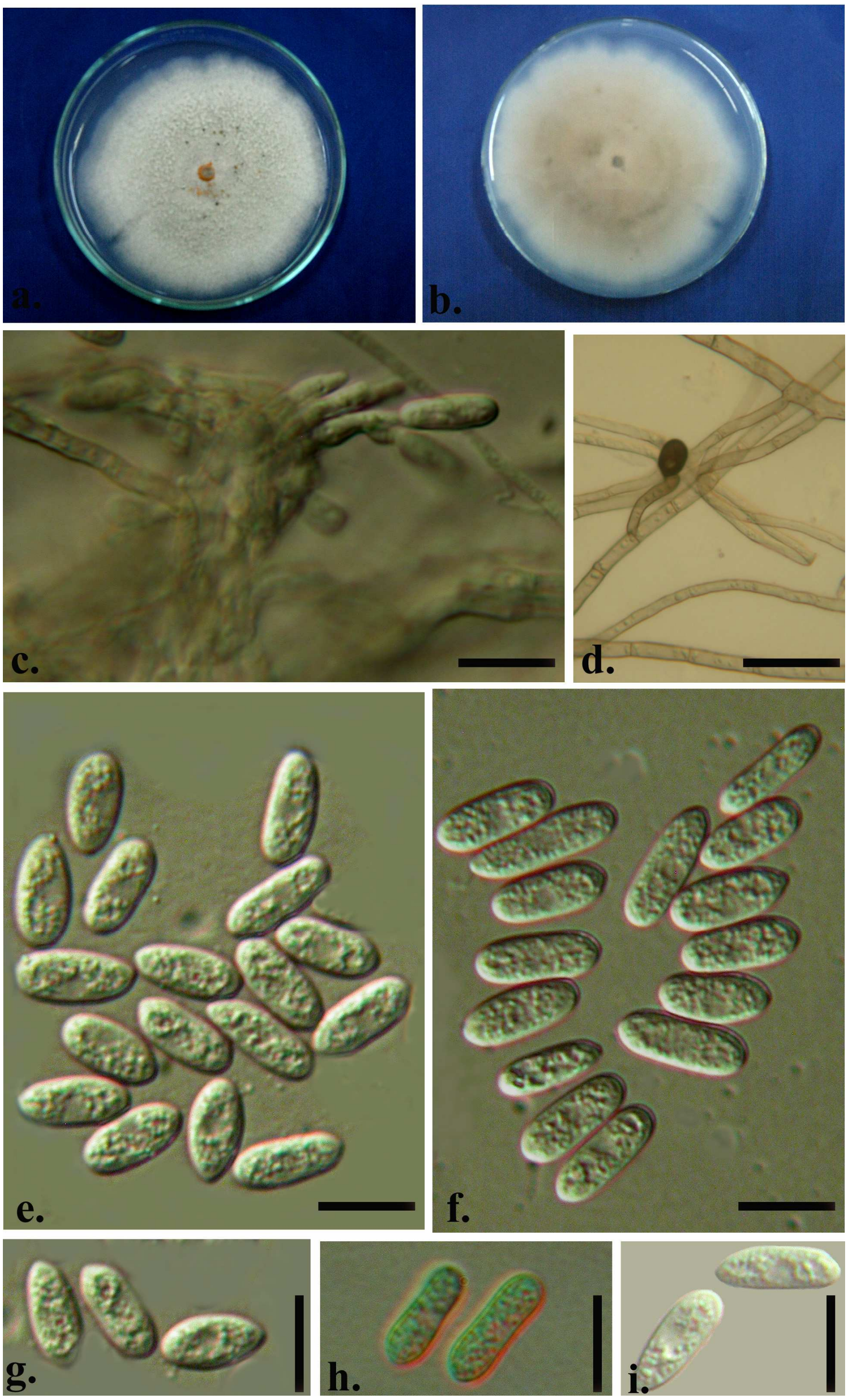

Figure 3.12 *Colletotrichum coffeae* (from holotype). Upper (A) and reverse (B) sides of cultures on PDA seven days after inoculation; (c) conidiogenous cell; (d) appressoria; (e – i) conidia (Bars = 10 μm).

Colletotrichum coffeigenum Prihastuti, L. Cai and K.D. Hyde, sp. nov. (Figure 3.13)

Etymology: *coffeigenum*, in reference to the habitat where the type was found.

Description: Colonies on PDA at first white and becoming grey to dark grey at the centre with age, dark circular around the growing margin at the centre in reverse, max. attaining 83 mm radial diam. in 7 days at 28°C, growth rate 10.58-11.5 mm/day ($\bar{x}$ = 11 ± 0.25, n = 9) (Figure 3.12). Aerial mycelia pale grey, dense, cottony, without visible conidial masses. *Sclerotia* absent. *Acervuli* absent in culture. *Setae* absent. *Conidia* 9.7-14 x 3-4.3 µm ($\bar{x}$ = 11.53 ± 1.03 x 3.55 ± 0.32, n = 180), common in mycelium, unicellular, smooth walled with a large guttulate at the centre and surrounded by smaller guttulates, hyaline, cylindrical with obtuse to slightly rounded ends, sometimes oblong. *Appressoria* 4.3-9.7 x 3.7-7.3 µm ($\bar{x}$ = 7.35 ± 1.28 x 4.49 ± 0.91, n = 30) in slide cultures, mostly formed from mycelia, brown to dark brown, ovoid, clavate and slightly irregular to irregular in shape and often becoming complex with age.

Teleomorph: *Glomerella* sp.

Ascoma 312-385 x 354–490 µm ($\bar{x}$ = 345.67 ± 36.83 x 431.33 ± 69.89, n = 10), light brown to brown, globose to subglobose, with hairs, semi-immersed or completely immersed on PDA. Peridium of *textura angularis*, thick-walled. *Asci* 30–55 x 6.5-8.5 µm ($\bar{x}$ = 41.22 ± 7.02 x 7.61 ± 0.58, n = 25), unitunicate, thin-walled, 6-8 spored, clavate or cymbiform,. *Ascospores* 9–14 x 3–4 µm ($\bar{x}$ = 11.91 ± 1.38 x 3.32 ± 0.35, n = 25), one-celled, hyaline, smooth walled with a large guttulate at the centre and surrounded by smaller guttulates, slightly curved to curved with obtuse to slightly rounded ends. Many formed within 3 months.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti. (MFU 090226).

Known distribution: Chiang Mai Province, Thailand.

Additional specimens examined: THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (paratype in MFU 090227); THAILAND, Chiang Mai Province, Mae Taeng District, Pha Daeng Village, near Mushroom Research Centre, on berry of *Coffea arabica*, 12 December 2007, H. Prihastuti (paratype in MFU 090228).

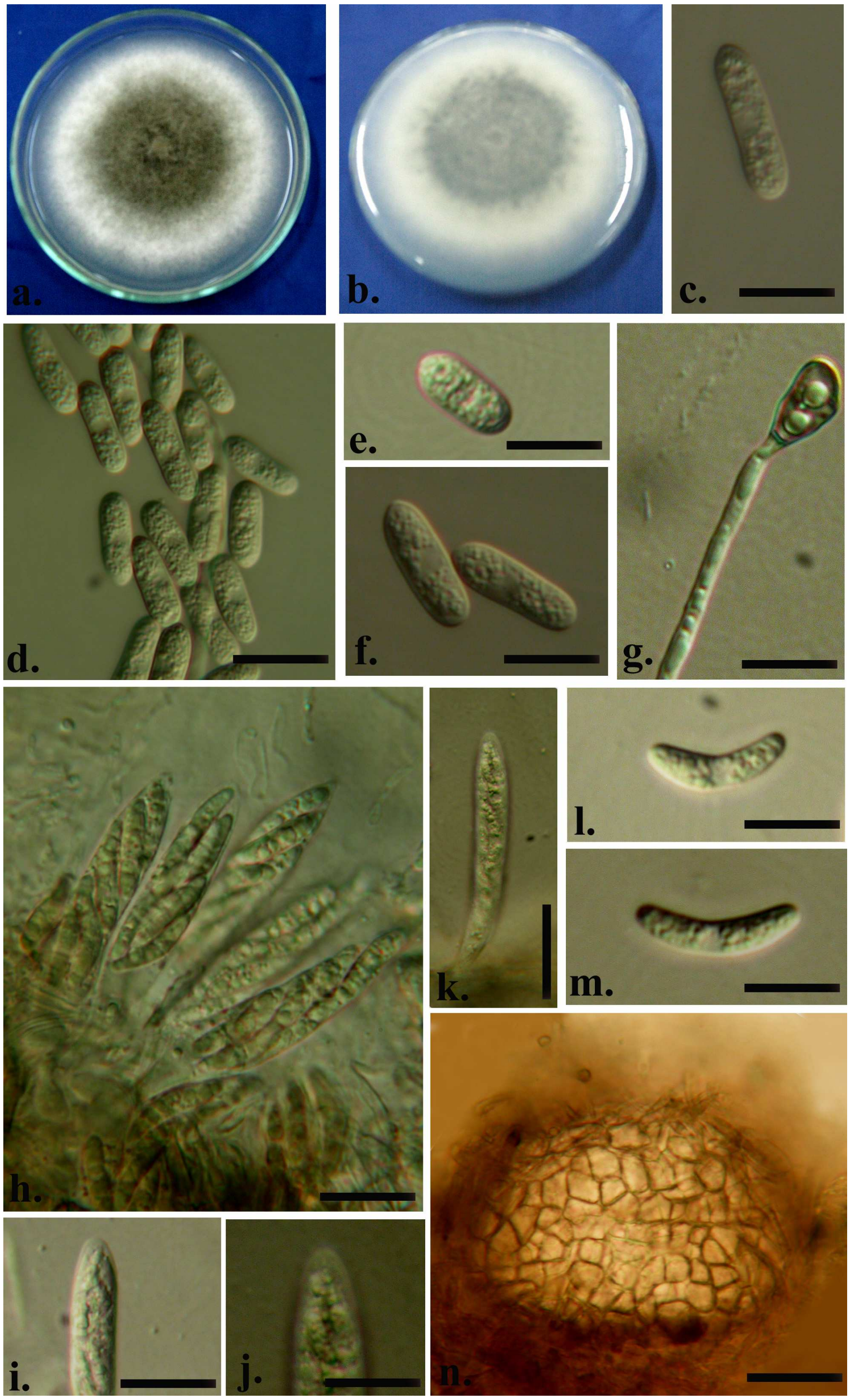

Figure 3.13 *Colletotrichum coffeigenum* (from holotype). Upper (A) and reverse (B) sides of cultures on PDA seven days after inoculation; (c - f) conidia; (g) appressoria; (h - k) asci; (l and m) ascospore; (n) ascomata (Bars: a-m = 10 µm, n = 100 µm).

3.5 Discussion

The main objective of this study was to characterize the species of *Colletotrichum* associated with coffee berries in Chiang Mai, Thailand, in addition to determine whether the various genes sequences were congruent with morphological characters. Phylogenies inferred from gene sequences support a close relationship of isolates with the same morphological characters in Groups 1, 2 and 3. This shows that DNA sequences analyses were useful for delimiting species of the genus *Colletotrichum*. DNA sequences analyses have therefore been suggested by various authors to overcome the inadequacies of morphological criteria (Sreenivasaprasad, *et al*., 1996; Abang, *et al*., 2002; Moriwaki, *et al*., 2002; Peres, *et al*., 2002; Guerber, *et al*., 2003; Photita, *et al*., 2005; Du, *et al*., 2005; Peres, *et al*., 2008; Shenoy, *et al*., 2007; Whitelaw-Weckert, *et al*., 2007; Than, *et al*., 2008a; Than, *et al*., 2008b, Crouch, *et al*., 2009).

3.5.1 Usefulness of characters used in *Colletotrichum* identification

Based on the shape and size of conidia the 34 isolates clustered into three morphogroups. The conidia size was statistically significant, both in length and wide of conidia. Differentiation among these *Colletotrichum* morphogroups therefore appears to be reliable based on traditional methods such as conidia shape and size. Similar results have been reported by Simmonds (1965), Sutton (1962, 1965, 1966, 1968, 1980) and Von Arx (1981).

The appressorial size data, both in terms of length and width showed that there was little distinction among the morphogroups. However, the appressorial shape was not different among the morphogroups in slide cultures. This shows that appressorial criteria are unreliable to distinguish *Colletotrichum* species. Similar results have been concluded that appressorial criteria are unreliable for *Colletotrichum* species differentiation (Sanders & Korsten, 2003; Than, *et al*., 2008a). However, appressorial characters were thought to warrant a more detailed examination (Simmonds, 1965).

Cultural characters and colony growth rates *in vitro* were one of the important characteristics for distinguishing between the three *Colletotrichum* morphogroups. Phylogenetic placement revealed a highly supported relationship between isolates with the same cultural characteristics and colony growth rates. Similar results have been reported for *Colletotrichum* by Than, *et al*. (2008a).

The inability of *C. kahawae* to utilize either citrate or tartrate as a sole carbon source provides a useful test to distinguish *C. kahawae* from the other closely related taxa of coffee. All *Colletotrichum* isolates from coffee berries in Chiang Mai, Thailand are distinct from *C. kahawae* because they produced a colour change either on citrate or tartrate. Several studies have reported that the inability of *C. kahawae* to utilize either citrate or tartrate may be indirectly related to pathogenicity, but may be directly related to a reduced saprobic capability (Waller, *et al*., 1993; Derso & Waller, 2003; Bridge, *et al*., 2008).

Pathogenicity testing using isolates from the three morphogroups of *Colletotrichum* and *C. kahawae* from infected coffee berries showed that all the isolates were pathogenic both in non-wound/drop and wound/drop inoculation of green and red berries. Waller, *et al*. (1993) have shown that *Colletotrichum kahawae* and *C. gloeosporioides* are distinct species that can be distinguished on the basis of the pathogenicity to green berries. The extent of lesion development may vary considerably with the interplay of factors, such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds, 1965; Freeman, *et al*., 1998). This result may not accurately reflect the true virulence potential.

The phylogenetic placement of *Colletotrichum* coffee berry isolates based on single genes resulted in different topology. This shows that single gene may be inadequate to identify the *Colletotrichum* species complex. The phylogenetic grouping based on the combined datasets were differentiated the coffee berry isolates into three highly-supported phylogenetic lineages which appears to be congruent with their morphological characters. Similar results have been reported from the *Colletotrichum* species complex associated with grasses (Crouch, *et al*., 2006). DNA barcoding analysis of combined multi-genes indicated that three new species occur on coffee in plantations in Chiang Mai, Thailand (Figure 3.10). Sreenivasaprasad, *et al*. (1993) showed that *C. kahawae* is very close to *C. gloeosporioides* on the basis of rDNA sequences. However, this was based on a single base difference in rDNA sequence data used to discriminate related taxa. Due to the limited number of informative sites identified the underlying difference is very small. Cannon, *et al*. (2008) showed that *C. kahawae* is belongs to the *C. gloeosporioides* aggregate based on ITS sequence analysis. The results presented here confer with Cannon, *et al*.

(2008) and demonstrate that multi-gene phylogenies can distinguish *C. kahawae* and *C. gloeosporioides* as distinct species. Crouch, *et al*. (2006) used data from multiple genes to attempt to estimate the limits of gene flow within and between populations of *Colletotrichum* species associated with grasses. Previously, *C. kahawae* was differentiated from *C. gloeosporioides* by morphological, biochemical and pathogenic characteristics (Waller, *et al*., 1993; Derso & Waller, 2003). Molecular techniques (e.g. restriction fragment length polymorphisms, variable number tandem repeats, amplified fragment length polymorphisms) can now be used to distinguish *C. kahawae* from other *Colletotrichum* species from tropical perennial crops (Martinez-Culebras, *et al*., 2003; Bridge, *et al*., 2008).

The taxonomy of most taxa within *Colletotrichum* has been based primarily upon variation in conidial size and shape, appressoria, and colony characters (Bailey & Jeger, 1992; Sutton, *et al*., 1992). These may not be accurate enough for species identification within *Colletotrichum*, which is vital for developing and implementing disease management, resistance breeding and pathogen control (Freeman, *et al*., 1998). A combination of molecular diagnostic tools with traditional morphological techniques is an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon, *et al*., 2000; Abang, 2003; Than, *et al*., 2008a).

3.5.2 Species of *Colletotrichum* known from coffee

The taxonomy of *Colletotrichum* is confused, both for the anamorphic species and its teleomorph *Glomerella*. Furthermore, accurate identification is important for breeding programs, developing effective control strategies and establishing cost effective quarantine programs (TeBeest, 1997; Sutton, 1992; Freeman, *et al*., 1998; Than, *et al*., 2008a). Therefore, in attempt to establish the accurate identification of *Colletotrichum* species associated with coffee berry from Chiang Mai, Thailand, this study has introduced three new species which can be identified based on morphological and cultural characters, biochemical, pathogenicity testing and phylogenetic analyses. A synopsis of species of *Colletotrichum* presently known from coffee is listed in Table 3.6. The following new species are introduced and justified in this chapter.

1. *Colletotrichum asianum* is similar to to *C. kahawae* in growth rate and colony colour. The conida shape of these species however is distinctly different (straight in *C.*

kahawae versus cylindrical with narrowing at the centre in *C. asianum*) (Table 3.6). Although these species are culturally similar, they differ both morphologically and genetically.

2. *Colletotrichum coffeae* may resemble to *C. acutatum* in cultural characters and conidia shape. The conidial of *C. coffeae* are fusiform, sometimes with obtuse slightly rounded ends. Generally, fusiform conidia are recognized in *C. acutatum* which have pinkish colonies. *Colletotrichum acutatum* has been recorded to be associated with coffee berries but *Colletotrichum coffeae* is introduced as a new species because it clustered in different clade from the type of *C. acutatum*.

3. *Colletotrichum coffeigenum* may resemble *C. coffeanum* Noack as they have overlapping conidia (12-18 x 4-5 μm in *C. coffeanum* versus 9.7–14 x 3–4.3 μm in *C. coffeigenum*). The conidia shape of these species however is distinctly different (ellipsoidal in *C. coffeanum* versus cylindrical in *C. coffeigenum*) (Table 3.6). It is also impossible to obtain a fresh culture of *C. coffeanum* and establish physiological and molecular data, therefore *Colletotrichum coffeigenum* is introduced as a new species.

Phylogenetically: *Colletotrichum asianum*, *C. coffeae* and *C. coffeigenum* appeared to be most closely related to *C. kahawae*, this may be due to the fact that they were isolated from the same host. This suggests that host-specificity may be relevant to taxonomic placement of *Colletotrichum* species. Similar results have been reported for *C. gloeosporioides* from almond in Israel (Freeman, 2000). The combined datasets of Actin, partial β-tubulin-2 (tub2), Calmoudulin, Glutamine synthetase, Glyceraldehyde-3-Phosphate Dehydrogenase and the complete rDNA-ITS region of three new species associated with coffee, however show them to be distinctly different from other species of *Colletotrichum*, and this is congruent with their morphological characters. Further studies, therefore, are needed to resolve the inter-specific relationships in the genus of *Colletotrichum* for establishing their correct taxonomy.

Table 3.6 Species of *Colletotrichum* known from coffee.

Characters	***Colletotrichum coffeanum***	***Colletotrichum coffaeophilum***	***Colletotrichum kahawae***	***Colletotrichum gloeosporioides***	***Colletotrichum asianum***	***Colletotrichum coffeae***	***Colletotrichum coffeigenum***
Colony	Undescribed	Undescribed	Colonies on PDA at first grey and becoming grey to dark olivaceous grey, dark greenish in reverse, max. attaining 32 mm radial diam. in 7 days at 28°C.	Colonies on PDA at first grey and becoming dark grey to black, black circular zones in reverse, max. attaining 83 mm radial diam. in 7 days at 28°C.	Colonies on PDA at first greenish-white and becoming grayish-green to dark green at the centre with age, dark green at the centre in reverse, max. attaining 42 mm radial diam. in 7 days at 28°C.	Colonies on PDA at first white and becoming pale brownish to pinkish, pale yellowish to pinkish colonies in reverse, max. attaining 82 mm radial diam. in 7 days at 28°C.	Colonies on PDA at first white and becoming grey to dark grey at the centre with age, dark circular around the growing margin at the centre in reverse, max. attaining 83 mm radial diam. in 7 days at 28°C.

Table 3.6 Species of *Colletotrichum* known from coffee (Cont.).

Characters	*Colletotrichum coffeanum*	*Colletotrichum coffaeophilum*	*Colletotrichum kahawae*	*Colletotrichum gloeosporioides*	*Colletotrichum asianum*	*Colletotrichum coffeae*	*Colletotrichum coffeigenum*
Conidia	Oblong and often curved, hyaline (12-18 x 4-5 µm).	Ellipsoidal, 1 guttulate, hyaline (13-15 x 6-8 µm).	Straight, cylindrical, invariably guttulate, obtuse at the apex (7.5-17 x 3.5-5 µm).	Cylindrical with obtuse end and slightly tapered, sometimes slightly rounded ends to oblong (8-11 x 3-4.5 µm).	Cylindrical with obtuse ends (oblong) with narrowing to slight narrowing at the center, one-unicellular celled, smooth walled, guttulate, hyaline (8.7-20.3 x 3–4.7 µm).	Fusiform, sometimes with obtuse to slightly rounded ends, sometimes oblong, one-unicellular celled, smooth walled, guttulate, hyaline (7-18.3 x 3–4.3 µm).	Cylindrical with obtuse to slightly rounded ends, sometimes oblong, one-unicellular celled, large guttulate at the centre and surrounded by small guttulate, hyaline (9.7-14 x 3-4.3 µm).

Table 3.6 Species of *Colletotrichum* known from coffee(Cont.).

Characters	***Colletotrichum coffeanum***	***Colletotrichum coffaeophilum***	***Colletotrichum kahawae***	***Colletotrichum gloeosporioides***	***Colletotrichum asianum***	***Colletotrichum coffeae***	***Colletotrichum coffeigenum***
Appressoria	Undescribed	Undescribed	Circular to slightly irregular, pale to medium brown, often becoming complex in slide cultures (4.5-10 x 4-7.5 μm).	Circular to slightly irregular, medium-brown to brown, sparse in slide cultures (3-5.1 x 2.1-3.3 μm)	Ovoid, clavate and slightly irregular to irregular in shape and often becoming complex with age, brown to dark brown, mostly formed from conidia in slide cultures (4.7-10.7 x 3.3-6.7 μm).	Ovoid, sometimes clavate and often becoming complex with age, brown, mostly formed from mycelia in slide cultures (4.7-8.3 x 3.5-5 μm).	Ovoid, clavate and slightly irregular to irregular in shape and often becoming complex with age, brown to dark brown, mostly formed from mycelia in slide cultures (4.3-9.7 x 3.7-7.3 μm).

Table 3.6 Species of *Colletotrichum* known from coffee (Cont.).

Characters	***Colletotrichum coffeanum***	***Colletotrichum coffaeophilum***	***Colletotrichum kahawae***	***Colletotrichum gloeosporioides***	***Colletotrichum asianum***	***Colletotrichum coffeae***	***Colletotrichum coffeigenum***
Teleomorph	Unknown	*Glomerella cingulata*	Unknown	*Glomerella cingulata*	Unknown	Unknown	*Glomerella* sp.
Substrate	Twigs and leaves	Undescribed	Berries	Leaves	Berries	Berries	Berries
Host	Coffee (*Coffea arabica*)	Coffee (*Coffea* sp, Rubiaceae)	Coffee (*Coffea arabica*)	Citrus (*Citrus sinensis*)	Coffee (*Coffea arabica*)	Coffee (*Coffea arabica*)	Coffee (*Coffea arabica*)
Origin Country	Brazil	Tuis, Costarica, America	Kenya	Italy	Chiang Mai	Thailand	Chiang Mai, Thailand
Material examined	F. Noack (1901)	C. L. Spegazzini (1919)	IMI 319418, IMI 363578	CBS 953.97	Chiang Mai isolates	Chiang Mai isolates	Chiang Mai isolates

CHAPTER 4

CONCLUSIONS

4.1 Overall conclusions

This study was performed to examine epiphytic and endophytic fungal communities associated with coffee berries of Arabica coffee in Chiang Mai, Thailand. A total of 352 fungal strains were obtained and could be grouped into 12 genera. Two genera were found only in Pha Daeng village, and *Moniliopthora* is a first record from a coffee plantation. The results show that variation of the fungal diversity between epiphytic and endophytic communities is insignificant ($0.05 < P < 0.10$). Two sites were investigated and it was found that fungal diversity in Pha Daeng village was significantly higher than that in Mae Lod village ($P < 0.001$). Epiphytic and endophytic fungi probably interact in ways that affect the host plant. Future studies are needed to enable a better understanding of interactions within and between these communities.

The characterization of *Colletotrichum* species associated with coffee berries was further studied using combination of morphological and cultural characters, biochemical, pathogenicity testing and DNA barcoding clustered the isolates into three morphogroups of *Colletotrichum*. *Colletotrichum asianum*, *C. coffeae* and *C. coffeigenum* are introduced as new species. This study showed that host-specificity may be relevant to taxonomic placement of *Colletotrichum* species. Therefore, the complexity related to host-specificity and host-range in *Colletotrichum* must be determined for each host at every given location.

Pathogenicity studies of selected *Colletotrichum* isolates from coffee berries resulted in fruit infection. The experiments were carried out under experimentally controlled conditions in the laboratory, thus these results may not accurately reflect their virulence potential. Future studies should attempt to determine pathogenicity of *Colletotrichum* according to natural infections rather than artificial inoculations.

The analysis of the combined multi-genes sequences datasets confirmed that the three new species associated with coffee berries are distinctly different from other species of *Colletotrichum* and congruent with their morphological characters. The taxonomy of *Colletotrichum* species is in a state of constant flux and remains unclear. The use of classical morphology and molecular tools may contribute to the understanding of complexity in *Colletotrichum*. Future studies, therefore, are needed to resolve the inter-specific relationships in the genus of *Colletotrichum* for establishing their correct taxonomy.

Since the PCR amplification methods are relatively simple, this approach should therefore be improved by exploring of further genes, designing appropriate primers and new species-specific primers of unique DNA fragments. This seems to be the most promising for differentiating the species of *Colletotrichum* in the future.

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APPENDIXES

APPENDIX A

REFERENCE SEQUENCE FROM GENBANK USED IN ANALYSIS

Colletotrichum Species	Isolates	Culture Collection	GenBank Accession Number					
			ACT	TUB-2	CAL	GS	GDPH	ITS
C. asianum	BML-I3	MFU 090232*	FJ 903188	FJ 907434	FJ 917501	Submitted	Submitted	Submitted
C. asianum	BPD-I4	MFU 090233	FJ 907424	FJ 907439	FJ 917506	Submitted	Submitted	Submitted
C. asianum	BML-I14	MFU 090234	FJ 907421	FJ 907436	FJ 917503	Submitted	Submitted	Submitted
C. coffeae	BML-I6	MFU 090229*	FJ 907420	FJ 907435	FJ 917502	Submitted	Submitted	Submitted
C. coffeae	BPD-I2	MFU 090230	FJ 907423	FJ 907438	FJ 917505	Submitted	Submitted	Submitted
C. coffeae	BML-I15	MFU 090231	FJ 907422	FJ 907437	FJ 917504	Submitted	Submitted	Submitted
C. coffeigenum	BPD-I18	MFU 090226*	FJ 907427	FJ 907442	FJ 917509	Submitted	Submitted	Submitted
C. coffeigenum	BPD-I12	MFU 090227	FJ 907425	FJ 907440	FJ 917507	Submitted	Submitted	Submitted
C. coffeigenum	BPD-I16	MFU 090228	FJ 907426	FJ 907441	FJ 917508	Submitted	Submitted	Submitted

Colletotrichum Species	Isolates	Culture Collection	GenBank Accession Number					
			ACT	TUB-2	CAL	GS	GDPH	ITS
C. acutatum (type specimen)	BRIP 28519	BRIP 28519	FJ 907428	FJ 907443	FJ 917510	Submitted	Submitted	Submitted
C. acutatum (type specimen)	CBS 294.67	CBS 294.67	FJ 907429	FJ 907444	FJ 917511	Submitted	Submitted	Submitted
C. falcatum Epitype	FAL		FJ 907431		FJ 917513	Submitted	Submitted	Submitted
C. gloeosporioides (type specimen)	CBS 953.97	CBS 953.97	FJ 907430	FJ 907445	FJ 917512	Submitted	Submitted	Submitted
C. kahawae (type specimen)	IMI 319418	IMI 319418	FJ 907432	FJ 907446	FJ 917514	Submitted	Submitted	Submitted
C. kahawae (type specimen)	IMI 363578	IMI 363578	FJ 907433	FJ 907447	FJ 917515	Submitted	Submitted	Submitted

Note: ACT: actin; TUB-2: partial ß-tubulin-2 (tub2); CAL: calmoudulin; GS: glutamine synthetase; GDPH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region; MFU: Mae Fah Luang University, Thailand; IFRD: International Fungal Research and Development Centre, China; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; IMI: CABI Europe – UK, Bakeham Lane, Egham, Surrey TW209TY, UK; *: holotype.

APPENDIX B

ABSTRACTS PERTAINING TO THESIS

Morphological and genetic diversity of *Colletotrichum* in Thailand

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Colletotrichum species infect several economically important crop plants and cause anthracnose which may cause heavy yield losses. We are studying the diversity of the genus *Colletotrichum* in Thailand. In one study we have isolated *Colletotrichum* species from coffee berry disease and anthracnose on chilli in Chiang Mai Province in northern Thailand. Four isolates of *C. acutatum*, two isolates of *C. gloeosporioides* and six isolates of *C. coffeanum* were isolated from coffee and identification confirmed based on morphological characters. We will also carry out phylogenetic analysis and pathogenicity testing. Chilli anthracnose was found to be caused by three species of *Colletotrichum* and this also will be reviewed. We are also interested in the genetic diversity *C. acutatum*. We isolated 60 strains from six hosts and are in the process of characterizing them morphologically. They will also be subjected to phylogenetic analysis and pathogenecity testing to establish which groups of *C. acutatum* occur in Thailand.

The 3rd Annual Meeting of Thai Mycological Association and
Mycology Conference in Thailand. 11 October 2008, p. 17.

Collection and Identification of *Colletotrichum* spp. Causing Anthracnose of Fruit crops in Laos.

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Abstract: *Colletotrichum* is an important plant pathogenic genus worldwide, affecting a wide host range, especially tropical and subtropical crops. *Colletotrichum* is main post harvest disease of fruit is anthracnose which can be found throughout the fruit storage period. Disease symptoms are commonly known as anthracnose. Sixty-nine strains of *Colletrotruchum* were isolated from fruit crops of Laos from the field and from markets. They were collected from Luangbrabang, Sayaboury (Northern Laos), Vientiane (Central Laos) and Savannaketh (Southern Laos). In this study we used morphological characteristics to identify species. Three species of *Colletotrichum* isolated are *C. acutatum*, *C. gloeosporioides* and *C. capsici* from seven hosts: chilli, guava, jujube, mango, papaya, rose apple and strawberry. Thirty-one were strains isolated from northern Laos, 46 from central Laos and 14 from southern Laos. Our next step will be focus on the genetic diversity and host specificity of *Colletrotrichum acutatum* in Laos and Thailand.

***Key words*: *Colletrotrichum*, Collection, Identification**

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APPENDIX C

CANDIDATE'S PUBLICATION LIST

Prihastuti, H., Cai, L. & Hyde, K.D. (2009). "Characterization of *Colletotrichum* species associated with coffee berries in Chiang Mai, Thailand" **Fungal Diversity**. Submitted.

Prihastuti, H. & Hyde, K.D. (2009). "Review: A history of *Colletotrichum*" **Fungal Diversity**. Submitted.

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Research Experience	Use of *Trichoderma* sp. and chitosan for inhibiting *Colletotrichum gloeosporioides* (penz.) Penz. & Sacc, the causative agent of anthracnose in sweet-oranges (*Citrus sinensis*). Bachelor Thesis. Department of Plant Protection. Brawijaya University. Indonesia.

Abstract

Prihastuti, H., Phoulivong, S., Than, P.P. & Hyde, K.D. (2008). "Morphological and genetic diversity of *Colletotrichum* in Thailand" **Abstract International Symposium on Fungal Diversity**. Hangzhou, China.

Phoulivong, S., Than, P.P., **Prihastuti, H**. & Hyde, K.D. (2008). "Collection and Identification of *Colletotrichum* spp. Causing Anthracnose of Fruit crops in Laos" **Abstract The 3rd Annual Meeting of Thai Mycological Association and Mycologgy Conference**. Khon Kaen, Thailand.

Publications

Than, P.P., **Prihastuti, H.**, Phoulivong, S., Taylor, P.W.J. and Hyde, K.D. (2008). Review: Chilli anthracnose disease caused by *Colletotrichum* species. Journal Zhejiang University 9 (10): 764-778. Doi: 10.1631/jzus.B0860007. [Online] Available: http://www.springerlink.com/content/w4887l2125j41686/; http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2565739.