Colletotrichum SPECIES CAUSING ANTHRACNOSE DISEASE IN LAOS AND THAILAND

SITTHISACK PHOULIVONG

DOCTOR OF PHILOSOPHY
IN
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Sitthisack Phoulivong

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ABSTRACT

Fruits and leaf samples exhibiting anthracnose disease were collected from different regions of Laos (i.e., Luang Prabang and Vientiane) and Thailand (i.e., Chiang Maiand Nakon Si Thammarat). In total, 236 strains were isolated and, based on morphological characteristics, they could be initially identified as *Colletotrichum gloeosporioides*. Molecular approaches were then used to confirm these fungal identities. The ITS results showed that the fungal isolateswere *C. gloeosporioides*. However, it is widely accepted that *C. gloeosporioides*is genetically diverse consisting of several species, the so-called "*C. gloeosporioides* species complex". Subsequent analyses using multi-genes (partial actin (ACT), β-tubulin (TUB2), glutamine synthetase gene (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region) were performed and our data revealed that the *C. gloeosporioides* strains were complex species. Interestingly, most of these fungal isolates (> 99%) did not group into the *C. gloeosporioides* clade represented by the *C. gloeosporioides* epitype. We also showed that the use of these five gene regions couldbe used to resolve the species identity of *C. asianum*, *C. fructicola*, *C. horii*, *C. kahawae* and *C. gloeosporioides* in the "gloeosporioides" complex as distinct phylogenetic lineages with high statistical support.

Cultural, conidial and appressorial characters can be used to differentiate taxa into species complexes, but cannot separate species within a complex. Four new species of *Colletotrichum* were discovered in this study based on distinct morphology as well as sequencing results. These new species include *C. brevisporum*, *C. codylinicola*, *C. tropicicola*, and *C. thailandicum*. In this study, all *Colletotrichum* strains were complex comprisingnine species namely, *C. asianum*, *C. brevisporum*, *C. cordylinicola*, *C. fructicola*, *C. gloeosporioides*, *C. siamense*, *C. tropicicola*, *C. thailandicum* and *C. simmondsii*. Pathogenicity testing was also used to determine whether the fungal isolates were host specific. Eleven strains of *Colletotrichum* representing five species were able to infect a broad range of hosts used. It can be concluded from the pathogenicity results that the fungal isolates of the *C. gloeosporioides* complex could cause a potential outbreak of anthracnose disease.

Keywords: Anthracnose/*Colletotrichum*/Identification/Multi-gene loci/
Pathogenicity/Taxonomy

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CHAPTER 1

INTRODUCTION

Colletotrichum is one of the most economically important pathogenic genera causing anthracnose of fruits and leaves, affecting a wide range of hosts in the tropics and subtropics (Sutton, 1992). The above-ground plant parts of crops as well as fruit trees can be affected by *Colletotrichum* anthracnose and in the case of fruit results in reduction in yield quantity or quality. *Collectotrichum* species have been reported to cause disease of many hosts in Thailand including chilli (*Capsicum* spp.), guava (*Psidium guajava*), jujube (*Zizyphus mauritiane*), mango (*Mangifera indica*), papaya (*Carica papaya*) and rose apple (*Eugenia Javanica*) (Freeman, Katan & Shabi, 1996). *Colletotrichum* species are cosmopolitan with either multiple species on a single host or a single species on multiple hosts (Sanders & Korsten, 2003). Fungus/host relationships are broad, imprecise and often overlapping (Freeman et al., 1996). *Colletotrichum* species can infect many hosts and may adapt to new environments (Sanders & Korsten, 2003), leading to serious cross infection problems in plant production. The study of pathogenic variability of *Colletotrichum* species is therefore important and the understanding of the host range of a particular pathogen may help in efficient disease control and management (Whitelaw-Weckert et al., 2007).

1.1 Historical Background of *Colletotrichum*

Colletotrichum was first reported by Tode (1790) in *Vermicularia*, but was later redescribed as *Colletotrichum* (Corda, 1837) in the order Melanconiales; class Coelomycetes; subdivision Deuteromycotina. *Colletotrichum* speciescomprise imperfect or asexual taxa which have a *Glomerella* teleomorph stage (Sutton, 1992). *Colletotrichum* comprises a number of endophytic, saprobic and plant pathogenic

species, the latter of worldwide importance on a wide range of economic crops and ornamentals (Cai et al., 2009; Hyde, Cai, Cannon et al., 2009; Hyde, Cai, McKenzie et al., 2009; Prihastuti, Cai, Chen, McKenzie & Hyde, 2009; Yang, Cai, Yu, Liu & Hyde, 2012, Yang, Liu, Cai & Hyde, 2012; Phoulivong, Cai, Chen et al., 2010).

The pathogenesis of *Colletotrichum* is diverse, arising from nutritional and ecological diversity within the genus, which varies from intracellular hemibiotrophy to subcuticular intramural or abiotrophic necrotrophy (Bailey & Jeger, 1992; Pring, Nash, Zakaria & Bailey, 1995). Specialized infection structures are produced by *Colletotrichum* species such as germ tubes, aprèssoria, intracellular hyphae and secondary necrotrophic hyphae (Perfect, Hughes, O'Connell & Green, 1999; Rojas, Rehner & Samuels, 2010). *Colletotrichum* infect hosts by either colonizing subcuticular tissues intramurally or being established intracellularly. The pre-infection stages of the both infection types in *Colletotrichum* are very similar, in which colonization of conidia of susceptible hosts included adhesion, germination, appressoria formation and penetration (Du, Schardl & Vaillancourt, 2005).

The pathogens colonize the intramural region beneath the cuticle, invading in a necrotrophic manner and spread rapidly throughout the tissues (O'Connell, Bailey & Richmond, 1985). There is no detectable biotrophic stage of parasitism form. In contrast, most anthracnose pathogens exhibit abiotrophic infection strategy initially by colonizing the plasmalemma and cell wall intracelularly. The biotrophic stage which is generally 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). *Colletotrichum* are regarded as hemiobiotrophs or facultative biotrophs (Kim, Lim, Kim, Kim & Kim, 2009). An example of hemiobiotrophy is found in infection of avocado, chili and citrus by putatively named strains of *C. gloeosporioides* which produce both intracellular biotrophy at an early stage and later intramural necrotrophy. Though, the infection process in *Colletotrichum* species is apparently similar in the prepenetration process, there are differences between species in the later process such as conidial adhesion, melanization and cutinizetion in penetration of the plant cuticle by the appressoria (Rojas et al., 2010; Dam, Cannon, Woudenberg & Crous, 2012).

1.2 Diversity and Modelife of *Colletotrichum* Species

Colletotrichum species such as *C. acutatum* J. H. Simmonds, *C. asianum* Prihastuti, L. Cai & K. D. Hyde, *C. boninense, C. fructicola* Prihastuti, L. Cai & K. D. Hyde, *C. gloeosporioides, C. siamense* and *C. yunnanense* Xiao Ying Liu and W.P. Wu, have been reported as endophytes from a wide range of plant species (Lu, Cannon, Reid & Simmons, 2004; Promputtha et al., 2007; Hyde, Cai, Cannon et al., 2009; Hyde, Cai, McKenzie et al., 2009; Prihastuti et al., 2009; González, Sutton & Correll, 2006). Of these, *C. asianum, C. fructicola* and *C. siamense* have been also isolated as epiphytes. This suggests that *Colletotrichum* species are able to survive within the healthy plant tissues as endophytes and are opportunistic pathogens (Prihastuti et al., 2009). When humidity and temperature are optimal for disease development, these endophytic species may cause anthracnose of their hosts. The term "endophyte" is used for those fungi that grow inside living plant tissues without causing apparent disease symptoms they may be latent pathogens (Photita, Lumyong, S., Lumyong, P., McKenzie & Hyde et al., 2004) and/or saprobes (Promputtha et al., 2007). This is slightly different from species isolated from coffee berries in Thailand (Prihastuti et al., 2009), where *C. asianum*, *C. fructicola* and *C. siamense* were isolated as pathogens, endophytes, as well as epiphytic fungi, and all strains tested were able to infect non-wounded coffee berries in pathogenicity tests. In contrast, a putative strain of *C. gloeosporioides* isolated as an endophyte from healthy *Musa acuminata* did not cause any disease of banana leaves following pathogenicity testing (Photita et al., 2004). Hence, the lifestyle of *Colletotrichum* species varies and is an area for further investigation.

Table 1.1 Representative of *Colletotrichum* were Isolated from Hosts

Modelife	*Colletotrichum* Species	References
Saprobe		
	C.anthrisci	Damm, Woudenberg, Cannon & Crous (2009); Yang, Liu et al. (2012)
	C.denatium	Damm et al. (2009)
	C.lineola	Damm et al. (2009)
Entophyte		
	C.gloeosporioides	Photita et al. (2004)
	C. musae	Photita et al. (2004)
Pathogen		
	C.brevisporum	Noireung et al. (2012)
	C.codylinicola	Phoulivong, Cai, Noireang et al. (2010)
	C.tropicicola	Noireung et al. (2012)
	C.thailandicum	Noireung et al. (2012)

1.3 Characterization of *Colletotrichum* Species

1.3.1 Morphological Characters

Morphological characterization used to identify *Colletotrichum* species are the shape and size of conidiomata (acervuli), conidia, conidiophores, setae, conidiophores, apressoria and setae in culture (Figure 1.1) (Sutton, 1992; Cai et al., 2009; Than, Prihastuti, Phoulivong, Taylor & Hyde, 2008). The conidia of *Colletotrichum gloeosporioides* are oblong with obtuse ends, and are generally broader than conidia of *C. fragariae* and *C. acutatum*. In general, conidia of *C. acutatum* are elliptic to fusiform, whereas conidia of *C. gloeosporioides* are oblong with obtuse ends (Freeman, Katan & Shabi, 1998).

From Damm, U., Cannon, P. F, Woudenberg, J. H. C & Crous, P.W. (2012). The *Colletotrichum acutatum* species complex. **Studies in Mycology**, **73**, 37-113.

Figure 1.1 *Colletotrichum johnstonii*, A–B. Conidiomata, C-H. Setae. D–G, I–J. Conidiophores. K–P. Appressoria. Q–R. Conidia

However morphological characters overlap between species and morphology alone does not provide sufficient information for a precise identification, especially for those species in the *C. gloeosporioides* and *C. dematium* complexes. (Crouch & Beirn, 2009) considered that conidial size and shape, along with conidial appressoria were taxonomically uninformative and of little use for species diagnosis in graminicolous *Colletotrichum* species. Species with similar morphological characteristics may have considerable variation at the physiological and pathogenic levels. Taxonomy based on morphology alone is likely to result in ambiguity and morphological characters should be

used in conjunction with other characters to establish species relationships within *Colletotrichum* (Cai et al., 2009; Prihastuti et al., 2009).

1.3.2 Molecular Approach

Previous studies have shown that anthracnose and fruit rot of tropical fruits is mainly caused by *C. gloeosporioides* and to a lesser extent *C. acutatum*. These identifications were, however, based on morphological identification or if gene sequence data were used comparisons were often made with wrongly applied names. Epitypification of many important *Colletotrichum* species has now occurred. The most important finding is that none of these *Colletotrichum* isolates from tropical fruits is *C. acutatum* or *C. gloeosporioides*. Thus, the previous understanding that anthracnose of most tropical fruits is caused by *C. acutatum* and *C. gloeosporioides* is incorrect. Cultural, conidial and appressorial characters can be used to differentiate taxa into species complexes, but cannot easily separate species within a complex. Certain species do however, have distinct morphological characters or growth rates that can be used in tandem with molecular data to distinguish species. The generally accepted barcoding gene for fungi, the internal transcribed spacer (ITS) region, does not adequately resolve species in *Colletotrichum*, however, this house keeping gene can resolve species complexes accepted barcoding gene for fungi, the internal transcribed spacer (ITS) region, does not adequately resolve species in *Colletotrichum*, however, this gene can resolve species complexes (Cai et al., 2009). The six gene regions presently recommended for resolving *Colletotrichum* species are actin (ACT), β-tubulin (TUB2), calmodulin (CAL), gluta-mine synthetase (GS), glyceraldehyde-3-phos-phate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region (Cai et al., 2009; Phoulivong, Cai, Noireang et al., 2010). Prihastuti et al. (2009) were able to resolve *Colletotrichum asianum*, *C fructicola*, *C. horii*, *C. kahawae* and *C. gloeosporioides* in the "gloeosporioides" complex using these five genes. Another novel species in the "gloeosporioides" complex, i.e. *C. siamense*, however, received only moderate support and further genes are needed to resolve phylogenetic relationships of this species. Ideally a single housekeeping gene needs to be found that can readily differentiate between *Colletotrichum* species (Noireung et al., 2012; Dam et al., 2012; Yang, Cai et al., 2012).

1.4 *Colletotrichum* and Plant Disease

1.4.1 Anthracnose Disease

The epidemiology of several anthracnose diseases of tropical fruits (Figure 1.2) has been studied at various stages of crop development (Freeman et al., 1998; Jeger & Jeffries, 1998; Kim, Yoon, Park, Park & Kim, 2004). In most *Colletotrichum* diseases, conidia are water-borne with the occurrence of quiescent infections being highest during the wettest periods of the growing season (Wharton & Deiguez-Uribeondo, 2004). In avocado, citrus, papaya and mango it has been shown that infected leaves in the tree canopy are the main source of inoculum, with conidia being rain-splash dispersed to unripe fruit (Hindorf, 2000). However, in mango and citrus disease, infected flowers also contribute to the conidia inoculum source (Chen et al., 2005; Abang et al., 2009). Infection by *Colletotrichum* can take place at all stages of fruit development. In blueberry, the fungus is thought to over winter as mycelium in and on blighted twigs, which act as the main source of inocula in spring (Sutton, 1992). However, recent data suggest that the primary source of overwintering inoculum may be from dormant flower buds (Van Der Vossen & Walyaro, 2009). In studies carried out on the cultivar "Bluecrop" in New Jersey, flower buds accounted for 72% of overwintering infections (Wharton & Deiguez-Uribeondo, 2004). In screening experiments carried out on the susceptible cultivar "Jersey" in Michigan, 57% of healthy looking flower buds were found to be infected, and of those infected, 82% of the infections were caused by *C. acutatum* (Wharton & Deiguez-Uribeondo, 2004). It was observed that flower buds broke dormancy, the fungus grew out of the buds and colonized the surrounding stem tissue, causing black lesions around the infected buds. These lesions gradually grow from small to large and cause the death of flower bud after about seven days to produce a pore on death tissue (Freeman, Shabi & Katan, 2000). In the field, the fungus sporulates on infected tissue during periods of extended wetness in the spring, and conidia of *C. acutatum* are dispersed by rain splash. As in citrus and strawberry, secondary conidiation, may play a role in early-season dispersal of *C. acutatum* conidia on blueberries (Wharton & Deiguez-Uribeondo, 2004).

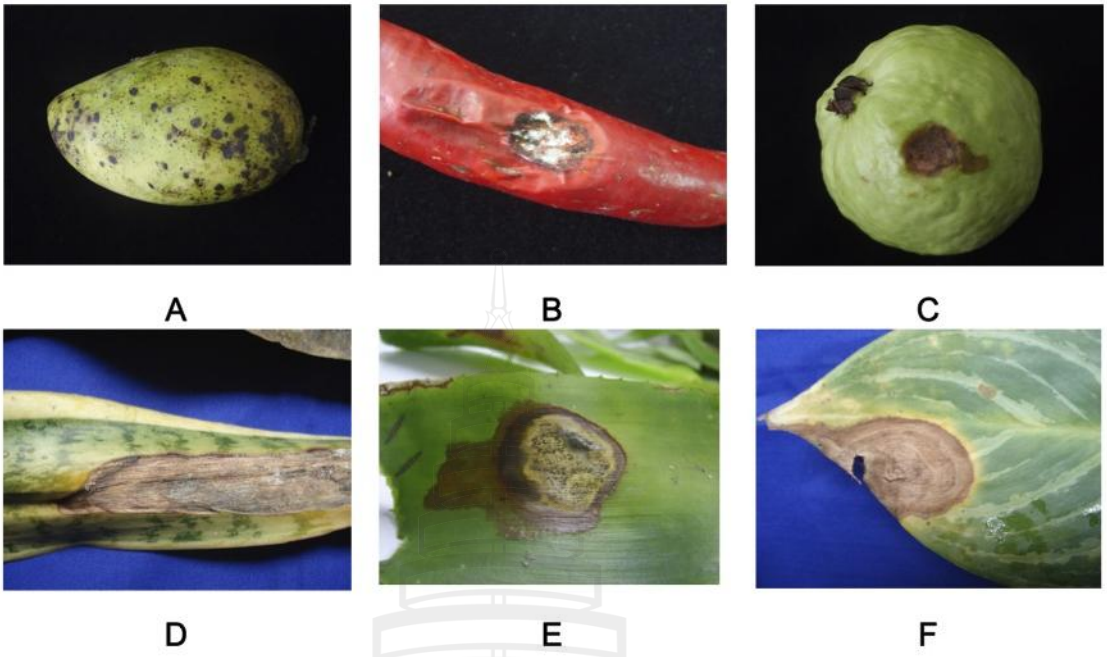


Figure 1.2 Symptom Representative of *Colletotrichum* Causing Anthracnose Disease on A.Mango Fruit, B.Chili Fruit, C.Guava Fruit, D.*Sansevieria* sp., E.*Ananas comosu*, F.*Dieffenbachia maculate*

Colletotrichum species survive in and on seeds as acervuli and micro-sclerotia (Pernezny, Roberts, Murphy & Goldberg, 2003). They may also persist on alternative hosts such as other solanaceous or legume crops. *Colletotrichum* may also be introduced into fields on infected transplants or it may survive between seasons in plant debris or on weed hosts (Peres, Kuramae, Dias & Ee Souza, 2002). Microsclerotia are naturally produced by *Colletotrichum* species to allow the fungus to lie dormant in the soil during winter or under stressed conditions. However, this mode of survival has not been confirmed for all species. Micro-sclerotia can survive for many years (Figure 1.3) even throughout a 2 or 3 years crop rotational though significant reductions in inoculum are quite likely (Zitter, 2004).

Conidia from acervuli and microsclerotia can be dispersed in water splash and thus spread to the foliage and fruit (Bailey & Jeger, 1992). Cuticular wax layers of plants are one of the first barriers to fungal infection. New spores which are produced within the infected tissue are then dispersed to other foliage or fruits (Pernezny et al., 2003).

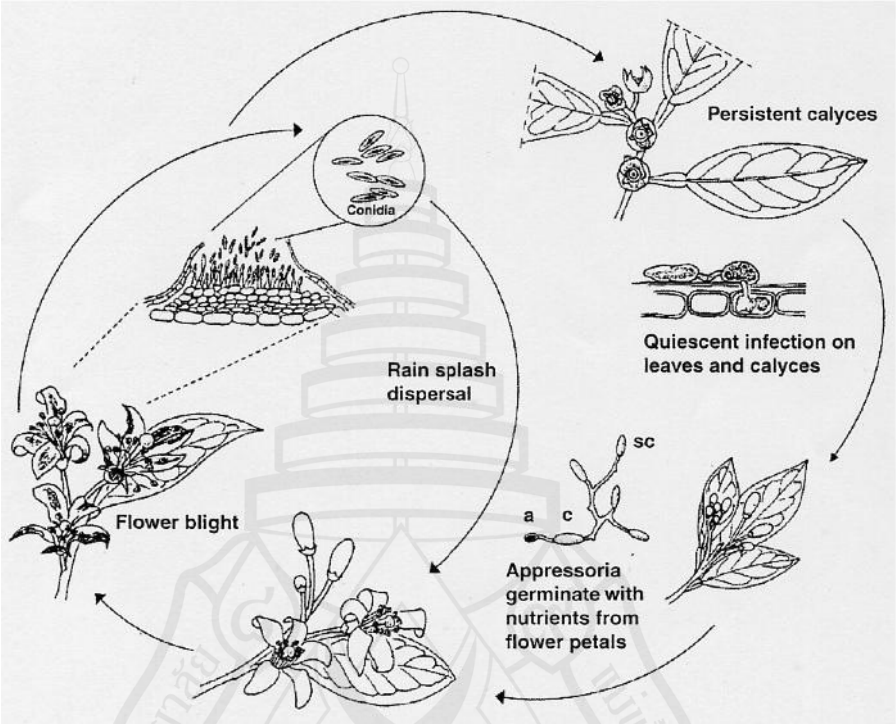
Adhesive appressoria serve as survival structure until an infection peg penetrates the surface (Ratanacherdchai, Wang, Lin & Soytong, 2010).

Colletotrichum capsici is a sub-cuticular intramural pathogen, indicating that it grows entirely beneath the cuticle and within the periclinical walls of the epidermal cells, causing dissolution of the wall structure (Bailey & Jeger, 1992; Pring et al., 1995). An intramural network of hyphae is then formed, which spreads rapidly throughout the tissue exhibiting.

1.4.2 *Colletotrichum* Causing Anthracnose

Colletotrichum species cause anthracnose of various hosts most frequently in humid and sub-humid tropical regions. Strains can often be isolated from disease tissues of stems, leaves, flowers and fruits of a wide range of crops and especially fruit trees (Freeman, Minq, Maymon & Zverbil, 2001; Peres et al., 2002; Kim et al., 2009; Crouch & Beirn, 2009; MacKenzie, Peres, Barquero, Arauz & Timmer, 2009). Crop loss is a result of reduction in quantity and/or quality of total yield. The pathogen is capable of affecting various plant parts such as root, twigs, leaves, blooms and fruit, causing a range of symptoms such as crow root rot, defoliation, bloom blight and fruit rot (Lubbe, Denman, Lamprechi & Crous, 2006). Symptoms on the fruit first appear as sunken, water-soaked lesions that expand rapidly on the fruit (Voorrips, Finkers, Sanjaya & Groenwold, 2004). Fully expanded lesions are soft, sunken and range in colour from dark red to tan to black, generally described as anthracnose disease (Wharton & Diéguez-Uribeondo, 2004). *Colletotrichum acutatum* mainly affects fruits, but branches, twigs and leaves can occasionally be affected and severe defoliation of trees has been reported (Chen et al., 2005; MacKenzie et al., 2009). Moreover *C. acutatum* is a major pathogen of various disease complexes where more than on *Colletotrichum* species is associated with a single host (Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008). In the case of strawberry, *C. acutatum*, *C. gloeosporioides* and *C. fragariae* cause anthracnose with up to 80% plant death in nurseries and yield losses of over 50% (Sreenivasaprasad & Talhinhas, 2005; Hyde, Cai, McKenzie et al., 2009), *Colletotrichum acutatum* mainly causes fruit rots on strawberry, but can also infect various other parts. An unusual strawberry root necrosis was observed in Israel in 1995–1996 during a major anthracnose outbreak (Freeman, Shalev & Katan, 2002). *Colletotrichum acutatum* isolates recovered

from these plants did not differ from isolates from plants with typical anthracnose symptoms (Freeman et al., 2000).



From Peres, N. A, Timmer, L. W., Adaskaveg, J. E. & Correll, J. C. (2005). Life styles of *Colletotrichum acutatum*. **Plant Disease**, **89**, 784-796.

Figure 1.3 Disease Life Cycle of *Colletotrichum* Causing Citrus a. Appresorium, c. Conidium, sc. Secondary Condition

Fruit infections caused by *C. acutatum* can lead to economically import losses on various crops. For example, *Colletotrichum acutatum* cause major losses in strawberry production worldwide, and is frequently responsible for important yield losses (Mertely & Legard, 2004). It is also responsible for poor olive oil quality (Rhouma, Triki & Msallem, 2010). Post-blossom fruit drop of citrus (*Citrus* spp.) is caused by *C. acutatum* and was first identified in Belize an then found throughout the humid tropical citrus areas of the Americas (Chen et al., 2005; Sreenivasaprasad & Talhinhas, 2005). Damage to tamarillo

(*Cyphomandra betacea*) fruit occurred with yield loss of more than 50% in Colombia (Sreenivasaprasad & Talhinhas, 2005).

During infection by *Colletotrichum codylinicola*, the first symptoms are multiple small lesions; these can rapidly cover most of the fruit (Roberts, Pernezny & Kucharek, 2001). As the infection progresses, the surface of the lesion becomes covered with wet, gelatinous, buff to salmon-coloured spores that exude from acervuli that may contain numerous black setae either scatered or in concentric rings within the lesions, Figure 1.2 (Esquerré-Tugayé, Mazau, Barthe, Lafitte & Touze, 2000). The formation of setae gives the overall lesion its black colouration (Zitter, 2004). Foliage and stem symptoms appear as small, irregularly-shaped gray-brown spots with dark brown edges (Asian Vegetable Research and Development Center [AVRDC], 2004), (Figure 1.2).

1.4.3 Host Range of *Colletotrichum*

Colletotrichum can affect host ranges with a worldwide diffusion and having a severe impact on crops (Cai et al., 2009; Hyde, Cai, Cannon et al., 2009; Phoulivong, Cai, Chen et al., 2010). It is common to find that a single species of *Colletotrichum* infects multiple hosts such as apple (*Malus pumila*), avocado (*Persea americanna*), banana (*Musa sapientum*), coffee (*Coffea arabica*), citrus (*Citrus* spp), guava (*Psidium guajava*), jujube (*Zizyphus mauritiana*), lime (*Citrus aurentiforia*), longan (*Euphoria longana*), papaya (*Carica papaya*), mango (*Mangifera indica*, olive (*Olea eupea*), papaw (*Carica papaya*), strawberry (*Fragria frageriae*), sugar apple (*Annoana squamosa*), tomato (*Lycopersicon esculentun*) (Bailey & Jeger, 1992; Simmonds, 1965; Wharton & Deiguez-Uribeondo, 2004). *Colletotrichum falcatum* is however, host-specific on sugar cane (*Saccharum officenarum*) (Kumar, Jhang, Satyavir & Sharma, 2010; Malathi, Viswanathan, Padmanaban, Mohanraj & Sundar, 2002).

Previous data on host ranges of *Colletotrichum* species must however be treated with caution (Freeman et al., 2000, 2001; Hyde, Bahkali & Moslem, 2010). Recent studies have shown that the ubiquitous species, *C. gloeosporioides* is not as common in the tropics as thought. In a study of *Colletotrichum* species causing anthracnose in Laos and Thailand no fruits were infected by *C. gloeosporioides*. In fact, molecular data has revealed that *C. gloeosporioides* is a species complex comprising between 20 and 50 species (Hyde, Cai, Cannon et al., 2009) The study of host range of *Colletotrichum*

species is therefore an area of research that needs in-depth study and most previous data must be treated with caution (Hyde, McKenzie & KoKo, 2011).

1.4.4 Disease Control Management

Effective control of *Colletotrichum* diseases usually involves the use of one or a combination of the following practices: using resistant cultivars, cultural control, chemical control and biological control using antagonistic organisms. The applicability of control strategies much depends on the characteristics of the crops on which they are being used as on the disease at which they are targeted (Wharton & Deiguez-Uribeondo, 2004).

1.4.4.1 Cultural Control: Cultural control is related to the range of methods use to control diseases, mostly using tactics aimed at disease avoidance through phytosanitation, manipulation of cropping patterns or by enhancing resistance and avoiding predisposition (Roberts et al., 2001; Agrios, 2005). The ubiquitous nature of inoculum sources of *Colletotrichum* under suitable conditions reduce the effectiveness of many pre-harvest general phytosanitary practices. However, general orchard hygiene has a place in integrated disease control, as removal of obvious inoculation sources such as diseased leaves and fruit can increase the efficiency of chemical control (Waller, 1988).

Cultural control refers to tactics aimed at disease avoidance through phytosanitation, manipulation of cropping patterns, or by enhancing resistance and avoiding predisposition. For chili peppers, only seeds and seedlings that are pathogen free should be planted (Pernezny et al., 2003). Otherwise, seeds should be disinfected with a 30 minute soak in water at 52°C followed by fungicide treatment (AVRDC, 2004). Healthy transplants should be used and transplant flats should be sanitized if they are to be reused (Kefialew & Ayalew, 2008; Sreenivasaprasad & Talhinhas, 2005; Sutton, 1992). Broad-spectrum fumigants may by used in soil to control the pathogens and soil solarization may also be effective (Bailey & Jeger, 1992). Proper plant spacing should be maintained to provide adequate movement of air around plants which helps reduce the severity of foliar diseases (Abang, 2003). If disease was previously present, chili peppers should be rotated with crops other than potato, soybean, beans, tomato, eggplant and cucurbits for three years (Pernezny et al., 2003; AVRDC, 2004).

Crop rotation is one of the best ways to promote healthy crops production, since it helps minimizing diseases especially those caused by soil borne pathogens (Bailey & Jeger, 1992). Mulch should be provided to reduce soil splash onto fruit and lower leaves. Overhead irrigation should be minimized or avoided to reduce periods of wetness. The field should have good drainage and be free from infected plant debris. Insects should be controlled to reduce fruit wounds as they provide entry points for *Colletotrichum* species (Roberts et al., 2001; Agrios, 2005; Than et al., 2008).

A crucial cultural control for minimizing disease is to harvest vegetables and fruits as soon as they ripen, as otherwise anthracnose develops very readily (Jeyalakshmi & Seetharaman, 1998; Kefialew & Ayalew, 2008). In addition, proper sanitation techniques during processing of the harvested fruit, transportation, packaging and storage should be adopted to minimize the resumption of growth of the dormant infection of the pathogen (Abang et al., 2009).

Covering fruit with paper bags is common place in many parts of the word. This method not only excludes insects from the fruit but also excludes *Colletotrichum* infection. When fruits are young the bags are placed over individual fruits or if small, over many fruits and left until mature. The type of bag used is important as fruits will rot in plastic and soft paper bags will disintegrate in heavy rain. This method is particularly useful for avocado, banana, guava, longan, mango, rose apple, santol and star fruit. (Nakasone & Paul, 1998).

1.4.4.2 Biological Control: Biological control methods for *Colletotrichum* diseases have not received much attention until recently even although as early as the potential of biological control through the use of phyllosphere antagonists was discussed. (Jeger & Plumbley, 1988) reviewed possibilities for biological control of post-harvest fruit diseases caused by *C*, *gloeosporioides,* when they found that an isolate of *Pseudomonas fluorescens* was successful in significantly reducing anthracnose development on mango as compared to the control fruit. However, the mechanism by which the bacterium was able to reduce anthracnose development is still unknown. These positive results indicated that there was considerable potential for the development of a biological control agent for control of mango anthracnose.

Most biological control methods are still at the research stage but recent progress has resulted in a number of new commercial products which have been

developed for post-harvest applications as this situation offers more advantages for biological control strategies (Korsten, De Villiers, Wehner & Kotzé, 1997).

Biological control of anthracnose fruit rot and die-back of chili peppers with plant products in laboratories and field trials showed that the crude extracts from rhizome, leaves and creeping branches of sweet flag (*Acorus calamus* L), palmorosa (*Cymbopogon martini*) oil, and neem (*Azadirachia indica*) oil could restrict growth of the anthracnose fungus (Jeyalakshmi & Seetharaman, 1998). Sweet flag extract in ethyl acetate showed good inhibitory effect. However, this and other biocontrol method need further research and validation before being promoted at the commercial scale.

1.4.4.3 Chemical Control: Chemical control of anthracnose has widely been used for controlling anthracnose of fruit crops because the increase in value of the product usually offsets the relatively expensive chemical inputs, in terms of pesticide cost, machinery, materials and labor, and transportation and storage. Moreover the availability and efficiency of chemical control is relatively greater than that of other control methods (Jeger & Plimbley, 1998). Generally, *Colletotrichum* disease can be controlled by a wide range of chemicals including copper compounds such as dithiocarbanates, benimidazole and triazole compounds, and other fungicides such as chlorothalonill, imazalil and prochloraz (Waller, 1992). Newer classes of fungicides such as the strobilurins are also proving highly effective against *Colletotrichum* species that infect fruits. However, the problem of fungicide tolerance may arise quickly if a single compound is relied upon too heavily (Wharton & Deiguez-Uribeondo, 2004).

Chemical controlinvolves the frequent applications of fungicides such as mancoceb, carbendazim, dipheconasol, dicolad and benomyl. However, there are negative effects on farmers income and health, particularly in developing countries (Voorrips et al., 2004) and even with the application of fungicides, pre- and post-harvest anthracnose fruit rot can cause severe loss (Hartman & Wang, 1992). Farmers may get into the habit of over-spraying their crops with fungicides that may lead to other forms of damage and the chemical applications would become costly.

For successful chemical control, timing and placement are of critical significance. Application of registered protectant fungicides to plants starting when the first fruit are set may be recommended for the control of anthracnose when environmental conditions are less than optimum for disease development or when a low level of

inoculum is present. This will prevent or minimize the occurrence of infections (Asian Vegetable Research and Development Center [AVRDC], 2003). However, poorly timed fungicide applications may actually lead to an increase in the severity of disease due to the disturbance of natural biological control mechanisms and increased crop susceptibility. Although treatment with fungicides can significantly reduce the incidence and severity of disease, eradication cannot normally be achieved (Adaskaveg & Förster, 2000). Thus, if treatments are stopped and conditions favorable for disease re-occur, then the disease in the crop may subsequently increase. Applications prior to conductive conditions are thus required and rotation programs between fungicides of different classes are highly recommended (Adaskaveg & Förster, 2000). Development of models to predict anthracnose risk due to environmental conditions can efficiently reduce the number of fungicide applications (Wharton & Diéguez-Uribeondo, 2004).

1.4.4.4 Using *Colletotrichum* in weed biocontrol: Numerous plant pathogens have been considered as potential biocontrol agents but in reality there has been little commercial success (Zidack & Quimby, 1999). However, with the move towards organic vegetables and restricted use of pesticides there is a need to develop more effective biocontrol bioherbicides. Biotrophs are usually host-specific but do not often cause serious disease and are thus not good herbicides (Goodwin, 2001). Necrotrophs on the other hand, are often severe pathogens but are generally not host-specific and thus also not suitable bioherbicides. As discussed earlier, *Colletotrichum* species are hemibiotrophs having an initial biotrophic phase with high host specificity followed by a necrotrophic phase with extensive tissue death; thus species have relatively high specificity and virulence (i.e., degree of pathogenicity). *Colletotrichum* species are therefore prime targets for use in weed control and there are presently several products on the market and several under investigation (Templeton, 1992).

There has been much research on using *Colletotrichum* species in weed control. *Colletotrichum gloeosporioides* f. sp. *malvae* (Penz.) Penz. and Sacc. has been developed as a mycoherbicide to control round-leaved mallow (*Malva pusilla*) weed in Canada (Goodwin, 2001). *Sesbania exaltata* (hemp sesbania) is a weed of soybean. Microsclerotia of a putative strain of *C. truncatum*, formulated in wheat gluten-kaolin granules called “Pesta” resulted in highly significant weed control (Boyette et al., 2007). Microsclerotia formulated in “Pesta” granules had an excellent shelf-life, retaining high

viability after storage for 10 years at 4˚C. These results suggest that microsclerotia of *C. truncatum* formulated in "Pesta" granules offer an effective method for controlling this important weed and preserving the activity of this bioherbicide. Another example of a *Colletotrichum* species with potential for use as a bioherbicide is *C. gloeosporioides* f. sp. *Aeschynomene*, which is highly virulent against the leguminous weed *Aeschynomene virginica* also know as northern jointvetch (Boyette et al., 2007).There have also been several patents using *Colletotrichum* species as biological herbicides. What is most interesting concerning the bioherbicides is that the names often used in publications or registered in patents have been outdated by recent developments in the taxonomy of species based on molecular data (Cai et al., 2009). This must throw doubt on the use of names and the validity of patents themselves. The taxa used in bioherbicides therefore should be reevaluated using a polyphasic approach and renamed where necessary.

1.4.4.5 Resistance Control: Use of resistant cultivars is perhaps the most desirable aspect for disease control in agriculture crops (Than, Jeewon et al., 2008; Wharton & Diéguez-Uribeondo, 2004). Such an approach has been less exploited in fruit crops mainly due to the longer time frame required for breeding and selecting for resistance and the shorter-term advantages of chemical control (Voorrips et al., 2004). Cultivar resistance in fruit crops is also complicated by the ability of most *Colletotrichum* fruit pathogens to form quiescent infections (Agrios, 2005).

The resistance varieties can be eliminated crops losses and eliminated chemical and mechanical expenses of disease control (Agrios, 2005; Than, Jeewon et al., 2008) Resistance is considered the most prudent means of disease control because of its effectiveness, ease of use, and lack of potential negative effects on the environment and its use is highly recommended.

One area that has received much research attention is that of developing chili varieties that are resistant to *Colletotrichum* anthracnose (list 3−4 refs from below). Genetic control of resistance to anthracnose in chili peppers has been studied for over 10 years and several cultivars resistant to *Colletotrichum* species have been reported (Asian Vegetable Research and Development Center [AVRDC], 2002; Agrios, 2005; Kim et al., 2009). At present, research is underway to identify resistance sources, to evaluate these sources for purity of resistance, and to introgress the resistance traits into cultivated chili pepper, *Capsicum annuum*. (Roberts et al., 2001).

AVRDC (2002) has identified five accessions of peppers (*Capsicum chinense*: CO4554, PBC932, *Capsicum baccatum*: PBC880, PBC81, PBC133) that are resistant to three species of *Colletotrichum*, i.e. *C. acutatum*, *C. gloeosporioides* and *C. capsici*. Resistance to all three pathogens is of great importance because it increases the likelihood that resistance will be expressed in the field where all three pathogens occur. However, breeding for resistance is complicated by the ability of most *Colletotrichum* species to form quiescent infections. AVRDC (2003) reported that not all accessions that express resistance in the green fruit stage express resistance at the ripe fruit stage, although accessions PBC 932 and PBC 81 express immune resistance at both stages of fruit development.

Characterization of resistance genes is being carried out to transfer and incorporate resistance into cultivars to develop anthracnose resistant *C. annuum* genotypes. AVRDC Pepper Breeding Unit has been introgressing resistance from PBC932 into advanced *C. annuum* chili lines and BC_1F_5 and BC_3F_4 lines resistant at both the green and ripe fruit stages have been identified. The BC_3F_4 lines are now being used as the resistant parent in the breeding program to develop advanced anthracnose resistant chili pepper lines, to generate populations to study inheritance of resistance studies and to develop molecular markers for use in marker-assisted breeding for anthracnose resistance (AVRDC, 2003). The studies have recently become complicated due to changes in understanding of the species that infect chili (Than, Jeewon et al., 2008). However, as long as resistant is bred against those strains causing disease it should not present great problems.

In most host-pathogen interactions, resistance involves the triggering of host defense responses that prevent or retard pathogen growth and may be conditioned by a single gene pair, a host resistance gene and a pathogen a virulence gene (Flor, 1971). However, the reports differ in the predictions of the number of genes involved in conferring resistance in *Capsicum* species. Some studies reported that resistance to *C. capsici* in *C. annuum* populations segregated in a Mendelian fashion and was likely to be controlled by a single dominant gene (Park, Kim & Lee,1990a; 1990b; Lin, Kanchana-udomkarn, Jaunet & Mongkolporn, 2002) while resistance to *C.gloeosporioides* was reported to be partially dominant or over dominant (Park et al., 1990a). Another study found that resistance to *C. gloeosporioides* in one cultivar was controlled by a single

dominant gene, and in the other two cultivars was controlled by a pair of dominant. In addition, polygenic resistance has been reported (Ahmed, Dey & Hundal, 1991). However, Cheema, Singh, Rawal & Deshpande (1984) found that resistance to *C. capsici* was inherited recessively, with significant epistatic interactions. The differences could have resulted from different cultivars being used in establishing the chili pepper population, different fungal strains used in the bioassays, difference in the level of resistance of the so called resistant lines and the different evaluation methods adopted (Pakdeevaraporn, Wasee, Taylor & Mongkolporn, 2005). However, Pakdeevaraporn et al. (2005) mentioned that none of the above mentioned resistant *C. annuum* cultivars were completely resistant. Resistance in *C. chinense* PBC932 was reported to be controlled by a single recessive gene, which has been designated '*co1*' (Pakdeevaraporn et al., 2005). The inheritance of resistance to *Colletotrichum capsici* and *C. gloeosporioides* in *C. chinense* (PRI95030) was studied using a quantitative trait locus mapping approach in an F_2 population derived from a cross between *C. chinense* and an Indonesian hot pepper variety (*C. annuum*). In laboratory tests where ripe fruits were artificially inoculated with either *C. gloeosporioides* or *C. capsici*, three resistance-related traits were scored, the infection frequency, the true lesion diameter (averaged over all lesions that actually developed), and the overall lesions diameter (averaged over all inoculation points, including those that did not develop lesions). One main quantitative trait locus was identified with highly significant and large effects on all three traits after inoculation with *C. gloeosporioides* and on true lesion diameter after inoculation with *C. capsici*. Three other quantitative trait locus with smaller effects were found for overall lesion diameter and true lesion diameter after inoculation with *C. gloeosporioides*, two of which also had an effect on infection frequency. The resistant parent carried a susceptible allele for a quantitative trait locus for all three traits that was closely linked to the main quantitative trait locus. Although the main quantitative trait locus was shown to have an effect on true lesion diameter after inoculation with *C. capsici*, no significant quantitative trait locus were identified for overall lesion diameter or infection frequency. The main quantitative trait locus is the most important genetic factor in all the resistant-related traits studied (Voorrips et al., 2004). A substantial part of the different resistance-related traits was controlled by one quantitative trait locus with mostly additive effects. Therefore, the

different resistant-related traits were inherited in an intermediary or partly dominant manner.

The results of the quantitative trait locus study deviated from those in earlier studies, which were based on intraspecific *C. annuum* crosses and did not use a quantitative trait locus approach. All the information on linkages and estimates of specific quantitative trait locus effects offers a new opportunity for resistance breeding against anthracnose fruit rot.

1.5 Pathogenicity Testing

Artificial inoculation methods *in vitro* are commonly used to test the pathogenicity of a fungal species, as it is easy to control environmental conditions (Photita et al., 2004). Common inoculation methods for pathogenicity testing include drop inoculation and wound/ drop inoculation (Kanchana-udomkarn, Taylor & Mongkolporn, 2004; Lee, Park & Jung, 2005), micro-injection, and spraying with high pressure guns (Freeman et al., 1996; Lin et al., 2002; AVRDC, 2003; Sharma, Kaur, , Sharma, Sharma & Pathanja, 2005; Than, Jeewon et al., 2008; Cai et al., 2009). The drop inoculation method involves dropping a spore suspension on to the surface of a fruit and the wound/drop method involves wounding the surface of the fruit by pricking it with a pin and then placing a drop of fungal spore suspension on the wounded tissue (Ratanacherdchai, Wang, Lin & Soytong, 2007). The wound/drop method is more favourable since wounding allows the pathogenic isolate internal access to the fruit and enhances infection (Cai et al., 2009). The wound/drop method has been shown to be useful to select resistant varieties of *Capsicum annuum* from susceptible varieties (Lin et al., 2002). However some researchers are of the opinion that the wound/drop method is paramount to damaging the plant so much, that infection is inevitable.

Different hosts and their stages of maturity are important for testing the expression of resistance to *Colletotrichum* species.The interaction between fruit maturity stage and infection of colonisation may depend on the species of *Colletotrichum* (AVRDC, 2002). Pathogenicity testing can provide data on the resistance of crops to the fungal taxon and is useful in plant breeding programs. It is also important for integrated

disease management programs because the use of resistant plant varieties can reduce the negative effects of chemical use on the environment (Peres et al., 2002; Wharton & Diéguez-Uribeondo, 2004; Ratanacherdchai et al., 2010).*Colletotrichum gloeosporioides sensulato*has previously been listed to cause disease of a very wide range of fruits and infect leaves of many hosts in Thailand and Laos (Ratanacherdchai et al., 2007; Than Prihastuti et al., 2008; Phoulivong, Cai, Noireang et al., 2010; Hyde et al., 2011). *Colletotrichum gloeosporioides* was epitypified in 2008 with a living strain that has been sequenced with sequence data deposited in GenBank (Cannon, Buddie, & Bridge, 2008). This has enabled researchers to compare their isolates of *Colletotrichum* with the *C. gloeosporioides* epitype. This has resulted in the description of several new species in the *C. gloeosporioides* species complex (Prihastuti et al., 2009; Yang et al., 2009; Phoulivong, Cai, Noireang et al., 2010; Noireung et al., 2012). With the introduction of several new species it is important to establish whether they are host-specific or have a wide host range as this will have important implications in disease control and management (Freeman et al., 2000; Sanders & Korsten, 2003; Ratanacherdchai et al. 2007).

1.6 Identification of *Colletotrichum* Species

Traditionally, *Colletotrichum* species were identified and delimited based on morphological characters (Cai et al., 2009). Several identifying features have been utilized by taxonomists, including the size and shape of conidia and appressoria, the presence or absence of setae and sclerotia, acervuli form and teleo-morph characters (Bailey & Jeger, 1992; Sutton, 1992; Cai et al., 2009; Phoulivong, Cai, Noireang et al., 2010). Cultural characters such as colony colour, growth rate and texture have also been utilized (Simmonds, 1965; Sutton, 1992; Photita et al., 2004; Than, Prihastuti et al., 2008; Cai et al., 2009; Hyde, Cai, McKenzie et al., 2009; Prihastuti et al., 2009; Yang et al., 2009; Phoulivong, Cai, Noireang et al., 2010). 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 and the fact

the many similar species were actually part of a species complex (Cai et al., 2009; Hyde, Cai, Cannon et al., 2009).

To overcome the inadequacies of these traditional schemes, molecular techniques have been used to characterize and identify taxa within *Colletotrichum* (Cai et al., 2009; Hyde, Cai, Cannon et al., 2009; Prihastuti et al., 2009, Yang et al., 2009; Phoulivong, Cai, Noireang et al., 2010; Noireung et al., 2012). Cannon, Bridge & Monte, (2000) stated that nucleic acid analyses should provide the most reliable framework to classify *Colletotrichum*, as DNA characters are 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; Cai et al., 2009). Photita et al. (2004) separated 34 isolates of *Colletotrichum* from banana, *Draceana sanderian*, *Eupatorium thymifolia*, ginger, longan, mango and soybean. 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 β-tubulin 2 gene. Than, Jeewon et al. (2008) differentiated the isolates of chili 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, however these have since been shown to represent other species (Prihastuti et al., 2009; Yang et al., 2009; Cai et al., 2009; Phoulivong, Cai, Noireang et al., 2010).

1.7 Should We Use *Colletotrichum* or *Glomerella*?

The dual nomenclature system adopted for naming of fungi has long been problematic as the same biological species can have two names (Shenoy et al., 2007). With molecular sequence data it is now often possible to link the anamorph with the teleomorph or to establish the relationship of the anamorphic genus within the teleomorph taxonomic frame work (Hyde et al., 2011) and this system should be changed to using just one name.

There are at least three ways in which anamorphic genera and species names should be dealt (Hyde et al., 2011). In *Colletotrichum* this includes using only one name *Glomerella*, which follows the sexual state, or the earliest introduced name *Colletotrichum*, or keeping the status quo of using two names In *Colletotrichum* the second approach has already been adopted by some researches who have introduced new species under the oldest *Colletotrichum* name and within the description describing the teleomorph state (e.g.). If the oldest name in *Colletotrichum* is adopted, then the name of species that are generally know as important in disease causing agents will be maintained. The teleomorph state is *Glomerella* and very few species in this geneus are known for their ability to cause serious disease. Therefore one name *Colletotrichum*, should be adopted for all species in *Glomerella* and *Colletotrichum* and all *Glomerella* species should be considered to be synonyms of *Colletotrichum*.

1.8 Names in Current Use-Updated

Hyde, Cai, Cannon et al. (2009) published a list of *Colletortrichum* names that they considered were in current use and provided a Table 1 of the ex-type cultures and GenBank sequence data from ex-type cultures. At this time 66 taxa are recognized based on name usage since 1980. Five new species of *Colletotrichum* have been described since the publication of Hyde, Cai, Cannon et al. (2009) and *C. theobromicola* Delacr. From cocao has been characterized with morphology and sequence data and the currently excepted species are updated in Table 1.1 with the location of ex-type cultures and ex-type related gene sequences. The species are *C.cordylinicola* Phoulivong, L. Cai and K. D. Hyde isolated from leaf spots of *Cordyline fruiticosa* (Phoulivong, Cai, Noireang et al., 2010), *C. ignotum* Rojas, Rehner and Samuels isolated from *Therbroma cacao* and *C. tropicale* Rojas, Rehner and Samuels from *Tetragastris panamensis* (Rojas et al., 2010) and *C. jasminigenum* Wikee, K. D. Hyde, L. Cai and McKenzie isolated and *C. jasminisambac* Wikee, K. D. Hyde, L. Cai and McKenzie isolated from *Jasminum sambac* (Wikee, Cai, Noireuang, McKenzie & Su, 2011).

1.9 Aim of This Study

The main objectives of this study were as follows.

1.9.1 To investigate characterize of *Colletotrichum* speciesassociated anthracnose in fruit rot in Laos and Thailand.

1.9.2 Study taxonomy and identification of *Colletotrichum* isolates from anthracnose infected fruits by their morphology and sequences based on multigenes.

1.9.3 To investigate the host specificity and virulence of the *Colletotrichum* isolates from a range of hosts and their ability to cross-infection potential to each other.

Table 1.2 List of Species of *Colletotrichum* Treated as Currently Used Following Hyde et al. (2011), and Location of Type Specimens and Their Sequenced Genes

Species	Type strain	ITS	Calmodulin	Actin	GAPDH	Tub2	GS	Mat1	Tub1	CHS-1	HIS3	5'- *tef1*
C. acutatum	IMI 117617	AF411700	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. agaves	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. anthrisci	CBS 125334	GU227845	x	GU227943	GU228237	GU228139	x	x	x	GU228335	GU22804	**x**
C. asianum	MFU090233	FJ 972612	FJ 917506	FJ 907424	FJ972576	FJ 907439	FJ 972595	x	x	x	x	**x**
C. axonopodi	IMI 279189	x	x	x	x	x	x	FJ377907	**x**	**x**	**x**	**x**
C. boninense	MAFF 305972	AB051400	x	x	GQ221769	x	x	**x**	**x**	**x**	**x**	**x**
C. capsici	CBS 120709	EF683603	x	x	x	EF683602	x	**x**	**x**	**x**	**x**	**x**
C. caudatum	MAFF 057001	EU5541101	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. cereale	KS 20BIG	DQ12617	x	x	x	x	x	DQ131946	**x**	**x**	**x**	**x**
C. chlorophyti	IMI 103806	GU227894	x	GU227992	GU228286	GU228188	x	**x**	**x**	GU228384	GU228090	**x**
C. circinans	CBS 221.81	GU227855	x	GU227953	GU228247	GU228149	x	**x**	**x**	GU228345	GU228051	**x**
C. cliviae	CBS 125375	GQ485607	GQ849464	GQ856777	GQ856756	GQ849440	**x**	**x**	**x**	**x**	**x**	**x**
C. coccodes	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
C. cordylinicola	BCC38864	HM470247	HM470238	FJ 907425	HM470241	HM470250	HM470244	**x**	**x**	**x**	**x**	**x**
C. crassipes	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. curcumae	IMI 288937	GU227893	x	GU227991	GU228285	GU228187	x	**x**	**x**	GU228383	GU228089	**x**
C. dematium	CBS 125.25	GU227819	x	GU227917	GU228211	GU228113	x	**x**	**x**	GU228309	GU228015	**x**
C. destructivum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. dracaenophilum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. echinochloae	MAFF 511473	AB439811	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C.ignotum	CBS125390	GU944376	x	x	x	GU94469	x	GU944**40**	**x**	**x**	**x**	GU94279
C. falcatum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**

Table 1.2 (continued)

Species	Type strain	ITS	Calmodulin	Actin	GAPDH	Tub2	GS	Mat1	Tub1	CHS-1	HIS3	5'- *tef1*
C. fuscum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. gloeosporioides	IMI 356878 =CBS 953.97	EU371022, AY376532, FJ976209	FJ917512	FJ907430	FJ972582	FJ907445	FJ972589	**x**	**x**	**x**	**x**	**x**
C. fiorinae	EHS 58	EF464594	x	x	x	EF593325	x	**x**	**x**	**x**	**x**	**x**
C. fragariae	CBS 142.31	GU174546	x	x	GU174564	x	x	**x**	**x**	**x**	**x**	**x**
C. fructi	CBS 346.37 = CCT 4806	GU227844	x	GU227942	GU228236	GU228138	x	**x**	**x**	GU228334	GU228040	**x**
C. fructicola	MFU 090228	FJ972603	FJ917508	FJ907426	FJ972578	FJ907441	FJ972593					**x**
C. gossypii	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. graminicola	M 1.001	DQ003110	x	x	x	x	x	FJ377994	**x**	**x**	**x**	**x**
C. hanaui	MAFF 305404	EU554101	x	x	x	x	x	FJ377922	**x**	**x**	**x**	**x**
C. higginsianum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. hippeastri	CBS 125376	GQ485599	GQ849469	GQ856788	GQ856764	GQ849446	x	**x**	**x**	**x**	**x**	**x**
C. horii	ICMP 10492	GQ329690	x	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
C. hymenocallidis	CBS 125378	GQ485600	GQ849463	GQ856775	GQ856757	GQ849438	x	**x**	**x**	**x**	**x**	**x**
C. jacksonii	MAFF 305460	EU554108	x	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
C. jasminigenum	LLTX-01	HM131513	HM131494	HM131508	H131499	HM153770	HM131504	**x**	**x**	**x**	**x**	**x**
C. jasmini-sambac	LLTA-01	HM131513	HM131494	HM131507	HM131499	HM153768	HM131504	**x**	**x**	**x**	**x**	**x**
C. kahawae	IMI 319418	GU174550	x	x	GQ329681	x	x	**x**	**x**	**x**	**x**	**x**
C. lilii	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. lindemuthianum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. lineola		GU227829	x	GU227927	GU228221	GU228123	x	**x**	**x**	GU228319	GU228025	**x**
C. linicola	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**

Table 1.2 (continued)

Species	Type strain	ITS	Calmodulin	Actin	GAPDH	Tub2	GS	Mat1	Tub1	CHS-1	HIS3	5'- *tef1*
C. liriopes	CBS 119444	GU227804	x	GU227902	GU228196	GU228098	x	**x**	**x**	GU228294	GU228000	**x**
C. lupini	BBA 70884	x	x	x	x	x	x	DQ174704	AJ301948	**x**	**x**	**x**
C. lupini var. *setosum*	BBA 70352	x	**x**	**x**	**x**	**x**	x	DQ174702	AJ301923	**x**	**x**	**x**
C. malvarum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. miscanthi	MAFF 510857	EU554121	x	x	x	x	x	EU365028	x	x	x	**x**
C. musae	**x**	**x**	**x**	**x**	x	x	x	**x**	x	x	x	**x**
C. navitas	CBS 125086	GQ919067	**x**	**x**	x	x	x	GQ919071	x	x	x	**x**
C. nicholsonii	MAFF 511115	EU554126	**x**	**x**	x	x	x	FJ377946	x	x	x	**x**
C. nymphaeae	**x**	**x**	x	x	x	x	x		x	x	x	**x**
C. orbiculare	**x**	**x**	x	x	x	x	x		x	x	x	**x**
C. paspali	MAFF 305403	EU554100	x	x		x	x	FJ377921	**x**	**x**	**x**	**x**
C. phaseolorum	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. phormii	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. phyllachoroides	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**
C. rusci	CBS 119206	GU227818	x	GU227916	GU228210	GU228112	x	**x**	**x**	GU228308	GU228014	**x**
C. sansevieriae	MAFF 239721	AB212991	**x**	x	x	x	x	x	x	x	x	**x**
C. siamense	MFU 090230	FJ972631	FJ917505	FJ907423	FJ972575	FJ907438	FJ972596	x	x	x	x	**x**
C. simmondsii	BRIP 28519	FJ972601	FJ917510	FJ907428	FJ972580	FJ907443	FJ97259	x	x	x	x	**x**
C. spaethianum	CBS 167.49 = BBA 4804	GU227807		GU227905	GU228199	GU22810	x	x	x	GU228297	GU228003	**x**
C. spinaciae	**x**	x	x	x	x	x	x	x	x	**x**	**x**	**x**
C. sublineola	S 3.001	DQ003114	x	x	x	x	x	x	x	**x**	**x**	**x**
C. tofieldiae	**x**	x	x	x	x	x	x	**x**	**x**	**x**	**x**	**x**

Table 1.2 (continued)

Species	Type strain	ITS	Calmodulin	Actin	GAPDH	Tub2	GS	Mat1	Tub1	CHS-1	HIS3	5'- *tef1*
C. trichellum	x	x	x	x	x	x	x	x	x	x	x	x
C. trifolii	x	x	x	x	x	x	x	x	x	x	x	x
C. tropicale	CBS124949	GU944336	x	x	x	GU944452	x	GU94423	x	x	x	GU94261
C. truncatum	CBS 151.35	GU227862	x	GU227960	GU228254	GU228156	x	x	x	GU228352	GU228058	x
C. verruculosum	IMI 45525	GU227806	x	GU227904	GU228198	GU228100	x	x	x	GU228296	GU228002	x
C. xanthorrhoeae	BRIP 45094	GU048667	x	x	GU174563	x	x	x	x	x	x	x
C. yunnanense	AS 3.9167	EF369490	x	x	x	x	x	x	x	x	x	x

CHAPTER 2

Colletotrichum gloeosporioides IS NOT A COMMON PATHOGEN ON TROPICAL FRUITS

2.1 Introduction

Fruit rots caused by *Colletotrichum* species are major pre-and/or post harvest diseases which seriously constrain the production, marketing and export of tropical fruits and may be have quarantine concern (Alahakoon & Brown, 1994; Bailey & Jeger, 1992; Hindorf, 2000; Hyde, Cai, Cannon et al., 2009; Johnston & Jones, 1997; Sreenivasaprasad & Talhinhas, 2005). Fruit spoilage ranges from a slight loss in quality, resulting in reduced sales, to total spoilage of the fruits (Agostini, Timmer & Mitchell, 1992; Bailey & Jeger, 1992; Giblin, Coates & Irwin, 2010; Hindorf, 2000). The fruit rots are mainly caused by *Colletotrichum gloeosporioides*, and to a lesser extent by *C.acutatum* (Table 2.1). However, these two taxa are thought to be species complexes and accurate information concerning the causal species within these complexes is lacking (Hyde, Cai, Cannon et al., 2009; Johnston, 2000). The "*gloeosporioides*" complex arose because of the artificially enlarged spore range (especially spore length) placed on *C.gloeosporioides* (von Arx, 1957) to overcome the instability of spore morphology under different conditions or from different hosts. This treatment has subsequently been followed by researchers worldwide so that numerous *Colletotrichum* strains with cylindrical conidia from various studies were identified as *C.gloeosporioides*. Molecular data used to identify strains in this complex has also been problematic, as >86% of the ITS sequences designated as *C.gloeosporioides* in GenBank were not conspecific to the *C.gloeosporioides* epitype (Cai et al., 2009; Hyde, Cai, McKenzie et al., 2009).

Table 2.1 Reports of *Colletotrichum* Species Infecting Tropical Fruits

Fruit	*Colletotrichum* species	References
Avocado (*Persea americana*)	*C. acutatum*, *C. gloeosporioides*	Hindorf (2000); Peres et al. (2002); Everett (2003); Giblin et al. (2010)
Banana (*Musa* spp.)	*C. musae*	Peres et al. (2002); Photita el al. (2004); Nuangmek, McKenzie & Lumyong (2008)
Chili (*Capsicum annuum*)	*C. acutatum*, *C. capsici*, *C. gloeosporioides*	Kanchana-udomkarn et al., 2004; Than, Jeewon et al. (2008); Kim et al. (2009); Ratanacherdchai et al. (2010)
Citrus spp.	*C. acutatum*, *C. gloeosporioides*	Hindorf (2000); Chen et al. (2005); Fischer, Ferreira, Spósito & Amorim (2009); MacKenzie et al. (2009)
Coffee (*Coffea arabica*)	*C. acutatum*, *C. asianum*, *C. boninense*, *C. capsici*, *C. fructicola*, *C. kahawae*, *C. gloeosporioides*, *C. siamense*	Nguyen, Sall, Bryngelsson & Liljeroth (2009); Prihastuti et al. (2009); Van der Vossen & Walyaro (2009)
Dragon fruit (*Hylocereus undatus*)	*C. gloeosporioides*	Masyahit, Sijam & Awang Satar (2009)
Durian (*Durio zibethinus*)	*C. gloeosporioides*	Alahakoon & Brown (1994); Pongpisutta & Sangchote (1994); Freeman et al. (1996)
Guava (*Psidium guajava*)	*C. acutatum*, *C. gloeosporioides*	Hindorf (2000); Alahakoon & Brown, (1994); Peres et al. (2002); Amusa, Ashaye, Amadi & Oladapo (2006)
Jasmine (*Jasminum sambac*)	*C. jasminigenum, C. jasmini-sambac*	Wikee et al. (2011)
Mango (*Mangifera indica*)	*C. acutatum*, *C. gloeosporioides*	Peres et al. (2002); Alahakoon & Brown, (1994); Kefialew & Ayalew (2008); Nelson (2008); Giblin et al. (2010)
Mangosteen (*Garcinia mangostana*)	*C. gloeosporioides*	Alahakoon & Brown (1994)

Table 2.1 (continued)

Fruit	*Colletotrichum* species	References
Passion fruit (*Passiflora* spp.)	*C. acutatum, C. gloeosporioides*	Hindorf (2000); Peres et al. (2002)
Papaya (*Carica papaya*)	*C. acutatum, C. capsici, C. gloeosporioides*	Peres et al. (2002); Rahman, Mahmud, Kadir, Rahman & Begum (2008)
Rose apple (*Syzygium jambos*)	*C. gloeosporioides*	Alahakoon & Brown (1994)
Rambutan (*Nephelium lappaceum*)	*C. gloeosporioides*	Farungsang, Farungsang & Sangchote, (1994); Wijeratnam, Dharmatilaka & Weerasinghe (2008)
Strawberry (*Fragria frageriae*)	*C. acutatum, C. gloeosporioides, C. fragariae*	Schiller, Luebeck, Sundeli & Melendex (2006); Jelev, Bobev, Minz, Maymon & Freeman (2008); Hyde et al. (2009); MacKenzie et al. (2009)
Therbroma cacao	*Colletotrichum ignotum*	Rojas et al. (2010)

Colletotrichum gloeosporioides, known as one of the world's most important pathogens, is a species complex comprising morphologically indistinguishable but genetically and biologically isolated species (Cai et al., 2009; Johnston et al., 2008). Lumping taxa into species complexes is of little practical use for plant pathologists because the complexes confer little information concerning pathogenicity, host range or other features. A milestone publication (Cannon et al., 2008) epitypified *C. gloeosporioides*, based on a collection from an orange in Italy, the original location of the type specimen cited in the prologue. Living strains and sequence data are therefore available from the epitype so that fresh isolates can be compared by DNA sequence analyses to confidently identify species, overcoming the inadequacies of traditional morphological identification (Cai et al., 2009; Hyde, Cai, Cannon et al., 2009). DNA characters are not directly influenced by environmental factors, and thus the combination of multigene sequence analysis along with traditional morphological identification has lead to a reliable

application for identifying species of *Colletotrichum* (Cai et al., 2009; Cannon et al., 2000; Prihastuti et al., 2009; Yang et al., 2009).

The objectives of this study were to characterize the species of *Colletotrichum* associated with fruit rots (anthracnose) in Laos and Thailand. The disease fruits were bough from markets and orchards and *Colletotrichum* were isolated. The fungal strains were studied morphologically characterized and molecular analysis with 5 genes sequencing, partial actin (ACT), β-tubulin-2 (TUB1, TUB2), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS), to establish their identities.

2.2 Materials and Methods

2.2.1 Fungal Isolation and Morphological Study

Colletotrichum strains were isolated from infected of fruits from gardens, orchards and local markets in Laos and Thailand (Figure 2.1). The four collecting sites were performed in Laos (Luang Prabang, Sayaboury, Vientiane, and Savannaketh Provinces) and three in Thailand (Chiang Mai, Bangkok and Nakhon Si Thammarat Provinces). Eight species of tropical fruits were collected comprising banana (*Musa* sp.), chilli (*Capsicum* sp.), guava (*Psidium guajava*), jujube (*Zizyphus mauritiane*), longan (*Dimocarpus longan*), mango (*Mangifera indica*), papaya (*Carica papaya*) and rose apple (*Syzygium jambos*).*Colletotrichum* were isolated using the protocol of Cai et al. (2009), The pathogens without visible sporulation: Plant tissues are cut into small pieces, surface sterilized by dipping in 1% sodium hypochlorite for 1 minute, in 70% ethanol for 1 minute and rinsed three times with sterilized water and finally dried on sterilized tissue paper. The plant tissues are then placed onto potato dextrose agar (PDA) containing 100 µg/ml streptomycin and 50 µg/ml tetracycline to allow fungal growth. The mycelia growing from pieces of plant tissues are transferred onto a new PDA plate for morphological or molecular study (Than, Prihastuti et al., 2008). Pathogens with visible sporulation: A single spore isolation technique should be applied to plant tissues where spore masses are formed. Spore masses are transferred with a sterilized wire loop or fine forceps and suspended in sterilized water. The spore suspension is diluted to a reasonable

concentration and spread onto the surface of PDA agar, followed by incubation overnight at 25˚C. Single germinated spores are picked up with a sterilized needle and transferred onto new PDA plate for further study.

Cultures were grown on potato dextrose agar (PDA) in which three 4 mm plugs were aseptically cut from the edges of actively growing areas of 5 days old cultures and transferred to new PDA plates. Cultures were incubated at 27˚C for 7 days. Colony characteristics of each fungal strain were done in triplicate with examination of colony diameter daily for 7 days for estimation of fungal growth rate, colony color, conidial mass. The conidial size and shape of approximately 20 conidia under microscopic examination were measured. The productions of appressoria were observed using slide culture technique as described by (Cai et al., 2009).

2.2.2 DNA Extraction, PCR Amplification and DNA Sequencing

Colletotrichum were grown on PDA and incubated for 7 days at 27˚C. Mycelia were scraped off from the surface of agar. Genomic DNA was extracted by using a Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the manufacturer's protocol. Quality and quantity of DNA were estimated visually by staining with ethidium bromide on 1% agarose gel electrophoresis. Partial actin (ACT), β-tubulin (TUB2), Glutamine Synthetase gene (GS), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) region from *Colletotrichum* strains were amplified by PCR as previously described (Prihastuti et al., 2009). Tubulin (TUB2) region was amplified by using primer Bt2a and Bt2b (Glass & Donaldson, 1995). The sequences of all primers used in this study were shown in Table 2.2.

The PCR conditions used were as follows: 3 minutes denaturing step at 95˚C followed by 34 cycles of 95˚C for 1 minute, 52˚C for 30 seconds, 72˚C for 1 minute and a final cycle of 10 minutes at 72˚C; for β-tubulin, rDNA-ITS region and actin gene; 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 for calmoudulin gene; 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 for glutamine synthetase and glyceraldehyde 3-phosphate dehydrogenase.

PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis and purified using the GFX PCR Purification Kit Amersham Biosciences) according to the manufacturer's protocol. Sequencing was carried out at the Shanghai Sangon Biological Engineering Technology and Services Co using forward and reverse primers.

Table 2.2 Primers Used in This Study

Names	Sequences	References
Actin		
ACT512F	ATGTGCAAGGCCGGTTTCGC	Cai et al. (2009)
ACT783R	TACGAGTCCTTCTGGCCCAT	
β-tubulin-2		
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Than, Prihastuti
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	et al. (2008)
rDNA-ITS region		
ITS 4	TCCTCCGCTTATTGATATGC	White, Bruns, Lee &
ITS 5	GGAAGTAAAAGTCGTAACAAGG	Taylor (1990)
Calmoudulin		
CL1	GARTWCAAGGAGGCCTTCTC	Cai et al. (2009)
CL2	TTTTTGCATCATGAGTTGGAC	
Glutamine Synthetase		
GSF1	ATGGCCGAGTACATCTGG	Guerber & Correll
GSR1	GAACCGTCGAAGTTCCAC	(2001)
Glyceraldehyde 3-phosphate		
dehydrogenase		Peres, Timmer,
GDF1	GCCGTCAACGACCCCTTCATTGA	Adaskaveg, Correll
GDR1	GGGTGGAGTCGTACTTGAGCATGT	(2005)

2.2.3 Phylogenetic Analyses

Sequences of *Colletotrichum* isolates from different hosts used in the phylogenetic analysis were shown in Table 2.3 Initially, all the sequences were aligned in Mega 4.1 (Tamura, Dudley, Nei & Kumar, 2007) and optimized manually to assure positional homology. Gaps were treated as missing data. Phylogenetic analyses were performed

using Mega 4.1 (Tamura et al., 2007). Maximum parsimony (MP) trees were obtained using the Close Neighbor-Interchange algorithm (Nei & Kumar, 2000) with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset. Clade stability of the tree resulting from maximum parsimony analysis was assessed by bootstrap analysis with 1000 replicates (Kishino & Hasegawa, 1989). The minimum evolution (ME) trees were also created and evaluated with 1,000 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei & Kumar, 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (30) at a search level of 1. The Neighbor-joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset. The model of evolution was estimated by using Mrmodeltest 2.2. Posterior probabilities (PP) (Bridge, Cannon, Buddie, Baker & Borman, 2008) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Nuangmek et al., 2008). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generation (resulting in 10,000 total trees). The first 2,000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 8,000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree.

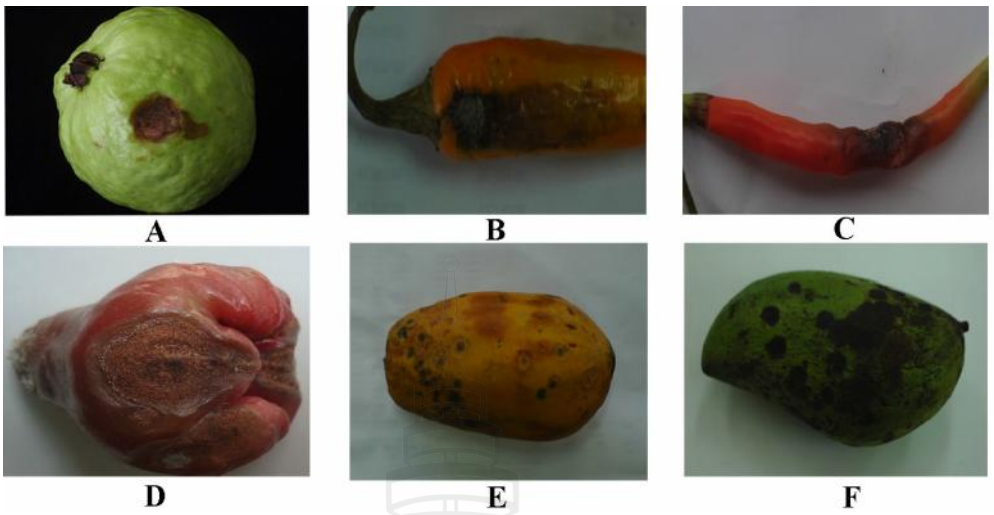


Figure 2.1 Representative of Anthracnose Disease on Tropical Fruits: A. Guava, B-C. Chilli, D. Rose Apple, E. Papaya, F. Mango

2.3 Results

2.3.1 Strain Isolation

Twenty-five *Colletotrichum* isolates from fruit rots of eight fruit hosts in Laos and Thailand (Table 2.3) were used in the phylogenetic analysis. The fruit rots varied from brown to black spots, and dark sunken lesions to light brown sunken areas (Figure2.1). All isolates are deposited in the culture collection of Mae Fah Luang Culture collection with some duplicates in the BIOTEC culture collection (BCC) and IFRDC (under MTA C01/2010). Hebarium material is deposited in Mae Fah Luang University (MFLU).

Sequence data from five gene fragments were analysed and the phylogenetic relationships of the 25 strains were inferred using the Maximum Parsimony (MP), Minimum Evolution (ME) and Bayesian analysis methods. One of the 72 equally parsimonious trees is shown in Figure2.2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The ME and Baysian tree is essentially similar to the MP tree and therefore not shown. MP bootstrap, ME bootstrap and Bayesian Posterior Probabilities are provided for the branches. The *Colletotrichum* isolates clustered into several different

lineages, which included named species and undetermined taxa. The phylogram in Figure 2.2 comprises two species complexes, i.e. "*acutatum*" complex (with five fusiform-spored strains), and the "*gloeosporioides*" complex (with 39 cylindrical spored strains). Each of these complexes has high bootstrap and posterior probability support.

Table 2.3 *Colletotrichum* Strains Causing Fruit Rot in Laos and Thailand

Fruit	MFU No.	Fungal Isolates	Conidia, Shape, Mean Length, Mean Width (µm)	Growth rate (mm day^{-1})
Chilli[1]	2091	*C. fructicola* (01)	Cylindrical, 17.26 ^{H}, 5.73 ^{D}, 10.35 ^{D}, 7.85 ^{A}	10.4^{F}
Chilli[2]	2092	Group A (02)	Cylindrical, 14.26 ^{I}, 4.8 ^{J}, 10 ^{D}, 5.41 ^{D}	11 ^{F}
Chilli[3]	2096	*C. asianum* (03)	Cylindrical, 15.86 ^{I}, 5.4 ^{F}, 10.69 ^{C}, 7.36 ^{B}	7.73^{F}
Chilli[4]	210	Group F (04)	Fusiform, 11.73 ^{J}, 4.13 ^{L}, 7.49 ^{H}, 6.53 ^{D}	7.53^{H}
Guava[2]	2105	Group A (05)	Cylindrical, 15.86 ^{I}, 5.2 ^{G}, 9.72 ^{D}, 7.77 ^{A}	11.4^{E}
Guava[5]	2107	Group A (06)	Cylindrical, 11.33 ^{J}, 4.5 ^{K}, 7.64 ^{H}, 5.41 ^{D}	6.13^{I}
Guava[5]	2107	Group A (06)	Cylindrical, 11.33 ^{J}, 4.5 ^{K}, 7.64 ^{H}, 5.41 ^{D}	6.13^{I}
Jujube[1]	2110	Group C (07)	Cylindrical, 17.2 ^{H}, 5.46 ^{F}, 9.73 ^{D}, 6.06 ^{D}	10.93 ^{F}
Jujube[5]	2112	Group C (08)	Cylindrical, 15.33 ^{I}, 5.13 ^{H}, 9.3 ^{G}, 6.11 ^{D}	10.6^{F}
Jujube[3]	2115	Group F (09)	Fusiform, 11.83 ^{J}, 5 ^{I}, 6.39 ^{H}, 5.83 ^{D}	4.26 ^{J}
Jujube[4]	2116	Group D (10)	Cylindrical, 17.60 ^{G}, 5.26 ^{G}, 10.2 ^{D}, 6.46 ^{D}	12^{C}
Jujube[4]	2155	Group C (24)	Cylindrical, 15.6 ^{I}, 5 ^{I}, 8.47 ^{G}., 5.9 ^{D}	9.73^{F}
Mango[2]	2120	*C. asianum* (11)	Cylindrical, 18 ^{G}, 5.53 ^{E}, 9.58 ^{E}, 6.67 ^{D}	10.4^{F}
Mango[5]	211	Group A (12)	Cylindrical, 22.26 ^{A}, 5.2 ^{G}, 8.61 ^{G}, 6.67 ^{D}	7.33^{H}
Mango[3]	2124	Group F (13)	Fusiform, 14.6 ^{I}, 4.33 ^{L}, 8.47 ^{G}, 6.59 ^{D}	5.33^{J}
Mango[6]	212	Group C (14)	Cylindrical, 20.6 ^{B}, 5.53 ^{E}, 10.13 ^{D}, 7.36 ^{B}	11.2^{F}
Papaya[1]	2131	*C. fructicola* (15)	Cylindrical, 15.06 ^{I}, 5 ^{I}, 9.2 ^{G}, 5.4 ^{D}	10.06^{F}
Papaya[2]	2133	Group B (16)	Cylindrical, 15.2 ^{I}, 4.7 ^{J}, 9.16 ^{G}, 6.25 ^{D}	11.1^{F}
Papaya[7]	213	Group A (17)	Cylindrical, 17.73 ^{G}, 6.4 ^{A}, 9.37 ^{F}, 7.22 ^{C}	12.6^{A}
Papaya[4]	2138	Group D (18)	Cylindrical, 20.2 ^{C}, 5 ^{I}, 11.53 ^{B}, 7.77 ^{A}	9.86^{F}
Rose apple[1]	2142	Group C (19)	Cylindrical, 18.8 ^{F}, 6.13 ^{B}, 9.44 ^{F}, 6.66 ^{D}	10.73^{F}
Rose apple[5]	214	Group D (20)	Cylindrical, 17.4 ^{G}, 6.26 ^{B}, 12.22 ^{A}, 6.87 ^{C}	9.66^{F}
Rose apple[4]	2149	Species E (21)	Cylindrical, 19.46 ^{D}, 5.53 ^{E}, 8.47 ^{G}, 5.9 ^{D}	12.53^{B}
Rose apple[6]	2151	Group C (22)	Cylindrical, 14.33 ^{I}, 4.66 ^{J}, 9.72 ^{D}, 7.22 ^{C}	11.4^{E}
Longan[1]	2154	*C. fructicola* (23)	Cylindrical, 19.2 ^{E}, 6 ^{C}, 8.61 ^{G}, 6.25 ^{D}	11.6 ^{D}
Banana[2]	2156	Group B (25)	Cylindrical, 19.2 ^{E}, 6 ^{C}, 7.29 ^{H}, 6.87 ^{C}	12^{C}
LSD (between group) 1.35, 0.3, 1.68, 1.18				

Note. [1] = Chiang Mai, Thailand; [2] = Bangkok, Thailand; [3] = Luangprabang, Laos; [4] = Vientiane, Laos; [5] = Nakhon Si Thammarat, Thailand; [6] = Savannakhet, Laos; [7] = Sayaboury, Laos

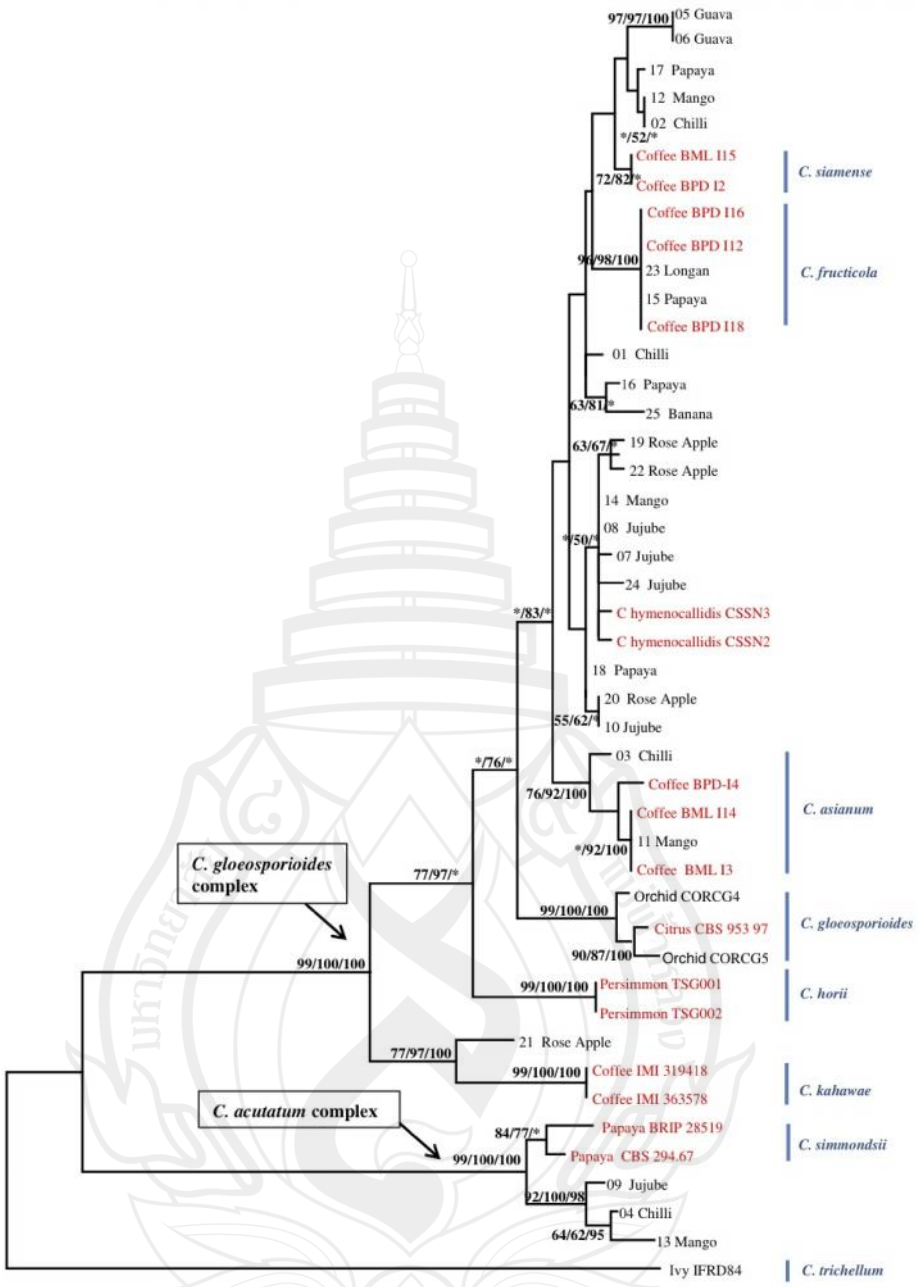


Figure 2.2 Phylogram of Tree Generated Maximum Parsimony Analysis Based on Combined ACT, TUB1, GPDH, ITS and TUB2 Sequences

Table 2.4 Sequences Used in the Phylogenetic Analysis

Isolate No.	Strain numbers	Species	GenBank Accession Number				
			ACT	TUB-1	GPDH	ITS	TUB-2
01	MFU090616	*Colletotrichum* sp.	HM038263	HM038269	HM038319	HM038348	HM038310
02	MFU090617	*Colletotrichum* sp.	HM038250	HM038270	HM038315	HM038354	HM038294
03	MFU090618	*C.asianum*	HM038242	HM038267	HM038313	HM038342	HM038292
04	MFU090619	*Colletotrichum* sp.	HM038264	HM038291	HM038335	HM038360	-
05	MFU090620	*Colletotrichum* sp.	HM038244	HM038285	HM038333	HM038353	HM038305
06	MFU090621	*Colletotrichum* sp.	HM038246	HM038286	HM038316	HM038341	HM038297
07	MFU090622	*Colletotrichum* sp.	HM038253	HM038276	HM038331	HM038340	HM038303
08	MFU090623	*Colletotrichum* sp.	HM038255	HM038283	HM038324	HM038338	HM038307
09	MFU090624	*Colletotrichum* sp.	HM038265	HM038289	HM038337	HM038362	-
10	MFU090625	*Colletotrichum* sp.	HM038258	HM038277	HM038327	HM038339	HM038306
11	MFU090626	*C.asianum*	HM038243	HM038268	HM038314	HM038343	HM038293
12	MFU090627	*Colletotrichum* sp.	HM038245	HM038282	HM038317	HM038351	HM038308
13	MFU090628	*Colletotrichum* sp.	HM038266	HM038290	HM038336	HM038361	-
14	MFU090629	*Colletotrichum* sp.	HM038249	HM038271	HM038328	HM038346	HM038304
15	MFU090630	*C.fructicola*	HM038251	HM038272	HM038321	HM038356	HM038302
16	MFU090631	*Colletotrichum* sp.	HM038261	HM038280	HM038322	HM038355	HM038295
17	MFU090632	*Colletotrichum* sp.	HM038254	HM038279	HM038332	HM038352	HM038296
18	MFU090633	*Colletotrichum* sp.	HM038256	HM038274	HM038325	HM038349	HM038299
19	MFU090634	*Colletotrichum* sp.	HM038247	HM038281	HM038323	HM038344	-
20	MFU090635	*Colletotrichum* sp.	HM038259	HM038287	HM038318	HM038345	HM038300
21	MFU090636	*Colletotrichum* sp.	HM038260	HM038288	HM038334	HM038359	HM038301
22	MFU090637	*Colletotrichum* sp.	HM038248	HM038284	HM038329	HM038347	HM038311
23	MFU090638	*C.fructicola*	HM038252	HM038273	HM038320	HM038357	HM038298
24	MFU090639	*Colletotrichum* sp.	HM038257	HM038275	HM038330	HM038350	HM038309
25	MFU090640	*Colletotrichum* sp.	HM038262	HM038278	HM038326	HM038358	HM038312
	CBS953.97b	*C.gloeosporioides*	FJ907430	-	FJ972582	FJ972609	FJ907445
	CORCG4a	*C.gloeosporioides*	HM034800	-	HM034806	HM034808	HM034810
	CORCG5a	*C.gloeosporioides*	HM034801	-	HM034807	HM034809	HM034811

The "*gloeosporioides*" complex consisted of 22 strains isolated from fruits in this study, and 17 reference strains that are either ex-types or voucher strains with confirmed identities. The latter includes *C.asianum, C.fructicola, C.kahawae, C.gloeosporioides* and *C. siamense* represented by ex-holotype, ex-paratype or ex-epitype strains, and *C.horii* represented by voucher strains (Figure 2.2). *C.gloeosporioides* sensu stricto is represented by the ex-epitype (IMI 356878=CBS 953.97) and two verified strains isolated in a

different study from *Vanda* sp. from China. None of the 22 strains from Asian fruits clustered with the ex-epitype of *C.gloeosporioides* and therefore should belong to other species. *C.asianum*, *C.fructicola*, *C.kahawae*, *C.gloeosporioides* and *C.horii* are well resolved in the phylogenetic tree with high bootstrap and posterior probabilities support, while *C.siamense* received only moderate support (Figure 2.2). Among the 22 strains from fruit, four strains could be confidently classified as *C.fructicola* (No. 15 and 23) or *C.asianum* (No. 3 and 11), while all other 18 strains could not be confidently identified and some may represent novel species (Figure 2.2).

The "*acutatum*" complex comprised the ex-holotype culture of *C.simmondsii* (BRIP 28519) from *Carica papaya* in Australia and three undetermined strains which occurred on chilli, jujube and mango (Figure 2.2). The three isolates had similar cultural characteristics, relatively short fusiform ascospores (11.7–14.6×4.1–5 μm) and a slow growth rate (6.4–8.5 mm per day) and is not conspecific with *C.acutatum* (Guerber & Correll, 2001; Vinnere, Fatehi, Wright & Gerhardson, 2002).

2.3.2 Morphology, Culture Characteristics and Growth Rate

Morphological features were determined and correlated with the species or complexes determined in the phylogenetic tree. Isolates in the "*gloeosporioides*" complex produced two types of conidia: those with cylindrical conidia with obtuse ends (oblong) and narrowing at the centre, and those with obtuse to slightly rounded ends and not narrowed. Isolates in the "*acutatum*" complex produced conidia that were fusiform with obtuse to slightly rounded ends. There were statistically significant differences in length and width of conidia among some strains (Figure 2.3), however, conidial morphology could not be confidently used alone to determine species within a complex. Similarly, appressoria were produced by all isolates in slide cultures and varied from ovoid, clavate, to irregular. The size and shape of appressoria also failed to distinguish amongst species (Figure 2.3).

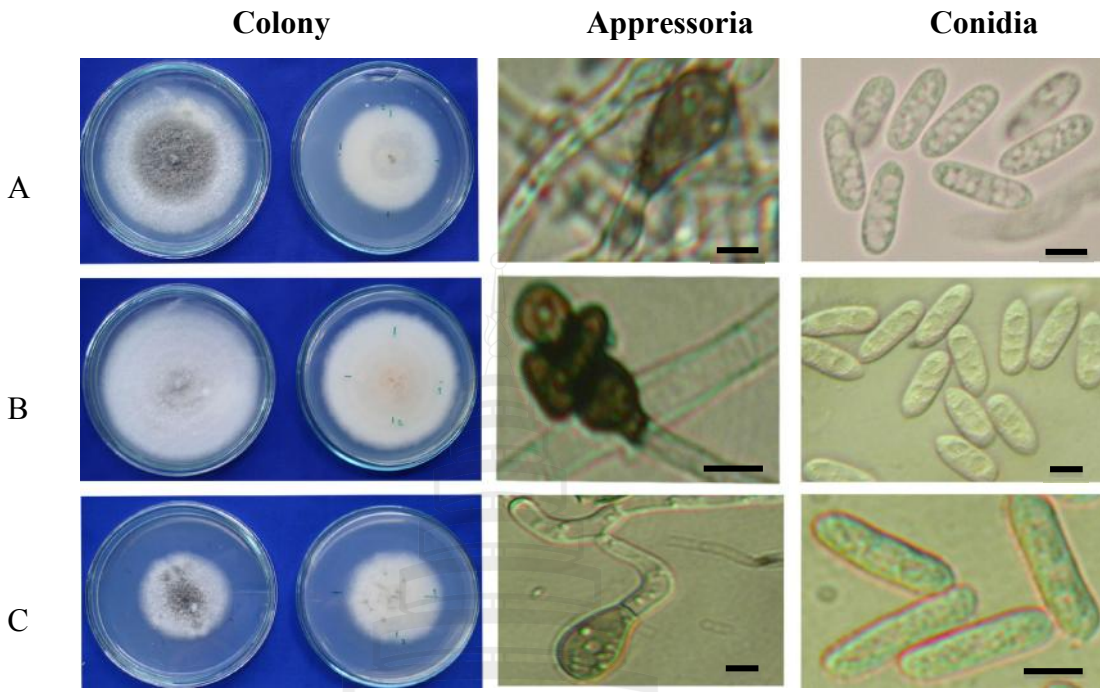


Figure 2.3 Morphological Structures of Some *Colletotrichum* Representatives (Bars = 10 µm), A.*C. cordylinicola*, B.*C. asianum*, *C. fructicola*, Left *Colletotrichum* Colony, Middle Appressoria, Right Conidias

Based on similarities in colony characteristics following growth on PDA for 7 days at 27˚C, the isolates could be grouped into seven morphotypes (Lacap, Liew & Hyde, 2003). The grouping of morphotypes had little relationship to the *Colletotrichum* species. There were differences in growth rates of individual strains, but it was not possible to differentiate between species and undetermined taxa based on growth rates.

2.4 Discussion

Although *C.gloeosporioides*, and to a lesser extent *C.acutatum*, has previously been shown to be the causal agent of tropical fruit rots, the most striking discovery of this study is that none of the 25 strains isolated from Laos and Thai fruits was either of these species. Previous understanding that anthracnose of most tropical fruits is caused by

C.gloeosporioides or *C.acutatum* should therefore be reconsidered. Phylogenetic analysis however, showed that most of the strains included in this study belong to the "*gloeosporioides*" complex. The 23 strains in the "*gloeosporioides*" complex clustered into several different clades, some of which were statistically well-supported monophyletic groups. Only four of these strains could be assigned to known species, i.e. *C.asianum* and *C.fructicola*, based on a conjunction of phenotypic characters and genealogical concordance species recognition (Cai et al., 2009). The remaining 19 strains may represent undescribed species that cannot be placed in any known species with the current data.

Strains isolated from the same host may contain more than one phylogenetic species. For example, four strains isolated from papaya clustered in four different phylogenetic clades; four strains isolated from mango also appeared in four different phylogenetic clades; five strains from jujube clustered in three different clades; four strains from rose apple were in three clades, while four from chilli scattered in four different clades. These results provide further evidence that one plant may often host more than one pathogenic *Colletotrichum* species and using host as a taxonomic criterion may result in significant misidentification and confusion.

ITS sequences of the three strains excluded from the "*gloeosporioides*" complex were incorporated into the backbone tree (Cai et al., 2009) and it was found that they are most closely related to *C.simmondsii* in the "*acutatum*" complex. Multigene phylogeny showed that these three strains clustered in a sister clade to *C.simmondsii* with strong statistical support and probably represent a novel species. The holotype of *C.acutatum* was described from papaya in Queensland (Than, Jeewon et al., 2008; Than, Prihastuti, 2008) and *C.acutatum* sensu stricto was not found on fruits in this study. Although *C.acutatum* has been reported to cause anthracnose of chilli fruits in Thailand (Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008; Than, Shivas et al., 2008) this was later revealed to be *C.simmondsii* following further molecular analysis (Shivas & Tan, 2009). Therefore it is unclear if *C.acutatum* occurs in Laos or Thailand.

The discovery of new species within the "*gloeosporioides*" complex is not surprising. *Colletotrichum* species on coffee berries have been extensively studied and numerous strains in the "*gloeosporioides*" complex have been isolated. Before 1993, *C.gloeosporioides* was initially thought to be the cause of all coffee berry disease, but a

new species, *C.kahawae*, for the highly pathogenic strain causing coffee berry disease in Africa was established based on differences in growth rate and biochemical characters (Waller et al., 1992). *C.kahawae* however, could not be differentiated from *C.gloeosporioides* using morphological characters. The validity of *C.kahawae* was therefore questioned for many years (Cannon et al., 2000; Correll, Guerber, Wasilwa, Sherrill & Morelock, 2000; Varzea, Rodrigues & Lewis, 2002) and only recently has it been confidently supported in various molecular phylogenetic studies (Bridge et al., 2008; Cai et al., 2009; Prihastuti et al., 2009). Previous studies of anthracnose on coffee berries in northern Thailand (Prihastuti et al., 2009) established three new species that bear both phenotypic distinctions and evolutionary distances from *C.gloeosporioides* and two of these species (*C.asianum* and *C.fructicola*) are resolved with strong support in Figure 2.2 These studies of *Colletotrichum* on coffee berries provide a good example of how *C.gloeosporioides* has previously been misunderstood.

The new *Colletotrichum* species isolated from coffee in Thailand (Prihastuti et al., 2009) were epiphytes, endophytes, or pathogens. In the present study, two of these species, *C.asianum* and *C.fructicola* were found to infect other fruits (Figure 2.2). However, *C.siamense* from coffee (Prihastuti et al., 2009) was not isolated in the present study, although Yang et al. (2009) isolated it from *Hymenocallis* sp. It therefore appears that some species of *Colletotrichum* such as *C. horii* (on persimmon) and *C.kahawae* (on coffee) may be restricted to certain hosts or families, while others have a wide host range. This is particular true of *C.fructicola* which has now been isolated from coffee, *Crinum asiaticum*, longan and papaya (Prihastuti et al., 2009; Yang et al., 2009; Figure 2.2). *Colletotrichum gloeosporioides* was not isolated from any of the fruits used in this study, but it is not restricted to citrus as two strains used in Figure 2.2 were isolated from *Vanda* sp.

The strains used in this study cluster into two species complexes ("*acutatum*" and "*gloeosporioides*") with high bootstrap support (Figure 2.2) and confirm that the taxa in these complexes are distinct. The "*gloeosporioides*" complex presently comprises *C.asianum*, *C.fructicola*, *C. horii*, *C. hymenocallidis*, *C.kahawae*, *C.gloeosporioides* and *C.siamense* (Cai et al., 2009; Johnston et al., 2008; Prihastuti et al., 2009; Yang et al., 2009), plus several further species that need epitypifying (e.g. *C.musae)*. This study has revealed that several novel species appear to occur on tropical fruits in Laos and Thailand

and therefore the number of novel species worldwide may be large. The "*acutatum*" complex currently comprises *C.simmondsii*, *C.fioriniae*, *C.lupinii* and *C.simmondsii* (Shivas & Tan, 2009), plus the potential new species isolated in this study (Figure 2.2). There is a lot more work needed before we can fully understand the genus.

Analysis of the usefulness of morphological characters and growth rate in distinguishing *Colletotrichum* species was carried out. Cultural characters, conidia and appressoria morphology and growth rate have previously been used in combination to differentiate species (Prihastuti et al., 2009; Yang et al., 2009). The combination of phenotypic characters is important in distinguishing genetically isolated but morphologically similar species, for example, *Colletotrichum gloeosporioides* and *C.kahawae*. Further study is needed to correlate the phenotypic characters with the phylogenetic lineages for those unclassified strains in this study.

ITS sequence data which has been generally adopted for barcoding fungi (Seifert, 2009), is inadequate for this genus as previously noted (Cai et al., 2009; Prihastuti et al., 2009; Yang et al., 2009). ITS sequence data is useful for placing species in a species complex and also providing an idea which species in a complex to which it can be assigned. However, other genes are needed to infer robust phylogenetic relationships and provide high confidence for species clusters. In this study, we used five gene fragments, and this has provided a relatively robust support for species within the '*gloeosporioides*' complex. *Colletotrichum asianum*, *C.fructicola*, *C. horii*, *C.kahawae*, *C.gloeosporioides* all receive high support as distinct phylogenetic lineages. Some strains that failed to cluster in any of above may contain novel species and further study is needed to confidently infer relationships. It is not the purpose of this paper to introduce new species but to show that our previous knowledge concerning infection of tropical fruits by *C.acutatum* and *C.gloeosporioides* in Laos and Thailand and probably elsewhere is incorrect. We will carry out further work concerning the resolution and designation of new species.

Correct identification of fungal pathogens is essential for quarantine decisions, in plant breeding, and in pathogen management and control (Cai et al., 2009). The systematic scheme for *Colletotrichum* species is now in a state of transition. Previously applied concepts using host and morphological characters to define species have been shown to be artificial and do not agree with evolutionary relationships. Numerous studies

have shown that one strain can often infect several hosts, whilst morphological characters are highly dependent on environmental factors (von Arx 1957; Sutton, 1992). The discovery in the current study is a reflection that host groups do not correlate with the phylogenetic groups. It has been proposed that species concepts in *Colletotrichum* should be based on multiple gene phylogeny and correlation between the genotype and phenotype (Cai et al., 2009). This is a sensible trend for future studies.

The findings presented here will have significant impact on many aspects of plant pathology, in particular, species identification, quarantine, plant breeding, and disease management and control. For example, the "*gloeosporioides*" complex on coffee berries has been well characterized and several distinct genetic and phenotypic species have been established (Prihastuti et al., 2009; Waller, Bridge, Black & Hakiza, 1993). Among these *C.kahawae* is a strongly aggressive pathogen specific to coffee in Africa (Waller et al., 1993) and should not be allowed to 'enter' into other continents by continuing with strict uarantine protocols. On the other hand, *C.asianum*, *C.fructicola* and *C.siamense* are opportunistic pathogens of coffee berries (Prihastuti et al., 2009) and appear to have a wide host range, thus are not of quarantine significance. The current study also indicates that morphologically similar isolates from chilli, mango, papaya, rose apple and jujube may also comprise more than one distinct species, and these species are currently poorly defined and characterized. Epitypification combined with analysis and comparison of sequence data will fundamentally change the understanding of species relationships, not only in *Colletotrichum*, but also in many other important plant pathogenic genera (e.g. Botryosphaeria, Fusarium, Pestalotiopsis, Phomopsis and Phyllosticta). Researchers must therefore transfer new knowledge to quarantine authorities so they can rapidly establish new quarantine protocols.

2.5 Conclusion

Colletotrichum gloeosporioides has been reported as one of the most important pathogens worldwide that infect at least 1000 plant species. Fruit rots (anthracnose) are often attributed to *C. gloeosporioides* and, to a lesser extent, to *C. acutatum.* These previous findings were, however, based on morphological identification or, if gene

sequence data were used, comparisons were often made with wrongly applied names. *Colletotrichum gloeosporioides* was recently epitypified so that living cultures and sequence data are, for first time available for comparison with fresh collections. Analysis of sequence data of 25 isolates from eight tropical fruits are compared with the *C. gloeosporioides* epitype. Contrary to previous understanding, none of the 25 *Colletotrichum* isolates from tropical fruits was *C. gloeosporioides*. The five gene regions used in this study resolved *Colletotrichum asianum, C. fructicola, C. horii, C. kahawae* and *C. gloeosporioides* in the "gloeosporioides" complex as distinct phylogenetic lineages with high statistical support. Some other likely novel species in the "gloeosporioides" complex and *C.siamense*, however, received only moderate or low support and further studies are needed to clarify their phylogenetic affinities and taxonomic placements. Cultural, conidial and appressorial characters can be used to differentiate taxa into species complexes, but cannot separate species within a complex. This discovery will have significant impacts on many aspects of plant pathology, pathogen diagnosis, quarantine decisions, plant breeding, and plant disease management and control and these are discussed.

CHAPTER 3

Colletotrichum SPECIES CAUSING ANTRACNOSE ON LEAVES IN LAOS AND THAILAND

3.1 Introduction

Colletotrichum is an important, cosmopolitan, phytopathogenic genus (Sutton, 1992; Freeman et al., 1998; Than et al., 2008; Crouch et al., 2009; Damm et al., 2009; Hyde, Cai, L., McKenzie et al., 2009; Prihastuti et al., 2009; Wikee et al., 2011). It is well known that a single host species can be infected by more than one *Colletotrichum* species and an individual *Colletotrichum* species may infect several different host species (Cai et al., 2009; Crouch & Beirn, 2009; Hyde, Cai, Cannon et al., 2009; Phoulivong, 2011; Yang et al., 2011). *Colletotrichum* species affect all above ground plant parts and cause yield and quality reduction. For example, *Colletotrichum* spp. cause extensive pre- and postharvest damage to chili fruits, with yield losses up to 50% (Manandhar, Hartman & Wang, 1995; Pakdeevaraporn et al., 2005). Roots may also be affected. For example, *C. acutatum* J.H. Simmonds has been isolated from necrotic roots of stunted, chlorotic strawberry plants and also from the rhizosphere of diseased plants (Freeman & Katan, 1997). *Colletotrichum graminicola* (Ces.) G.W. Wilson may also infect the roots of maize as a soil-borne pathogen but is symptomless on above-ground plant parts (Sukno, Garcia, Shaw & Thon, 2008).

Colletotrichum species have been reported to cause the anthracnose disease on leaves of several plants (Table 3.1).

Table 3.1 *Colletotrichum* Species Causing Anthracnose on Leaves

***Colletotrichum* species**	**Plant leaves**	**References**
C. acutatum	*Aspalathus linearis*	Dam et al. (2012)
C. asianum	*Coffee arabica*	Prihastuti et al. (2012)
C. boninense	*Crinum asiaticum*	Weir, Johnston & Damm (2012)
C. fructicola	*Ficus edelis*	Weir et al. (2012)
C.gloeosporioides	*Carya illinoinensis*	Weir et al. (2012)
C. godetiae	*Mahonia aquifolium*	Weir et al (2012)
C. lupini	*Lupinus albus*	Dam et al. (2012)
C. phormii	*Phornium tenax*	Dam et al. (2012)
C. sloanei	*Theobroma cacao*	Dam et al. (2012)

Colletotrichum classification is presently undergoing substantial revision and several species have been introduced following typification of species in some of the important species complexes, such as *C. gloeosporioides* (Penz.) Penz. and Sacc. (Cannon et al., 2008), *C. falcatum* Went (Prihastuti et al., 2010), *C. musae* (Berk and M. A. Curtis) Arx (Su et al., 2011) and *C. coccodes* (Wallr.) S. Hughes (Liu, Hyde & Cai, 2011). Molecular characteristics have become increasingly important in the identification of *Colletotrichum* species (Cai et al., 2009; Hyde et al., 2010; Phoulivong et al., 2010). The internal transcribed spacer (ITS) is the most widely sequenced region, but ITS sequences alone cannot be used for confident species delineation, especially for the *C. gloeosporioides* complex (Cai et al., 2009). (Gazis, Rehner & Chaverri, 2011). Multi-locus phylogeny has been widely applied to decrease subjectivity in species identification and based on this several new species have been described, e.g., *C. asianum* Prihast., L. Cai and K. D. Hyde, *C. codylinicola* Phoulivong, L. Cai and K. D. Hyde (Phoulivong et al., 2009), *C. fructicola* Prihast., L. Cai and K. D. Hyde, *C. siamense* Prihast., L. Cai and K. D. Hyde (Prihastuti et al., 2009), *C. cliviae* Y. L. Yang, Zuo Y. Liu, K. D. Hyde and L. Cai, *C. hippeastri* Yan L. Yang, Zuo Y. Liu, K. D. Hyde and L. Cai *C. hymenocallidis* Yan L. Yang, Zuo Y. Liu, K. D. Hyde and L. Cai (Yang et al., 2009), *C. simmondsii* R.

G. Shivas and Y. P. Tan (Shivas & Tan, 2009), and *C. jasminigenum* Wikee, K. D. Hyde, L. Cai and McKenzie (Wikee et al., 2011). In the present study, we introduce four new species from Thailand based on multi-locus phylogenetic analysis and morphology.

Figure 3.1 Symptoms of *Colletotrichum* sp. Causing Anthracnose Disease on Leaves: A.*Alocasia indica*, B.*Aglaonema* sp., C.*Scindapsus aureus*, D.*Dracaena fragrans* and E.*Sansevieria* sp.

3.2 Materials and Methods

3.2.1 *Colletotrichum* Isolates From Plant Leaves Disease

Colletotrichum isolates were collected from the leaf spots in various hosts and different collecting sites including Luang Prabang, Sayaboury, Vientiane, Savannaketh Provinces in Laos and Chiang Mai, Chiang Rai and Nakhon Si Thammarat provinces in

Thailand. The samples were incubated in moist chambers to assist sporulation. The fungi (see Table 3.2) were isolated by picking conidia directly from conidial masses on lesions, suspending in sterilized water and streaking onto WA media. After overnight incubation at room temperature, single germinated conidia were transferred to PDA plates.

3.2.2 Identification of *Colletotrichum* Strain

All *Colletotrichum* obtained were characterized based on morphological characters including colony characteristics, growth rate, conidial size and shape, appressoria as described previously (Section 2.2.1). To confirm the *Colletotrichum* species identification, the multi-gene analysis including partial actin (ACT), β-tubulin (TUB2), glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and the complete rDNA-ITS (ITS) was performed as described (Section 2.2.2).

Table 3.2 *Colletotrichum* Strains Causing Anthracnose Disease on Leaves

No.	MFU Code	Host Name	Collecting Sites
1	MFU09 0481	*Cordyline fruticosa*	Maejo, Chiang Mai
2	MFU09 0482	*Cordyline fruticosa*	Doi Luang, Chiang Rai
3	MFU09 0483	*Caryota mitis*	Doi Luang, Chiang Rai
4	MFU09 0484	*Arachis hypogaea*	Wieng Chiang Rung, Chiang Rai
5	MFU09 0486	*Morinda citriforia*	Wieng Chiang Rung, Chian Rai
6	MFU09 0487	*Morinda citriforia*	Wieng Chiang Rung, Chiang Rai
7	MFU09 0488	*Artocarpus heterophyllus*	Wieng Chiang Rung, Chiang Rai
8	MFU09 0489	*Artocarpus heterophyllus*	Maejo, Chiang Mai
9	MFU09 0490	*Dracaena sanderiana*	Maejo, Chiang Mai
10	MFU09 0491	*Dracaena sanderiana*	Wieng Chiang Rung
11	MFU09 0492	*Dracaena sanderiana*	Bangkok
12	MFU09 0493	*Dracaena sanderiana*	Bangkok
13	MFU09 0494	*Sansevieria* sp.	Wieng Chiang Rung, Chiang Rai
14	MFU09 0495	*Sansevieria* sp.	Wieng Chiang Rung, Chiang Rai

Table 3.2 (continued)

No.	MFU Code	Host Name	Collecting Sites
15	MFU09 0496	*Aglaonema tenuipes*	Maejo, Chiang Mai
16	MFU09 0497	*Aglaonema tenuipes*	Wieng Chiang Rung, Chiang Rai
17	MFU09 0498	*Alocasia calidora*	Doi Tung, ChiangRai
18	MFU09 0499	*Alocasia calidora*	Mae Fah Luang University
19	MFU09 0500	*Dieffenbachia maculate*	Wieng Chiang Rung, Chiang Rai
20	MFU09 0501	*Dieffenbachia maculate*	Bangkok
26	MFU09 0507	*Bauhinia variegate*	Wieng Chiang Rung, Chiang Rai
27	MFU09 0508	*Dracaena loureiri*	Bangkok
28	MFU09 0509	*Dracaena loureiri*	Maejo, Chiang Mai
29	MFU09 0510	*Dracaena loureiri*	Bangkok
30	MFU09 0511	*Dracaena loureiri*	Wieng Chiang Rung, Chiang Rai
31	MFU09 0512	*Saccharum officinarum*	Wieng Chiang Rung, Chiang Rai
32	MFU09 0513	*Saccharum officinarum*	Wieng Chiang Rung, Chiang Rai
33	MFU09 0514	*Cactus* sp.	Maejo, Chiang Mai
34	MFU09 0515	*Piper nigrum*	Maejo, Chiang Mai
35	MFU09 0516	*Piper nigrum*	Maejo, Chiang Mai
36	MFU09 0517	*Cactus* sp.	Doi Mae Fah Lung
37	MFU09 0518	*Morus alba*	Wieng Chiang Rung, Chiang Rai
38	MFU09 0519	*Houttuynia cordata*	Wieng Chiang Rung, Chiang Rai
40	MFU09 0521	*Hevea brasiliensis*	Maejo, Chiang Mai
41	MFU09 0522	*Hevea brasiliensis*	Maejo, Chiang Mai
42	MFU09 0523	*Dracaena fragrans*	Wieng Chiang Rung, Chiang Rai
43	MFU09 0524	*Dracaena fragrans*	Bangkok
44	MFU09 0525	*Coccinia grandis*	Wieng Chiang Rung, Chiang Rai
45	MFU09 0526	*Iris* sp.	Maejo, Chiang Mai
46	MFU09 0528	*Spathoglottis*	Kounkorn water fall, Chiang Rai
47	MFU09 0529	*Diplaziium esculentum*	Wieng Chiang Rung, ChiangRai
48	MFU09 0530	*Scindapsus aureus*	Bangkok
49	MFU09 0531	*Scindapsus aureus*	Bangkok
50	MFU09 0532	*Scindapsus aureus*	Bangkok
51	MFU09 0537	*Ananas comosu*	Nakhon Si Thammarat

Table 3.2 (continued)

No.	MFU Code	Host Name	Collecting Sites
52	MFU09 0538	*Citrus maxima*	Chiang Mai
53	MFU09 0540	*Citrus aurantifolia*	Chiang Mai
54	MFU09 0542	*Hibiscus rosa*	Mae Fah Luang University
55	MFU09 0543	*Allium ascalonicum*	Wieng Chiang Rung, Chiang Rai
56	MFUCC090551	*Codyline fruiticosa*	Maejo, Chiang Mai, Thailand

3.3 Results

3.3.1 *Colletotrichum* Strains Isolated From Anthracnose Disease on Leaves

Fifty-six isolates of *Colletotrichum* species causing disease on leaves were isolated from 22 hosts from Laos and Thailand (Table 3.2). All of these *Colletotrichum* strains were morphologically characterized and could be classified in the *Colletotrichum* genus. Besides, their species identity was further confirmed using the ITS-rRNA region in which only a few species were *C.gloeosporioides*. Based on the ITS-gene sequence analysis, the *Colletotrichum* species causing anthracnose on these plant leaves included *C. acutatum*, *C. capsici* and *C. gloeosporiodes* (data not shown).

Interestingly, there were four new species of *Colletotrichum* which were isolated from the plant leaves of *Cordyline fruiticosa*, *Hibiscus rosasinensis*, *Citrus maxima*, *Neoregalia* sp. (Table 3.2) These four new species were initially recognized by their distinct morphologies and the data obtained from the ITS-rRNA gene sequence also reveled that these species were grouped in the distinct clade. Subsequent study was then performed using the muli-gene analysis to further confirm their identity.

3.3.2 *Colletotrichum cordylinicola* Phoulivong, L. Cai &K.D. Hyde, sp. nov.

MycoBank 518577

Coloniae crescentes post 7 dies in PDA ad 27˚C 75 mm diam. Conidiogenae producentia in acervulis, tubulosa. Conidia 11–20 × 4–5 µm, unicellularae, hyalinae, cylindrici, laeviaad apicem obtuse. Appressoria 13–13.4 × 7.2–7.3 µm, brunnea vel atrobrunnea, irregulariter ovoidea vel clavati (Figure 3.3).

Holotype: Thailand, Chiang Mai Province, San Sai District, Maejo Village, on *Cordyline fruticosa* (L.) A. Chev. (Agavaceae), 15 March 2009, Sitthisack Phoulivong, MFLU10 0289; extype living culture MFUCC 090551, BCC 38872 and CGMCC.

Etymology: Referring to the host genus *Cordyline*.

Colonies on PDA attaining 75 mm diam. in 7 days at 27˚C, growth rate 10.8–11.6 mm/day (mean= 11.2, n = 5), white, sparse, with grey-orange visible conidial masses and with floccose aerial mycelia in centre, reverse slightly greenish. Sclerotia absent. Setae absent. Conidiophores congregative, straight or geniculate, produced in the acervuli. Conidia 11–20 × 4–5 µm (mean = 15.37 ± 0.6 × 4.5 ± 0.56, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, guttulate. Spore germination on PDA mostly observed near the apex of the conidia, sometimes from the centre. Appressoria in slide culture 13–13.4 × 7.2–7.3 µm (mean = 13.20 ± 0.94 × 7.25 ± 0.61, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age.

Teleomorph - not produced in culture.

Known distribution - Thailand and Laos.

Additional Specimens Examined: Laos, Vientiane, Pakngum District, Don-ngaeng Village, on rose apple fruit (*Eugenia javanica* Lam. (Myrtaceae), 26 September 2007, Sitthisack Phoulivong, MFLU10 0290, living culture MFLU 09 0636, IFRD 2149, BCC38864, CGMCC 3.14199. Thailand, Chiang Rai, Doi Tung, on *Cordyline fruticosa*, Noireung Parinn, MFLU10 0291, living cultures MFLU 100132, CGMCC 3.14200.

Phylogenetic study

The dataset of six combined genes comprised 2506 characters after alignment, of which 545 characters were parsimony informative (21.7%). The KH test showed that the two trees inferred from parsimonious analysis were not significantly different. One of the

most parsimonious trees (TL = 1377, CI = 0.895, RI = 0.881, RC = 0.798, HI = 0.105) generated from dataset of six combined gene regions is shown in Figure 3.2 The phylograms inferred from single genes ACT, GS, TUB2, ITS, CAL and GPDH show similar topology as that from combined datasets but with much lower statistical support for branches. In the phylogenetic tree, three strains of *C. cordylinicola* clustered in a distinct lineage and appeared as a sister clade to *C. kahawae* (100% bootstrap and posterior probability) (Figure 3.2). Other reference taxa employed in the analysis include type strains of *C. simmondsii, C. asianum, C. fructicola, C. gloeosporioides, C. kahawae, C. siamense* and authentic strains of *C. horii* (Table 3.3).

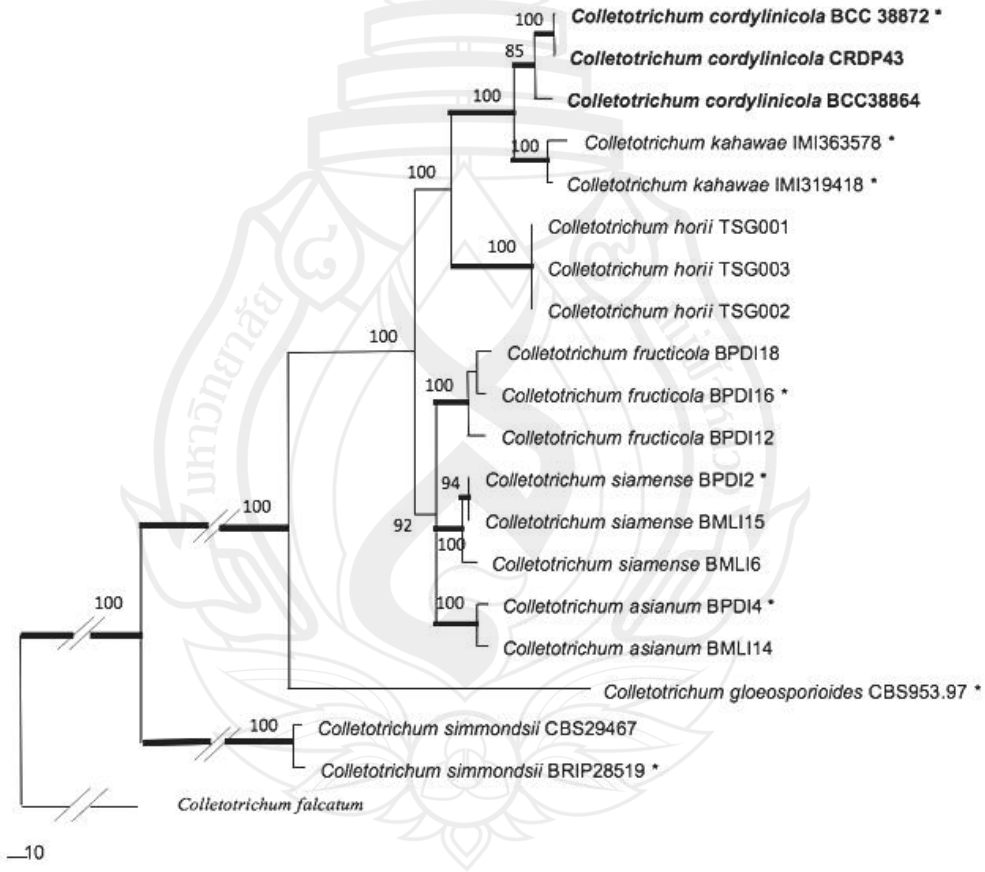


Figure 3.2 Maximum Parsimony Phylogram Showing Phylogenetic Relationships

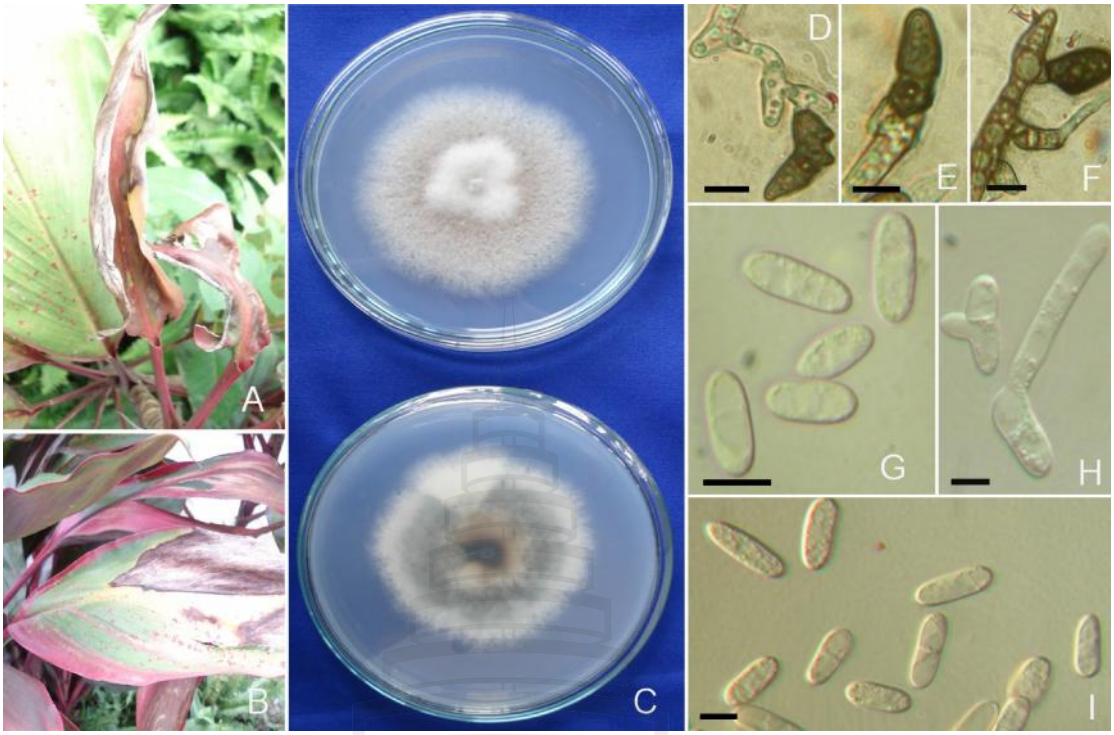


Figure 3.3 *Colletotrichum cordylinicola* (from BCC 38872, holotype) A-B. Symptoms on *Cordyline fruticosa*, C. Upper and Lower View of Cultures on PDA after 7 Days Growth, D-F. Appressoria, G-I, Conidia, H. Conidia Germination (Bars: D–I = 10 μm)

Table 3.3 *Colletotrichum* Species Strain

Colletotrichum species	Strain code	GenBank Accession Number					
		ACT	TUB-2	CAL	GS	GPDH	ITS
C. anthrisci	CBS 125334*	GU227943	GU228139	-	-	GU228237	GU227845
	CBS125335	GU227944	GU228140	-	-	GU228238	GU227846
C. asianum	MFU 090232*	FJ 903188	FJ 907434	FJ 917501	FJ 972586	FJ 972571	FJ 972605
C. asianum	MFU 090233	FJ 907424	FJ 907439	FJ 917506	FJ 972595	FJ 972576	FJ 972612
C. asianum	MFU 090234	FJ 907421	FJ 907436	FJ 917503	FJ 972598	FJ 972573	FJ 972615
C. boninense	CSSN1	GQ856774	GQ849437	-	-	GQ856743	GQ485597
	CSSX8	GQ856771	GQ849433	-	-	GQ856742	GQ485596
C. brevisporum	BCC 38876*	JN050216	JN050244	-	-	JN050227	JN050238
	MFLUCC100182	JN050217	JN050245	-	-	JN050228	JN050239
C. chlorophyti	IMI 103806*	GU227992	GU228188	-	-	GU228286	GU227894
	CBS 142.79	GU227993	GU228189			GU228287	GU227895
C. circinans	CBS 221.81	GU227953	GU228149	-	-	GU228247	GU227855
	CBS 111.21	GU227952	GU228148			GU228246	GU227854
C. cliviae	CSSS1	GU085861	GU085869	-	-	GU085867	GU109479
	CSSS2	GU085862	GU085870			GU085868	GU109480
C. coccodes	CBS 164.49	HM171666	-	-	-	HM171672	HM171678
	CBS 369.75*	HM171667	-	-	-	HM171673	HM171679
	CPOS1	GQ856787	GQ849444			GQ856744	GQ485588
C. coccodes	CBS 164.49	HM171666	-	-	-	HM171672	HM171678
	CBS 369.75*	HM171667	-	-	-	HM171673	HM171679
	CPOS1	GQ856787	GQ849444			GQ856744	GQ485588

Table 3.3 (continued)

Colletotrichum species	Strain code	GenBank Accession Number					
		ACT	TUB-2	CAL	GS	GPDH	ITS
C. cordylinicola	BCC 38872	HM470234	HM470249	HM470237	HM470243	HM470240	HM470246
C. cordylinicola	BCC38864	HM470233	HM470248	HM470236	HM470242	HM470239	HM470245
C. cordylinicola	MFLU 100132	HM470235	HM470250	HM470238	HM470244	HM470241	HM470247
C. curcumae	IMI 288937*	GU227893	GU228187	-	-	GU228285	GU227991
C. dematium	CBS 125.25*	GU227917	GU228113	-	-	GU228211	GU227819
	CBS 125340	GU227918	GU228114	-	-	GU228212	GU227820
C. gloeosporioides	CBS 953.97*	FJ 907430	FJ 907445	FJ 917512	FJ 972589	FJ 972582	FJ 972609
C. hippeastri	CSSG1	GQ856788	GQ849446	-	-	GQ856764	GQ485599
	CSSG2	GQ856789	GQ849445	-	-	GQ856765	GQ485598
C. horii	TSG001	GU133374	GU133375	GU133376	GU133377	GQ329682	AY787483
C. horii	TSG002	GU133379	GU133380	GU133381	GU133382	GQ329680	AY791890
C. kahawae	IMI 319418*	FJ 907432	FJ 907446	FJ 917514	FJ 972588	FJ 972583	FJ 972608
C. kahawae	IMI 363578*	FJ 907433	FJ 907447	FJ 917515	FJ 972587	FJ 972584	FJ 972607
C. lineola	CBS 125333	GU228126	GU227832	-	-	GU228224	GU227930
	CBS 125337*	GU227927	GU228123	-	-	GU228221	GU227829
C. liriopes	CBS 119444*	GU227902	GU228294	-	-	GU228196	GU227804
	CBS 122747	GU227903	GU228099	-	-	GU228197	GU227805
C. rusci	CBS 119206*	GU227916	GU228112	-	-	GU228210	GU227818
C. spaethianum	CBS 167.49*	GU228101	GU227905	-	-	GU228199	GU227807
	CBS 100063	GU228102	GU227906	-	-	GU228200	GU227808

Table 3.3 (continued)

Colletotrichum species	Strain code	GenBank Accession Number					
		ACT	TUB-2	CAL	GS	GPDH	ITS
C. siamense	MFU 090230*	FJ 907423	FJ 907438	FJ 917505	FJ 972596	FJ 972575	FJ 972613
C. siamense	MFU 090231	FJ 907422	FJ 907437	FJ 917504	FJ 972597	FJ 972574	FJ 972614
C. tropicicola	BCC 38877*	JN050218	JN050246	-	-	JN050229	JN050240
	MFLUCC100167	JN050219	JN050247	-	-	JN050230	JN050241
C. truncatum	CBS 151.35*	GU227960	GU228156 GU227964	-	-	GU228254	GU227862
	CBS 182.52	GU228160	GU228170	-	-	GU228258	GU227866
	CBS 136.30	GU227974		-	-	GU228268	GU227876
C. verruculosum	IMI 45525	GU227904	GU228100	-	-	GU228198	GU227806
C. yunnanense	AS3.9616	-	-	-	-	-	EF369491
	AS3.9617*	-	-			-	EF369490

Note. ACT: actin; TUB-2: partial ß-tubulin; CAL: calmoudulin; GS: glutamine synthetase; GDPH: glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA- ITS region. The newly generated sequences in this study are shown in bold. CGMCC: China General Microbial Culture Collection. MFU: Mae Fah Luang University, Thailand; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; IMI: CABI Europe – UK, Egham, Surrey TW209TY, UK; *: ex-type culture

3.3.3 *Colletotrichum brevisporum* Phoulivong, Noireung, L. Cai & K. D. Hyde, sp. nov.

MycoBank: MB564156

Etymology: *brevisporum* refers to the short conidia.

Conidiogenous cells enteroblastic, hyaline to pale brown, cylindrical to clavate. Conidia 12-17 x 5-6 μm (*mean* = 14.9 ± 3.3 x 5.9 ± 0.4, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, guttulate. Spore germination on PDA mostly form near apex of conidia (Figure 3.3). Appressoria in slide culture 10-13 x 8- 11 μm (mean= 11.3 ± 1.5 x 9.8 ± 4.4, n = 10), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age. Teleomorph not produced in culture after 2 months.

Habitat: leaf disease of *Neoregelia sp*. and *Pandanus pygmaeus* Thouars. Known distribution: Thailand.

Holotype: THAILAND, Nakhon Si Thammarat Province, Thasala District, Walailak University, on *Neoregelia* sp., 17 January 2008, Sitthisack Phoulivong (MFLU 110011); culture ex-type L57-CgPa1NK = LC0600 = MFLUCC 110115 = BCC 38876.

Additional specimen examined: THAILAND, Chiang Mai Province, San Sai District, San Sai Noi Village, on *Pandanus pygmaeus*, 9 July 2009, Parinn Noireung, (MFLU 110012); living culture BTL23 = LC0870 = MFLUCC 100182.

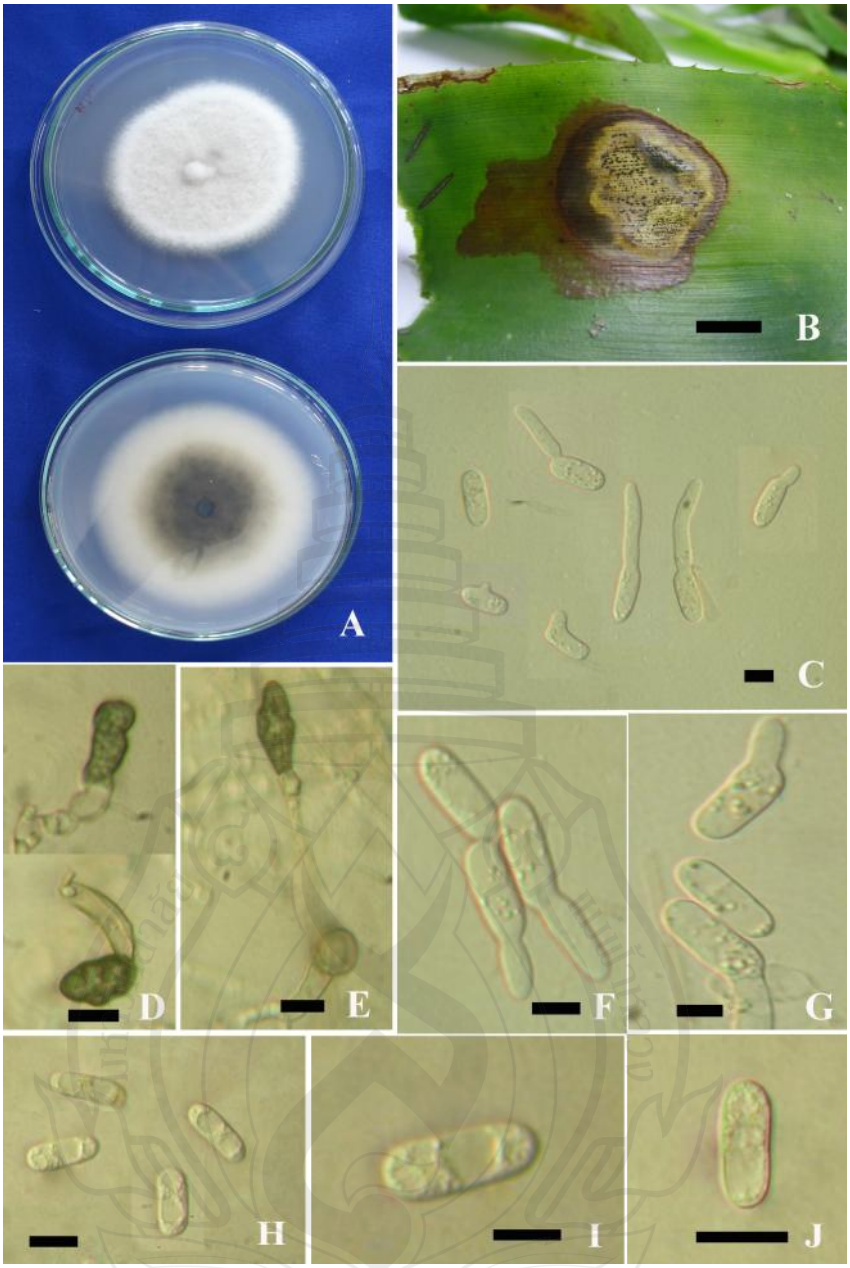


Figure 3.4 *Colletotrichum brevisporum* From Holotype. A. Upper and Reverse Sides of Cultures on PDA After 7 Days, B. symptom on *Neoregalia* sp. Leaf (Bars = 1 cm) D-E. Appressoria, F-G. Germinating Conidia H-J. Conidia (Bars: C–G=5 μm, H–J=10)

3.3.4 *Colletotrichum tropicicola* Phoulivong, Noireung, L. Cai & K. D. Hyde, sp. nov.

MycoBank: MB564159

Etymology: *tropicicola*, refers to the tropical region where the type specimen was collected.

Colonies on PDA attaining 75 mm diam. in 7 days at 27˚C, growth rate 6.7-7.2 mm/day (mean= 6.9 ± 0.2, n = 5), white, reverse white to grey. Aerial mycelium sparse, in small tufts, with orange conidial masses. Sclerotia absent. Acervuli absent. Setae absent. Conidiogenous cells enteroblastic, hyaline to pale brown, cylindrical to clavate. Conidia 15-19 x 6-7 μm (mean= 16.6 ± 2.6 x 6.5 ± 0.2, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled. Spore germination on PDA mostly from near the apex of the conidia. Appressoria in slide culture 13-24 x 7-8 μm (mean= 18.5 ± 9.2 x 7.1 ± 1.07, n = 10) (Table 3.4), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age. Teleomorph not produced in culture after 3 months.

Habitat: on leaf of *Citrus maxima* Merr. and *Paphiopedilum bellatulum* (Reichb. f.) Stein.

Known distribution: Thailand.

Holotype: THAILAND, Chiang Mai Province, Mae Taeng District, Phadeng village, on *Citrus maxima*, 14 March 2009, Sitthisack Phoulivong (MFLU 110013); culture ex-type L58 = CaPe3CM = LC0598 = MFLUCC 110114 = BCC 38877. Additional specimen examined: THAILAND, on *Paphiopedilum bellatulum*, 16 March 2009, Parinn Noireung (MFLU 110014); living culture BTL07 = MFLUCC 100167.

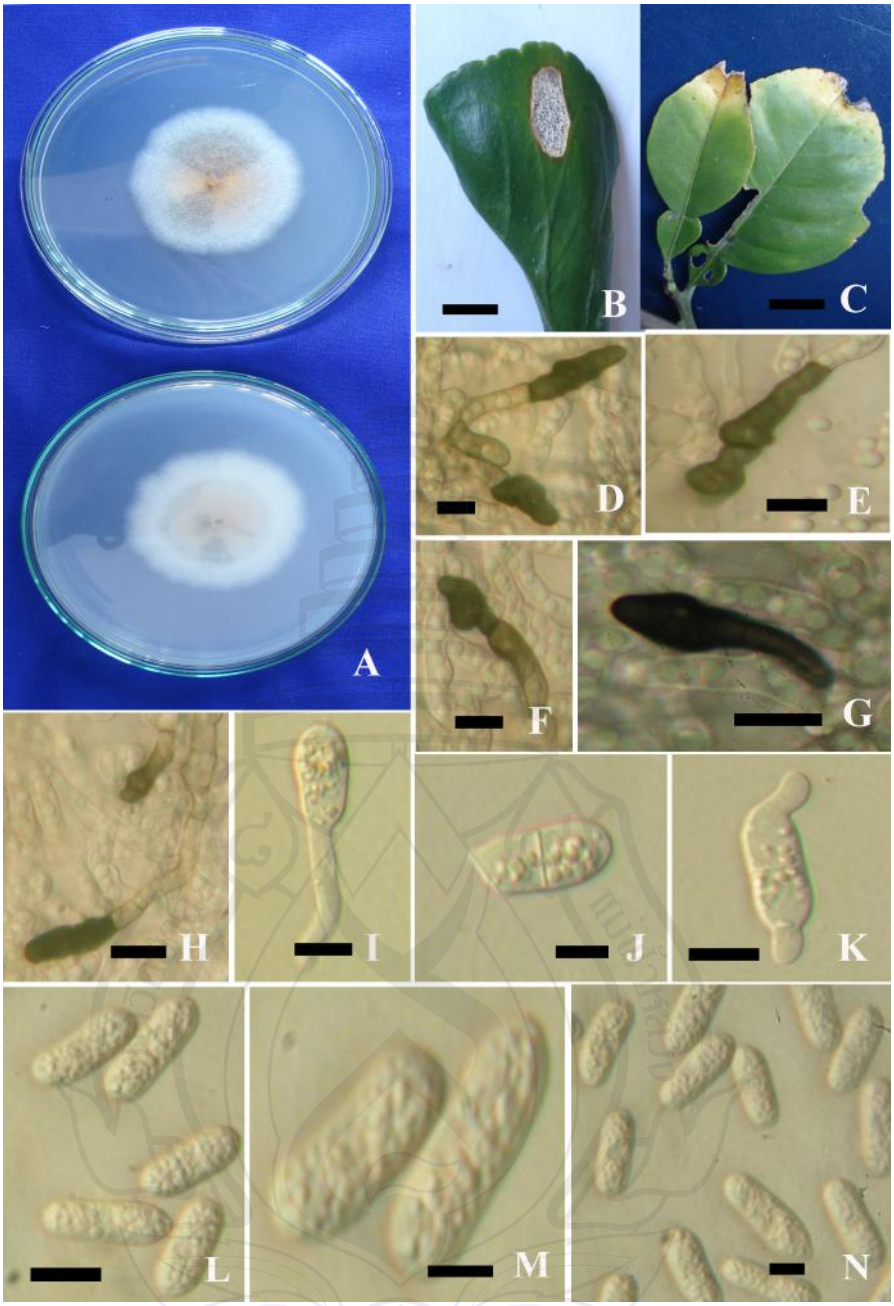


Figure 3.5 *Colletotrichum tropicicola* from Holotype A. Top and Reverse Sides of Cultures on PDA after 7 Days, B-C. Symptoms on *Citrus maxima* leaves (Bars = 1 cm), D-H.Appressoria, L-N. Conidia, I-K Germinating Conidia (Bars: D–H,K–L= 10 µm, I–J, M–N= 5µm)

3.3.5 *Colletotrichum thailandicum* Phoulivong, Noireung, L. Cai & K. D. Hyde, sp. nov.

MycoBank: MB564160

Etymology: *thailandicum*, refers to the country where the type specimen was collected.

Colonies on PDA attaining 75 mm diam. in 7 days at 27˚C, growth rate 3.8-8.8 mm/day (mean = 6.0 ± 1.5, n = 5), white, reverse green to dark green. Aerial mycelium sparse, in small tufts, with grey conidial masses. Sclerotia absent. Acervuli present in culture. Setae on PDA 65-185 μm in length (mean= 95 ± 50.30, n = 10). Conidiogenous cells enter oblastic, hyaline to pale brown, cylindrical to clavate. Conidia 27-30 x 9-10 μm (mean= 28.6 ± 0.16 x 9.9 ± 0.46, n = 30), one-celled, hyaline, cylindrical with round ends, smooth-walled, without guttules. Spore germination on PDA mostly near apex of conidia. Appressoria in slide culture 15-30 x 7-14 μm (mean= 21.8 ± 5.3 x 10.5 ± 3.0, n = 10) (Table 3.2), mostly formed from conidia, brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age. Teleomorph not produced in culture after 3 months.

Habitat: in leaf of *Alocasia* sp. and *Hibiscus rosasinensis* L.

Known distribution: Thailand. Holotype: THAILAND, Chiang Rai Province, Thasud Village, Mae Fah Luang University, on *Hibiscus rosasi-nensis*, 14 May 2009, Sitthisack Phoulivong (MFLU 110015); culture ex-type L62 = HR01MFU = LC0596 = MFUCC 110113 = BCC 38879.

Additional specimens examined: THAILAND, Chiang Mai Province, Sarapee District, on *Alocasia* sp., 20 February 2009, Parinn Noireung (MFLU 110016); living culture CMSP34 = MFLUCC 100192.

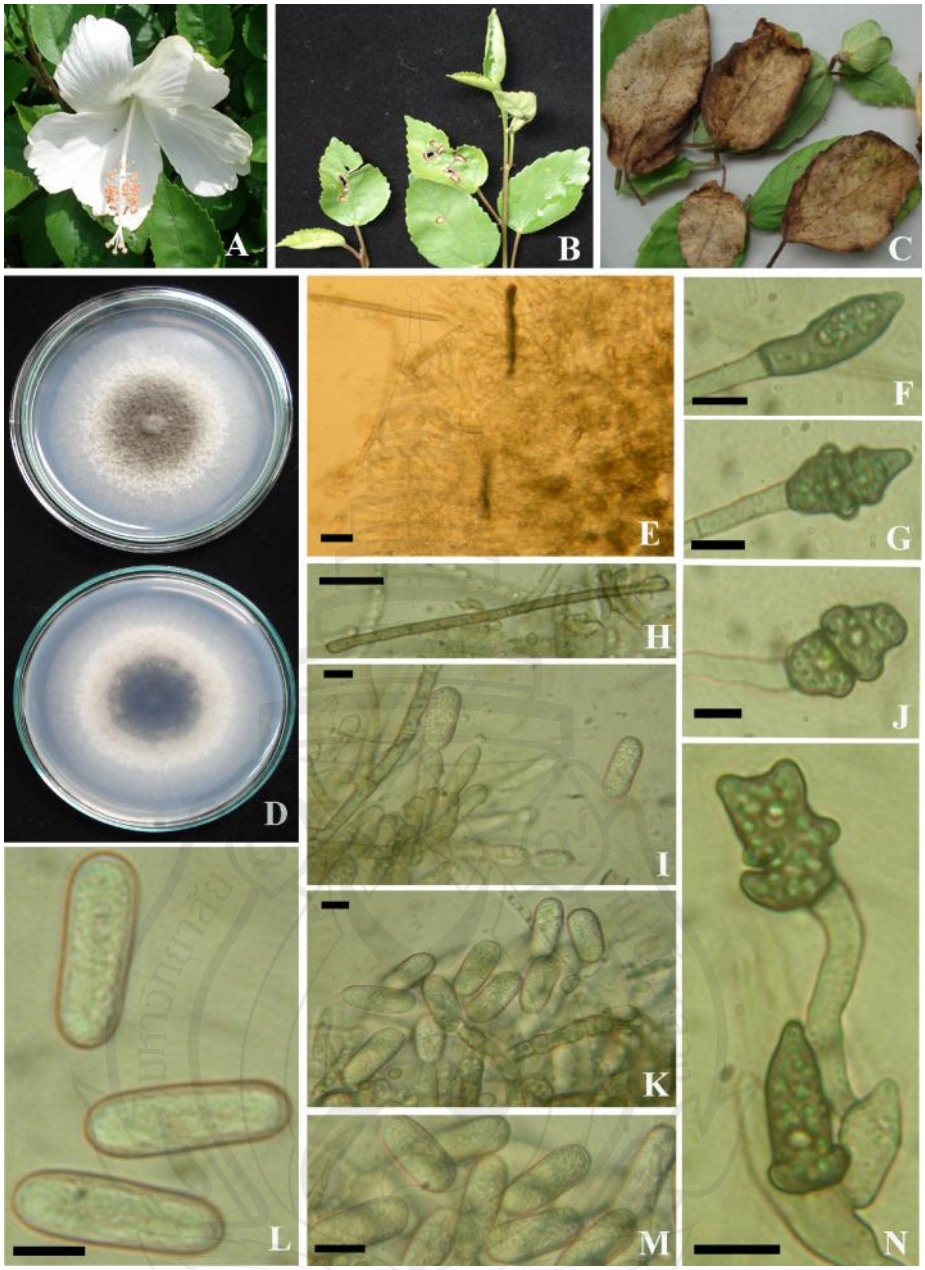


Figure 3.6 *Colletotrichum thailandicum* from Holotype A-C. Host and Symptoms on *Hibiscus rosa-sinensis* (Bars = 1 cm), D. Top and Reverse Sides of Cultures on PDA After 7 Days, F-N. Appressoria, H-M. Conidia, E. Setae (Bars: E–N = 10 μm)

Phylogenetic analysis

Six new strains were isolated from different hosts. Morphological studies classified the six isolates into three morphological groups. Sequences generated from this study were deposited in GenBank (Table 3.3).

The combined dataset of ACT, TUB2, GPDH and ITS comprised 1723 characters after alignment. Ambiguously aligned regions were excluded from all analyses. The Kishino-Hasegawa test showed that the four trees generated from parsimonious analysis were not significantly different. One of the most parsimonious trees (TL = 685, CI = 0.604, RI = 0.841, RC = 0.508, HI = 0.396) is shown in Figure 3.7. The phylogram from the combined dataset showed all the three new taxa appear as distinct lineages and clustered with *C. cliviae* Y.L. Yang, Zuo Y. Liu, K.D. Hyde and L. Cai, *C. dracaenophilum* D.F. Farr and M.E. Palm and *C. yunnanense* Xiao Ying Liu and W.P. Wu, respectively. This result was similar to the tree generated from Baysian analysis (data not shown). Two *C. brevisporum* strains formed a sister clade to *C. cliviae* supported by high bootstrap confidence (100%) and Bayensian posterior probabilities (100%). *C. tropicicola* appears basal to *C. brevisporum* and *C. cliviae* with 57% boostrap support.*C. thailandicum* also appeared as a distinct lineage basal to *C. yunnanense*, *C. dracaenophilum*, *C. cliviae*, *C. brevisporum* and *C. tropicicola* (Figure 3.7). The three new species group in a monophyletic clade that also includes *C. yunnanense*, *C. dracaenophilum* and *C. cliviae,* with moderate support.

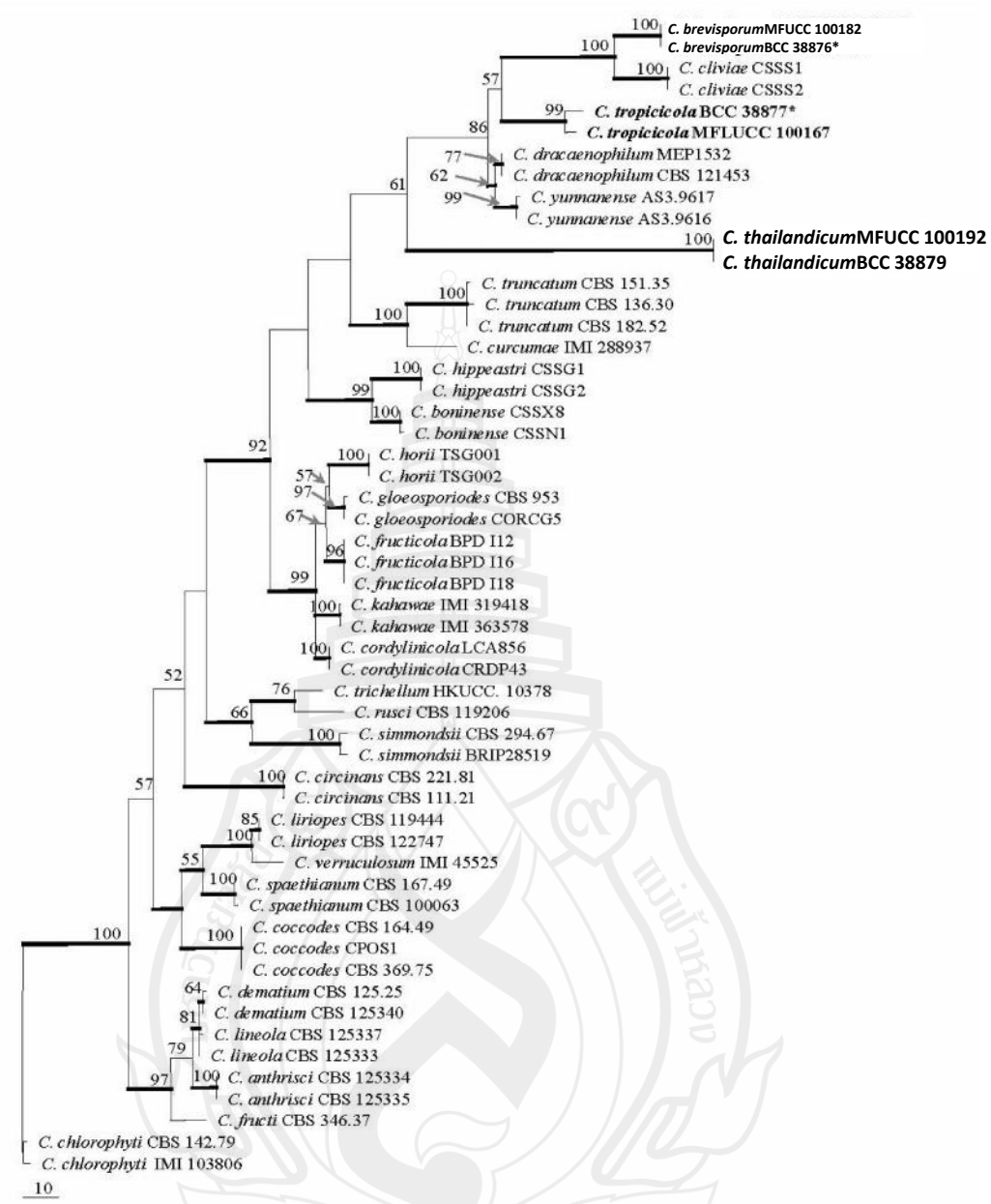


Figure 3.7 Phylogram Generated From Maximum Parsimony Analysis

Table 3.4 Morphological Characters of New Species Compared With Phylogentically Related Species

Taxa	Colonies	Conidia shape and size (µm)	Appressoria shape and size (µm)	Growth rate (mm per day)	References
C. brevisporum	Aerial mycelium in small tufts, white, sparse, with conidial masses, reverse dark green	Cylindrical with round ends, smooth-walled, hyaline, guttulate, 12-17 × 5-6 µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 10-13 × 8-11µm	7.5-9.8, $\bar{x}$ = 8.5	This study
C. cliviae	White to grey, white at margin, reverse dark brown to greenish black	Cylindrical, straight or slightly curved, obtuse at the ends, 19.5-24.5 × 4.5-7µm	Dark brown, irregular, crenate or lobed, 10.5-14.5 × 6-11, $\bar{x}$ = 11.7 × 8.6	15.2-16 , $\bar{x}$ = 15.6	Yang et al., 2009
C. dracaenophilum	Pale pink, reverse speckled from profuse sporulation, sparse aerial mycelium, rosy buff to saffron in centre, rosy buff to saffron in reverse	Broadly clavate to cylindrical, frequently slightly curved, hyaline, guttulate, 22-34 ×6.5-9.5 µm	(No information)	(No information)	Farr et al., 2006

Table 3.4 (continued)

Taxa	Colonies	Conidia shape and size (µm)	Appressoria shape and size (µm)	Growth rate (mm per day)	References
C. thailandicum	Aerial mycelium in small tufts, white, sparse, with grey conidial masses, reverse green to dark	Cylindrical with round ends, smooth-walled, hyaline, 27-30 × 9-10 µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 15-30 × 7-14 µm	3.8-8.8 ($\overline{x}$ = 6.0)	This study
C. tropicicola	Aerial mycelium in small tufts, white, sparse, with white orange conidial masses, reverse slightly white to grey	Cylindrical with round ends, smooth-walled, hyaline, guttulate, 15-19 × 6-7µm	Brown to dark brown, variable in shape including ovoid, clavate or slightly irregular, often becoming complex with age, 13-24 × 7-8 µm	6.7-7.2 µm, $\overline{x}$ = 6.9	This study

3.4 Discussion

3.4.1 *Colletotrichum cordylinicola*

Colletotrichum cordylines Pollacci (from Italy) is the only species of *Colletotrichum* described from *Cordyline* (*C. indivisa*). Conidial sizes were not provided in the protologue (Saccardo & Sydow, 1899) and the name has not recently been used (Hyde, Cai, McKenzie et al., 2009). It is impossible to establish whether our collections have any relationship to the type of *C. cordylines,* as there are no living extype cultures and it is presently impossible to isolate DNA from such an old type specimen. It is, therefore, prudent to introduce our collections as a new species.

Colletotrichum cordylinicola is morphologically similar to several species in the *C. gloeosporioides* complex. Species in this complex are difficult to differentiate based solely on morphology. Phylogenetic analysis using ITS sequences could not confidently resolve its systematic placement but showed that this fungus is well clustered in the *C. gloeosporioides* complex (details not shown). A multi-locus phylogeny based polyphasic approach was therefore employed to infer interspecific relationships in this group of fungi (Cai et al., 2009). In the six-gene combined phylogeny, the species relationships are well defined with all the major clades supported by parsimony bootstrap support and Bayesian posterior probabilities (Figure 3.2). The conidial morphology of *C. cordylinicola* is similar to that of *C. siamense*. However, *C. cordylinicola* can be distinguished from this species by its appressoria, which are irregular in shape (Figure 3.2). In the phylogenetic tree, *C. cordylinicola* does not group with *C. siamense*, but clusters as a sister clade to *C. kahawae* (Figue 3.2). Although similar in conidial morphology, *C. cordylinicola* can be differentiated from *C. kahawae* by its significantly larger appressoria (13–13.4 × 7.2–7.3 vs 4.5–10 × 4–7 μm) and smaller conidia. This is the first report of *Colletotrichum* species causing anthracnose on *Cordyline fruticosa* in Thailand.

Identification of species within the *C. gloeosporioides* complex has been a difficult issue as these species are morphologically very similar (Bailey & Jeger, 1992; Sutton, 1992). Morphology of conidia and appressoria, colony characters, host association, growth rate, and biochemical data should be used in conjunction with a multilocus phylogeny to identify a *Colletotrichum* species accurately (Cai et al., 2009; Prihastuti et al., 2009). In this study, a phylogenetically well-defined lineage is associated with distinct morphological and other phenotypic characters. It is therefore given species rank and described as a new species.

The strain of *C. cordylinicola* isolated from rose apple failed to infect *Cordyline fruticosa*, while that from *Cordyline fruticosa* failed to infect rose apple. In morphology, the two strains are essentially similar except the one from rose apple produced conidia that are slightly acute at one end, while the conidia in the strain from *Cordyline fruticosa* are rounded at both ends. The strains are, however, shown to be related based on multigene phylogenetic analysis with 100% support (Figure 3.2). The strain from rose apple infected more fruits than that from *Cordyline fruticosa*. This finding supports the

statement of Johnston (2000) that "there are no general rules concerning host relationships within *Colletotrichum* the group so recognized cannot be assumed genetically equivalent, even when appearing to be biologically similar". It will be interesting to establish whether these strains represent two pathotypes in nature (Bailey & Jeger, 1992). Pathogenicity may be affected by several environmental factors such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds, 1965; Freeman et al., 1998). The result reported here may not accurately reflect the true virulence potential. Future research should attempt to determine the pathogenicity of these strains according to natural infections rather than artificial inoculations. On the other hand, if more phenotypic divergence of these two strains could be identified following further collections or study, the systematic relationship between the two strains may need a re-evaluation.

3.4.2 *Colletotrichum brevisporum, Colletotrichum tropicicola* and *Colletotrichum thailandicum*

Species complexes in *Colletotrichum* include *C. acutatum* J.H. Simmonds, *C. boninense* Moriwaki, Toy. Sato and Tsukib., *C. gloeosporioides* (Penz.) Penz. and Sacc. and *C. dematium* (Pers.) Grove, plus distinct individual species such as *C. coccodes* (Wallr.) S. Hughes, *C. circinans* (Berk.) Voglino, *C. trichellum* (Fr.) Duke, *C. truncatum* and *C. curcumae* (Syd.) E. J. Butler and Bisby (Damm et al., 2009; Shivas & Tan 2009; Phoulivong et al., 2010).The new species described in this paper can be differentiated from these species complexes by morphological characters, such as conidial size and shape (Table 3.3), and the distinctions as indicated in the phylogenetic tree (Figure 3.7).

The new species are most similar in conidial shape to *C. cliviae* Y. L. Yang, Zuo Y. Liu, K. D. Hyde and L. Cai, *C. dracaenophilum* D.F. Farr and M.E. Palm and *C. yunnanense* Xiao Ying Liu and W.P. Wu respectively. However, they differ in conidial size. *C. dracaenophilum* has very long conidia (mean length > 28 μm) and overlap with those of *C. thailandicaum* (Farr, Aime, Rossman & Palm, 2006). Conidia of *C. thailandicum* are, however, wider (9-10 μm vs 6.5–9.5 μm). Conidial shape is cylindrical with round ends in *C. thailandicum*, as compared to broadly clavate to cylindrical, frequently slightly curved in *C. dracaenophilum*. In the phylogenetic tree, *C.*

thailandicum forms a separate clade with long branch length, indicating certain distance from *C. dracaenophilum* and *C. yunnanense* (Figure 3.7). *C. brevisporum* and *C. tropicicola* have short conidia, however, their appressoria are significantly different in size and shape (10-13 × 8-11 µm in *C. brevisporum* vs. 13-24 × 7-8 µm in *C. tropicicola*). In the phylogenetic tree *C. brevisporum*, *C. tropicicolum* and *C. clivae* cluster in a moderately supported clade and each represented by well support lineages (Figure 3.7). The conidia of *C. brevisporum* and *C. tropicicolum* are shorter than those of *C. cliviae* (12-17 µm in *C. brevisporum*, 15-19 µm in *C. tropicicola* and 19.5-24.5 µm in *C. cliviae*), the latter also grows faster in culture (Yang et al., 2009). *C. brevisporum* and *C. cliviae* cluster as sister group with high bootstrap support. In *brevisporum* morphology, they differ as conidia in *C. cliviae* are larger and the species grows much faster in culture (Table 3.3). The conidia of *C. thailandicum* are much larger than *C. yunnanense,* while those of *C. yunnanense* are similar to *C. brevisporum* and *C. tropicicola.* The appressoria of *C. yunnanense* are regularly lobed which distinguishes it from these species (Liu, Duan & Xie, 2007).

In this Chapter we introduce three new species based on morphological and molecular characters. A synopsis of the three new species and similar taxa is provided in Table 3.2 We have not epitypified older names that are from a same host of our new species because the new species colonize more than one host, while the older names were based on host specific associations and thus it would be difficult to decide on an earlier name for these new species. More importantly, living strains do not exist for the older names and characters in the original protologues of species on the hosts colonized differ as detailed below.

C. brevisporum is recorded from *Neoregelia* sp. and *Pandanus pygmaeus* Hook. which belong to related plant families. We could not find any species of *Colletotrichum* that are described from these hosts, but *C. gloeosporioides* was reported from *Pandanus utilis* Bory (Farr & Rossman, 2010), which is obviously not conspecific with *C. brevisporum*.

C. tropicicola was found on *Citrus maxima* Merr. and *Paphiopedilum bellatolum*Thouars (lady's slipper orchid) and cannot be conspecific to *C. orchidearum* Allesch. because the conidia are overall wider (6-7 µm in *C. tropicicola* vs. 4-6 µm in *C.*

orchidearum) (Saccardo, P. A. & Saccardo, D., 1906). There are also numerous varieties of *C. orchidearum* but none come from the same host of *C. tropicicola*. Therefore, we prefer to introduce a new species as it is also recorded from *Citrus maxima* and differs from *C. orchidearum* and *C. cliviae* in producing much smaller conidia (Saccardo, P. A. & Saccardo, D., 1906; Yang et al., 2009).

Several species of *Colletotrichum* have been recorded from *Hibiscus* such as *C. capsici* (Syd. and P. Syd.) E.J. Butler and Bisby, *C. gloeosporioides* (Penz.) Penz. and Sacc., *C. hibisci* Pollacci and *C. hibiscicola* Rangel (Farr & Rossman, 2010). However, *C. thailandicum* from *Hibiscus rosa-sinensis* differs from *C. hibisci* and *C. hibiscicola* in producing larger conidia (27-30 × 9-10 μm in *C. thailandicum*; 11-25 × 4.2 μm in *C. hibisci*; 12-20 × 4.6 μm in *C. hibiscicola*) (Saccardo & Sydow, 1899; Saccardo, P. A. , Saccardo, D., Traverso & Trotter, 1931).

3.5 Conclusion

Colletotrichum isolates were collected on various host from Laos and Thailand were found 56 isolates on 22 hosts were found on symtomps in this study, 56 isolates of *Colletotrichum* were obtained from plant leaves of different hosts ci.e. *Alocasia indica*, *Aglaonema* ssp., *Scindapsus aureus* and *Dracaena fragrans*.

Strains were isolated from diseased parts and their morphological characters were examined. DNA was extracted from isolates and the ITS rDNA regions sequenced for all strains. ITS sequence data was aligned against data from type strains in the genus in order to establish if they can be assigned to any known species. Strains that could not be given names were further sequenced for partial actin (ACT), β–tubulin (TUB2) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes and employed in a phylogenetic analysis to reveal their relationships with other closely related taxa. The multilocus sequence analysis, together with critical examination on phenotypic characters, revealed three new species and these are introduced as *C. brevisporum*, *C. tropicicola* and *C. thailandicum* in this paper. The new species are formally described, illustrated and compared with similar taxa.

A new species *C. cordylinicola*, isolated from *Cordyline fruticosa,* is characterized by morphological and molecular characters. The species would previously have been considered as a member of the *Colletotrichum gloeosporioides* complex. Combined six gene analysis using ACT, GS, TUB2, ITS, CAL and GPDH shows that three strains of *C. cordylinicola* clustered in a distinct lineage as a sister clade to *C. kahawae*. Other reference taxa employed in the analysis include type strains of *C. asianum, C. fructicola, C. gloeosporioides, C. kahawae, C. siamense,C. simmondsii,* and authentic strains of *C. horii.* This is also the first report of a *Colletotrichum* species causing disease of *Cordyline fruticosa* in Thailand.

CHAPTER 4

CROSS INFECTION *Colletotrichum* SPECIES FROM DIFFERENT HOSTS

4.1 Introduction

Colletotrichum is one of the most economically important pathogenic genera causing anthracnose of fruits, affecting a wide range of hosts in the tropics and subtropics (Cai et al., 2009; Cannon et al., 2012; Damm et al., 2012; Fujinaga et al., 2011; Hyde, Cai, Cannon et al., 2009; Phoulivong, Cai, Chen et al., 2010; Noireung et al., 2012; Weir et al., 2012; Yang, Cai et al., 2012; Yang, Liu et al., 2012). The above-ground plant parts of crops as well as fruit trees can be affected by *Colletotrichum* anthracnose and in the case of fruit infection, there is a reduction in yield quantity or quality (Phoulivong, Cai, Chen et al., 2010). Hosts of *Colletotrichum* species in Thailand include fruits such as chili (*Capsicum* sp.), guava (*Psidium guajava*), jujube (*Zizyphus mauritiane*), mango (*Mangifera indica*), papaya (*Carica papaya*) and rose apple (*Eugenia javanica*) (Freeman et al., 2000; Peres et al., 2002; Ratanacherdchai et al., 2010; Sreenivasaprasad & Talhinhas, 2005). *Colletotrichum* species are cosmopolitan with either multiple species occurring on a single host or a single species occurring on multiple hosts (Sander & Korsten, 2003). Fungus/host relationships are broad, imprecise and often overlapping (Freemand et al., 2000). *Colletotrichum* species can infect many hosts and may adapt to new environments (Sanders & Korsten, 2003; Photita et al., 2004), leading to serious cross infection problems in plant production. The study of pathogenic variability of *Colletotrichum* species is therefore important and the understanding of the host range of a particular pathogen may help in efficient disease control and management (Whitelaw-Weckert et al., 2007).

Artificial inoculation methods *in vitro* are commonly used to test the pathogenicity of a fungal species, as it is easy to control environmental conditions. Common inoculation methods for pathogenicity testing include drop inoculation, wound/drop inoculation (Kanchana-udomkan et al., 2004), micro injection, and spraying with high pressure guns (Cai et al., 2009; Lin et al., 2002, Sharma et al., 2005; Than, Jeewon et al., 2008). The drop method involves transferring a spore suspension on to the surface of fruit and the wound/drop method involves wounding the surface of the fruit by pricking with a pin then placing a drop of fungal spore suspension on the wounded tissue. The wound/drop method is more favorable since wounding allows the pathogenic isolate internal access to the fruit and enhances infection. The wound/drop method has been shown to be useful to select resistant varieties of chili (*Capsicum annuum*) from susceptible varieties (Lin et al., 2002; Than, Jeewon et al., 2008). Different hosts and stages of maturity are important to test the expression of resistance to *Colletotrichum* species. The interaction between fruit maturity stage and infection of colonisation may depend on the species of *Colletotrichum* (AVRDC, 2002). Pathogenicity testing can provide data on the resistance of fungi to crops in plant breeding programs and is important to integrated disease management programs because using the resistant varieties can reduce the negative effects of chemical use on the environment (AVRDC, 2002; Freeman et al., 1998; Wharton et al., 2004).

Colletotrichum gloeosporioides sensu lato has been listed to cause disease of a very wide range of hosts (Table 4.1) in Laos and Thailand (Cannon et al., 2012; Damm et al., 2012; Fujinaga et al., 2011; Ratanacherdchai et al., 2007; Than, Jeewon et al., 2008; Than, Shivas et al., 2008; Weir et al., 2012). This species has recently been epitypified with a living strain that has been sequenced with sequence data deposited in GenBank (Cai et al., 2009). This has enabled researchers to compare their isolates of *Colletotrichum* with the *C. gloeosporioides* epitype. This has resulted in the description of several new species in the *C. gloeosporioides* species complex (Cai et al., 2009; Cannon et al., 2012; Damm et al., 2012; Fujinaga et al., 2011; Noireung et al., 2012; Phoulivong, Cai, Chen et al., 2010; Prihastutiet al., 2009; Weir et al., 2012; Wikee et al., 2011; Yang et al., 2009; Yang, Cai et al., 2012). With the introduction of several new species it is important to establish whether they are host-specific or have a wide host range as this will have important implications in disease control and management. The objective of this

study is to understand the host range and cross infection of *Colletotrichum* species that were isolated from fruit lesions in Laos and Thailand.

4.2 Materials and Methods

4.2.1 Isolation of *Colletotrichum* Species

Colletotrichum strains were isolated from anthracnose of infected fruits from orchards and local markets in Laos and Thailand. Isolation was carried out by two methods depending on fungal sporulation on the sample. Conidia were picked directly from sporulating samples and then cultured on water agar (WA). The *Colletotrichum* isolates were then transferred to plates of potato dextrose agar (PDA) (Abang, 2003). Alternatively, isolates were obtained from fruit without visible sporulation by culturing three 5×5 mm^2 pieces of tissue taken from the margin of infected tissue on WA. Before culturing on WA, the surface of infected tissues was sterilized by dipping in 1% sodium hypochlorite for 3 minutes, and rinsing three times with sterile water. The growing edge of any fungal hyphae developing from the disease tissue was then transferred aseptically to PDA. Single spore isolation was carried out from sporulating lesions. Spore masses were picked up with a sterilized wire loop and streaked onto the surface of water agar followed by inoculation overnight. A germinated single spore was picked up with a sterilized needle and transferred onto PDA to obtain a pure culture following the procedure described by (Cai et al., 2009).

4.2.2 Pathogenicity Testing

Preparation of inoculum *Colletotrichum* isolates from a range of hosts used for pathogenicity test and their cross infection potential are listed in Table 4.2. Pure cultures of each isolate were grown on PDA for 14 days at 27-28°C under fluorescent light (12 hour light/dark cycle), to induce sporulation (Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008; Cai et al., 2009). The spores were harvested by placing about 10 ml sterile water onto the culture and filtering the spore and mycelium suspension with two layers of cheese cloth. The spore density was adjusted to a concentration of 1×10^6 spore/ml using a haemocytomet.

Table 4.1 *Colletotrichum* Species Causing Anthracnose in Laos and Thailand on Host Range

Species	Hosts	Causing Anthracnose	References
C. acutatum	*Capcicum annuum*	Fruit	Dam et al. (2012)
	Carica papaya	Fruit	Dam et al. (2012)
	Coffea arabica	Fruit	Dam et al. (2012)
	Fragaria ananassa	Fruit	Dam et al. (2012)
C. asianum	*Capcicum annuum*	Fruit	This paper
	Eugenia javanica		
C. brevispora	*Neoregalia* sp.	Leaf	Noireung et al. (2012)
	Pandanus pygmaeus	Leaf	
C. brisbanense	*Capsicum annuum*	Fruit	Dam et al. (2012)
C. coccodes	*Solanum tuberosum*	Fruit	Lees & Hilton (2003)
C. cordylinicola	*Capcicum annuum*	Fruit	Phoulivong, Cai, Noireang et al. (2010), This paper
	Carica papaya	Fruit	
	Cordyline fructicosa	Leaf	
	Eugenia javanica	Fruit	
	Mangifera indica	Fruit	
	Syzygium jambos	Fruit	
C. cuscutae	*Malus sylvestris*	Fruit	Dam et al. (2012)
C. dematium	*Eryngium campestre*	Leaf	Noireung et al. (2012)
	Apiaceae		
C. floriniae	*Vaccinium* sp.	Fruit	Dam et al. (2012)
C. fructicola	*Capcicum annuum*	Fruit	Prihastuti et al. (2009), This paper
	Carica papaya	Fruit	
	Coffea arabica	Fruit	
	Eugenia javanica	Fruit	
	Mangifera indica	Fruit	
C. gloeosporioides	*Citrus sinensis*		Cannon et al. (2012)
C. godetiae	*Citrus aurantium*	Fruit	Dam et al. (2012)
C. horii	*Diospyros kaki*	Fruit	Wikee et al. (2011)
C. horii	*Diospyros kaki*	Leaf	Phoulivong, Cai, Noireang et al. (2010)

Table 4.1 (continued)

Species	Hosts	Causing Anthracnose	References
C.ignotum	*Jasminum sambac*	Leaf	Wikee et al. (2011)
C. jasminigenum	*Jasminum sambac*	Leaf	Wikee et al. (2011)
C. kahawae	*Coffea arabica*	Leaf	Prihastuti et al. (2009)
C. melonis	*Cucumis melo*	Fruit	Dam et al. (2012)
C. musae	*Musa* sp.	Fruit	Weir et al. (2012)
C. nymphaeae	*Fragaria* sp.	Fruit	Dam et al. (2012)
C. pyricocola	*Pyrus communis*	Fruit	Dam et al. (2012)
C. queenslandicum	*Carica papaya*	Fruit	Weir et al. (2012)
C. simmondsii	*Capcicum annuum*	Fruit	Giblin et al.(2010),
			Weir et al. (2012),
	Carica papaya	Fruit	This paper
	Citrus reticulata	Fruit	
	Cordyline fructicosa	Leaf	
	Eugenia javanica	Fruit	
	Mangifera indica	Fruit	
	Syzygium jambos	Fruit	
C. tamarilloi	*Solanum betaceum*	Fruit	Dam et al. (2012)
C. thailandicum	*Hibiscus rosa-sinensis*	Leaf	Noireung et al. (2012)
	Alocasia sp.	Leaf	
C. tropicicola	*Citrus maxima*	Leaf	Noireung et al. (2012)
	Paphiopedilum	Leaf	
	bellatolum	Leaf	
C. truncatum	*Phaseolus lunatus*	Leaf	Yang et al. (2009)
	Glycine max		
	Crotalaria juncea		

Preparation of hosts freshly harvested untreated, unwaxed, physiologically mature and unripe fruits were collected from the field or purchased from the market (Sanders & Korsten, 2003). The detached fruits were washed under running tap water for 60 seconds followed by surface sterilization by immersing the fruits in 70% ethanol for 3 minutes,

1% sodium hypochlorite solution for 5 minutes and then rinsing three times in sterile distilled water for 2 minutes and drying with sterile tissue paper and then air drying.

Inoculation Surface sterilized fruits were placed in a plastic box with tissue paper then sprayed with sterilized water to maintain at least 95% relative humidity (Than, Jeewon et al., 2008). The samples were inoculated using the wound/drop inoculation method (Lin et al., 2002) which included pin-pricking the fruits to a 1 mm depth with a sterile needle in the middle portion of fruit and then placing 6 µl of conidia suspension onto the wound (Freeman et al., 1996; Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008). Control fruits were inoculated with 6 µl of sterile distilled water. The inoculated samples were incubated in the containers at 28-30˚C in a 12 hour light/dark cycle.

Fruits used in inoculation tests were chili (*Capsicum* spp.), guava (*Psidium guajava*), mango (*Mangifera indica*), papaya (*Carica papaya*) and rose apple (*Eugenia javanica*) with ten treatments (numbered A-J) and three replicates per fruit. Incubation duration was dependent on the nature of the fruit lesion development on fruits. Fruits were examined at five days for rose apple and papaya, seven days for chili, guava, orange and varying period for other fruits. The infection was measured based on lesion development on the symptom on fruit.

Lesion developments on fruit were assessed by measuring the disease area in centimeter on each fruit; data were analysed used analysis of variance ($P< 0.05$) with DMRT for multiple range tests from statistic software (Cai et al., 2009; Choi, Kim, Kim, Choi & Lee, 2011; Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008).

4.3 Results

All of the isolates were identified using morphological characters, colony growth rate, and confirmed with molecular sequence data analysis of combined multi-gene loci (Phoulivong, Cai, Chen et al., 2010; Phoulivong, Cai, Noireang et al., 2010).

The development of anthracnose symptoms on different fruits was statistically compared based on percentage of lesion area from the fruit (Table 4.2). All strains of *Colletotrichum* infected the original host from which they were isolated.

The strain of *Colletotrichum asianum* isolated from coffee infected chili and rose apple, whereas the strain isolated from mango infected chili and mango. *Colletotrichum cordylinicola* strain from rose apples infected a wide host range whereas that isolated from *Cordyline fruticosa* infected only papaya. Strains of *C. fructicola* from coffee and papaya had the same host range whereas the isolate from longan infected mango but not orange. The *C. siamense* isolate from coffee infected five hosts including orange and papaya although the isolate from chili did not infect the latter two fruits. The two isolates of *C. simmondsii* were both from papaya and both infected mango, chili, rose apple and papaya. However, one isolate also infected guava whereas the other infected orange but not guava.

4.4 Discussion

The *Colletotrichum* species infected a wide host range, however, the strains behaved differently. For example, the strain of *C. cordylinicola* isolated from rose apple failed to infect leaves of *Cordyline fruticosa* (Phoulivong, Cai, Noireang et al., 2010) while the strains of *C. cordylinicola* isolated from *Cordyline fruticosa* failed to infect rose apple fruit. The strain from rose apple however infected various other fruits. This study is consistent with inoculation studies by (Sanders & Korsten, 2003) who showed that isolates of *C. gloeosporioides* from mango could produce symptoms on other hosts such as guava, chili pepper and papaya. Although mango isolates of *C. gloeosporioides* were highly pathogenic when re-inoculated onto mango fruits, it is unclear why no symptom was produced on chili fruits by the mango isolates. This could possibly have been due to a lack of pathogenesis factors that could recognize chili fruit cells for infection and colonization (Than, Jeewon et al., 2008; Than, Prihastuti et al., 2008; Sanders & Korsten, 2003). The latter finding is extremely interesting as it shows that the same species isolated from different hosts, has different cross infection ability and this should be considered when establishing new species. There have been several studies concerning cross infection of *Colletotrichum* species especially with *C. acutatum* and *C. gloeosporioides* species complexes (Abang, 2003; Freeman et al., 2001; Kim et al., 2009; Peres, MacKenzie, Peever & Timmer, 2008; Sanders & Korsten, 2003). Cross-infection

of different hosts has not only been shown in the laboratory, but may also occur in the field (Afanador-Kafuri, Minz, Maymon & Freeman, 2003). Freeman et al. (2001) found that *C. acutatum* from strawberry was able to cause lesions on various fruits. *In vitro* infection studies by (Whitelaw-Weckert et al., 2007) revealed low host-specificity among isolates of *C. acutatum.* Cross inoculation studies by Sanders and Korsten (2003), showed that putative isolates of *C. gloeosporioides* from mango could produce symptoms on other hosts such as guava, chili and papaya. These studies showed that *Colletotrichum* strains can infect more than one host and one host also can be infected with many *Colletotrichum* species.

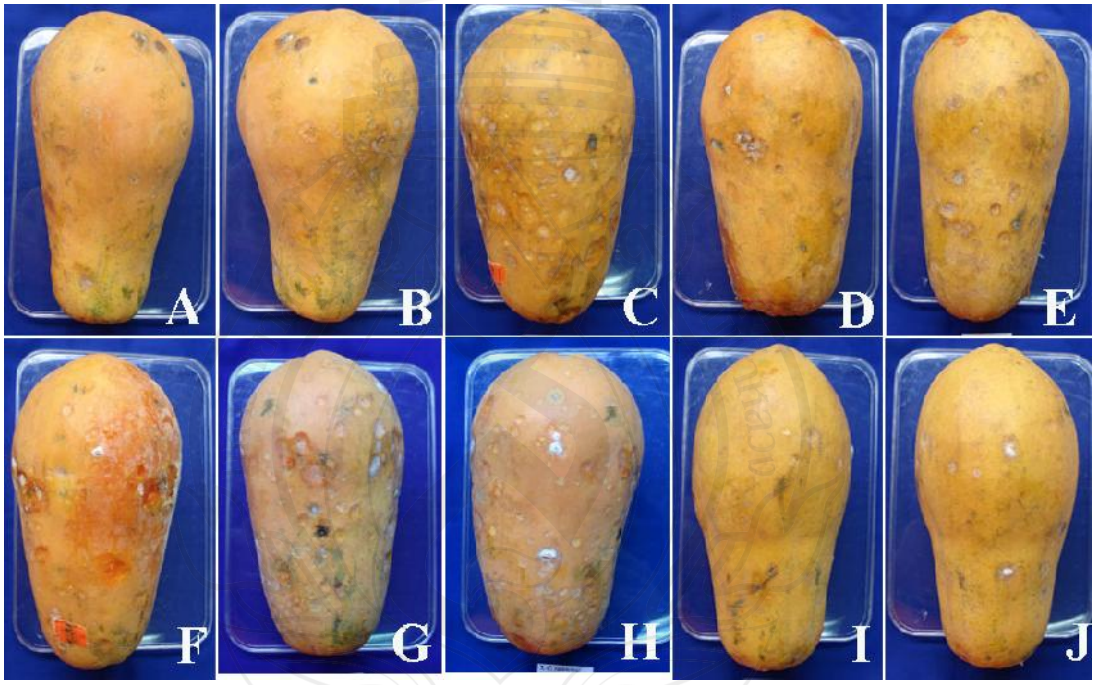


Figure 4.1 Anthracnose Symptoms on Papaya after 5 Days Inoculation A. *Colletotrichum asianum* Isolated from Coffee Berries, B.*C. asianum* From Mango Fruit, C.*C. cordylinicola* From Rose Apple Fruit, D.*C. fructicola* From Coffee Berries, E *C. fructicola* From Papaya Fruit, F.*C. fructicola* From Longan Fruit, G *C. siamense* From Coffee Berries, H.*C. siamense* From Chili Fruit, I.*C. simmondsii* From Papaya Fruit, J.*C. simmondsii* From Papaya Fruit

Identification of strains in post 2010 cross infection studies and even many since were based on names given using data available at the time. It has now been shown that *C. acutatum* (Cannon et al., 2012; Damm et al., 2012; Fujinaga et al., 2011) *C. boninense* (Weir et al., 2012) *C. gloeosporioides* (Cannon et al., 2012; Damm et al., 2012; Fujinaga et al., 2011; Weir et al., 2012; Živkovic, Stojanovic, Ivanovic, Gavrilovic & Balazn, 2010) and several other taxa are species complexes (Damm et al., 2012, Stankova et al., 2011; Weir et al., 2012). We therefore cannot compare our results with previous studies, as it is unlikely we were studying the same species.

Some recent studies have used strains identified based on combined sequence data. Phoulivong et al. (2010) showed that *C. asianum*, *C. fructicola*, *C. siamense* and *C. simmondsii* can infect chili, guava, jujube, mango, papaya and rose apple; Yang, Cai et al. (2012) showed that *C. orchidearum, C. karstii* and *C. siamense* are not host-specific as they infected fruit of apple, chili and tomato following pathogenicity testing. Peng, Youlian, Hyde, Bahkali and Liu, (2012) showed that *C. boninense*, *C. brevisporum*, *C. fructicola*, *C. gloeosporioides*, *C. karstii*, *C. simmondsii* and *C. murrayae* infected citrus leaves, while Noireung et al. (2012) found that *C. brevisporum*, *C. tropicicola* and *C. thailandicum* caused anthracnose on leaves of *Pandanus pygmaeus*, *Citrus maxima* and *Hibiscus rosa-sinensis.* Most studies, including the present one, confirm that most *Colletotrichum* species have wide host ranges (Cai et al., 2010; Noireung et al., 2012; Phoulivong, Cai, Noireang et al., 2010; Yang, Liu et al., 2012). Infection of fruits may be dependent on environmental factors such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds, 1965; Freeman et al., 1998), rather than which *Colletotrichum* species colonizes it.

Table 4.2 Pathogenicity Testing and Potential of Cross Infection of *Colletotrichum* Species on a Range of Hosts

Species	Isolate Number	Species type	Hosts	Location	Infection on inoculated fruits					
					Orange	Guava	Mango	Chili	Rose apple	Papaya
					Infected fruit area (cm^2)					
C. asianum	MFU090229	Holotype	coffee	Chiang Mai, Thailand	-	-	-	0.4BC*	1.33AB	-
C. asianum	MFU09 0556	-	mango	Bangkok, Thailand	-	-	1.25AB	0.2C	-	-
C. cordylinicola	MFU090551	Holotype	Cordyline	Chiang Mai, Thailand	-	-	-	-	-	0.35
C. cordylinicola	MFU090638	-	rose apple	Vientiane, Laos	-	0.5A	0.7AB	1A	1.73A	1.95A
C. fructicola	MFU090227	Holotype	coffee	Chiang Mai, Thailand	1.75A	-	-	0.75AB	1.40AB	1.5B
C. fructicola	MFU09 0560	-	papaya	Chiang Mai, Thailand	2A	-	-	0.75AB	1.07BC	1.45B
C. fructicola	MFU09 0568	-	longan	Chiang Mai, Thailand	-	-	1.95A	0.75AB	0.93BC	1C
C. siamense	MFU090230	Holotype	coffee	Chiang Mai, Thailand	1B	0.65A	0.3B	0.5BC	-	1C
C. siamense	MFU09 0548	-	chili	Luang Pra Bang, Laos	-	0.4A	0.4B	1A	-	-
C. simmondsii	BRIP28519	Holotype	papaya	Australia	-	0.35A	1.7AB	0.5BC	0.83C	1C
C. simmondsii	CBS.294.67	Epitype	papaya	Australia	1.5A	-	1.1AB	0.5BC	1.00BC	1C
LSD (between group)					**0.49**	**0.31**	**1.41**	**0.44**	**0.44**	**8.1**

Note. Means with the same letter in each column are not significantly different from each other based on DMRT test in Sirichai statistics version 6; -, no infection

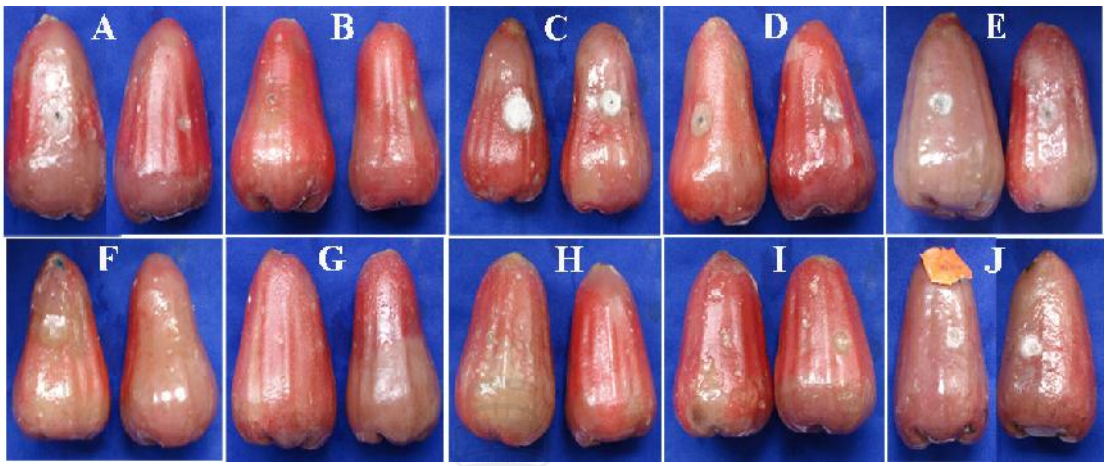


Figure 4.2 *Colletotrichum* Symptoms on Rose Apple 5 Days After Inoculation A. *C. asianum* Isolated From Coffee Berries, B.*C. asianum* From Mango Fruit, C.*C. cordylinicola* From Rose Apple Fruit, D. *C. fructicola* From Coffee Berries, E.*C. fructicola* From Papaya Fruit, F.*C. fructicola* From Longan Fruit, G.*C. siamense* From Coffee Berries, H.*C. siamense* From Chili Fruit, I.*C. simmondsii* From Papaya Fruit, J.*C. simmondsii* From Papaya Fruit

Because pathogenicity testing involves wounding fruits, the results of this study may not accurately reflect the virulence potential of the strains (Phoulivong, Cai, Noireang et al., 2010; Weir et al., 2012).

This study provides further evidence that most *Colletotrichum* species are not host-specific. However, some species of *Colletotrichum* have narrow host ranges. For example *C. kahawae* infects only coffee, *C. coccodes* infects on tomato and potato, *C. falcatum* infects only sugarcane, and *C. musae* infects only banana (Canon et al., 2008; Freeman et al., 2001; Kim et al., 2009; Prihastuti et al., 2009; Sreenivasapradad & Talhinhas, 2005; Yang, Liu et al., 2012). Only some isolates of *C. kahawae*, a disease of biosecurity importance, are able to cause coffee berry disease (Silva, Talhinas, Várzea et al., 2012; Silva, Talhinhas, Cai et al., 2012) and these isolates could be distinguished using GS sequences (Weir et al., 2012), Apn25L and MAT 1-2-1 (Silva, Talhinhas, Cai et al., 2012).

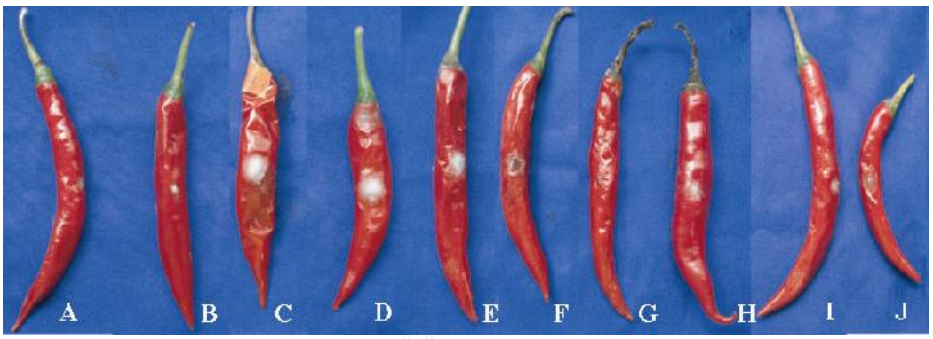


Figure 4.3 Anthracnose Symptom on Chili 7 Days After Inoculation A. *Colletotrichum asianum* Isolated From Coffee Berries, B.*C. asianum* From Mango Fruit, C.*C. cordylinicola* From Rose Apple Fruit, *C. fructicola* From Coffee Berries, E.*C. fructicola* From Papaya Fruit, F.*C. fructicola* From Longan Fruit, G.*C. siamense* From Coffee Berries, H.*C. siamense* From Chili Fruit, I.*C. simmondsii* From Papaya Fruit, J.*C. simmondsii* From Papaya Fruit

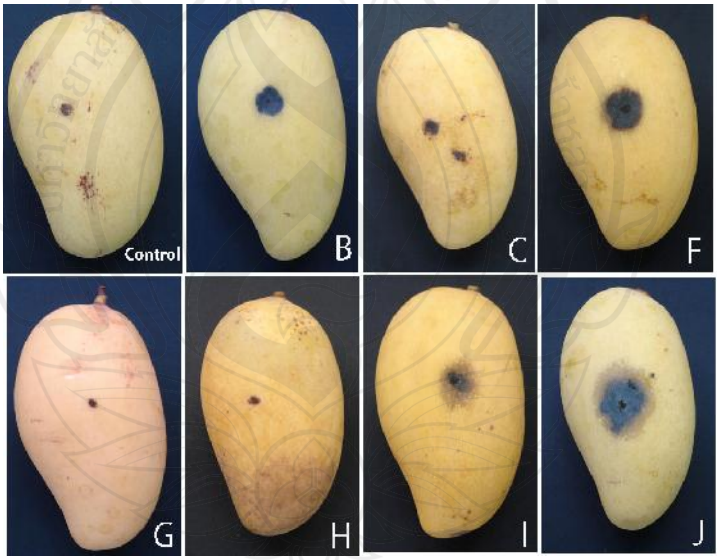


Figure 4.4 Anthracnose Symptom on Mango 7 Days After Inoculation: A. Control, B.*C. asianum* From Mango Fruit, C.*C. cordylinicola* From Rose Apple Fruit, F.*C. fructicola* From Longan Fruit, G.*C. siamense* From Coffee Berries, H.*C. siamense* From Chili Fruit, I.*C. simmondsii* From Papaya Fruit, J.*C. simmondsii* From Papaya Fruit

From a quarantine perspective, it is important to establish the host range of a specific *Colletotrichum* species, as spread of host-specific taxa such as *C. kahawae* should be restricted. *Colletotrichum simmondsii*, *C. fructicola* and *C. siamense* can infect many fruits including chili, coffee, dragon fruit, guava, mango, papaya, rose apple and strawberry (Phoulivong, Cai, Chen et al., 2010, Table 4.2) and are thus not of quarantine threat.

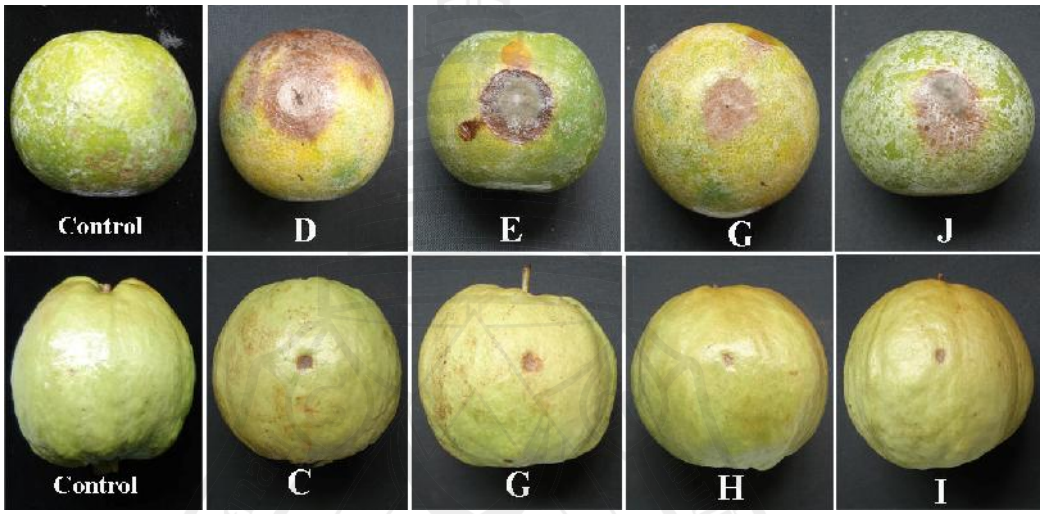


Figure 4.5 Anthracnose Symptoms on Selected Orange (i.e. D, E, G, J and control) and Guava (i.e. C, G, H, I and control) 7 Days After Inoculation: D. *C. fructicola* From Coffee Berries, E.*C. fructicola* From Papaya Fruit, G.*C. siamense* From Coffee Berries, J.*C. simmondsii* From Papaya Fruit, C.*C. cordylinicola* From Rose Apple Fruit, G.*C. siamense* From Coffee Berries, H.*C. siamense* From Chili Fruit, I.*C. simmondsii* From Papaya Fruit

In Table 4.1 we list the species used in this study and their hosts established in recent studies where species were identified based on phylogeny. Strains of *Colletotrichum asianum* infected chili, mango and rose apple host and strains of *C. fructicola* infected chili, citrus, rose apple, and papaya. *Colletotrichum cordylinicola* was specific to *Cordyline fruticosa* leaves. It is therefore apparent that *C. asianum*, *C. fructicola*, *C. siamense* and *C. simmondsii* have wide host ranges, while *C. cordylinicola*

has a narrow host range. This is important for understanding the virulence *Colletotrichum* species on different hosts (Phoulivong, McKenzie & Hyde, 2012).

4.5 Conclusion

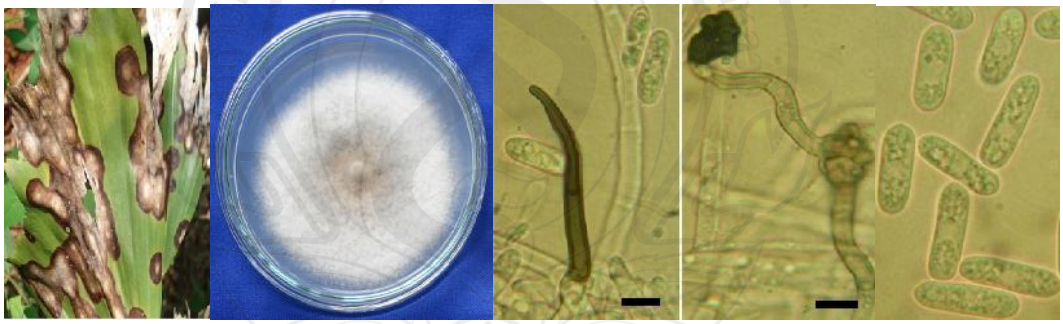
Strains of *Colletotrichum* were isolated from the fruits of chili, coffee, longan, mango, papaya and rose apple, collected from orchards and markets in Laos and Thailand. Isolates were identified using morphological characters, colony growth rate, and confirmed with molecular sequence data analysis of combined multi-gene loci. Pathogenicity testing of ten strains representing five species of *Colletotrichum* was carried out on *Capsicum* sp. (chili), *Carica papaya* (papaya), *Citrus reticulata* (orange), *Eugenia javanica* (rose apple), *Mangifera indica* (mango) and *Psidium guajava* (guava) using a wound drop technique. Pathogenicity and potential for cross infectivity of *Colletotrichum asianum*, *C. cordylinicola*, *C. fructicola*, *C. saimense* and *C. simmondsii* were tested on the hosts. The *Colletotrichum* species tested were generally shown to infect a wide host range.

CHAPTER 5

OVERALL CONCLUSION

5.1 Diversity and Distribution of *Colletotrichum* Causing Anthracnose

Colletotrichum species isolated from infected fruits and leaves with anthracnose were collected from Luang Prabang, Sayaboury, Vientiane and Savannaketh Provinces in Laos, and Chiang Mai, Chiang Rai, Bangkok and Nakon Si Thammarat Provinces in Thailand. All of the *Colletotrichum* strains were identified based on morphological characters including colony characteristics, growth rate, conidial size and shape, and appressoria (Figure 5.1 and Table 5.1).



A B C D E

Figure 5.1 General Characteristics of the Fungus Isolated From Plant Leaves: A. Anthracnose Disease on Leaves, B. Mycelium, C. Setae, D. Appressoria, and E. Conidia

Some general characteristics of *C. gloeosporioides* are given as follows. The fungus produces hyaline, one-celled, ovoid to oblong, slightly curved or dumbbell-shaped conidia, 11.9-17 µm in length and 3.6-5.8 µm in width. Masses of conidia appear pink or salmon colored. The waxy acervuli, that are produced in infected tissue, are subepidermal, typically with setae, and simple, short, erect conidiophores. An example of cell and colony morphology of *C. gloeosporioides* complex is presented in Figure 5.1.

Anthracnose, caused by *C. gloeosporioides* is the most important and widepread disease affecting fruits and leaves. The symptoms vary between different hosts, and are characterised by dark, depressed lession on ripe fruit often accompanied by pink, slimy spore masses which develop as acervuli mature. Infection on leaves are manifested as subcircular or angular black lession which enlarge and coaleses, frequently destroying leaf edged or entire inflorescences.

Table 5.1 Morphological Characteristics *Colletotrichum gloeosporioides*

Characteristics	Description	References
Colony	Colonies at first grey and becoming dark grey to black, black circular zones in reverse, max.	Cai et al. (2009); Phoulivong et al. (2009); Prihastuti et al. (2009); Rojas et al. (2010); Than et al. (2009); Weir et al. (2012)
Growth rate (mm/day)	10.9±5	
Conidia measurement	11.9-17.0 x 3.6-5.8	
Shape type	Cylindrical with obtuse end and slightly tapered, sometimes slightly rounded ends to oblong	
Appessoria measurement	4.9-24.6 x 3.7-12.3	
Shape type	Circular to slightly irregular, medium-brown to brown, sparse in slide cultures	
Setae	Present and absent	
Sclerotia	Present and absent	

In this study, we isolated 236 strains of *Colletotrichum*. Based on morphology, these strains were initially characterized as *C. gloeosporioides* since all characteristics were in agreement with those described in Table 5.1. They were obtained from different fruits and plant leaves (data not shown) the distribution of these fungal isolates based on geographical location was provided in Table 5.2.

Table 5.2 Locations and Numbers of *Colletotrichum* Strains

Regions	Numbers of isolates
Laos	
Luang Prabang and Sayaboury	31
Vientiane	46
Savannaketh	14
Thailand	
Chiang Mai and Chiang Rai	63
Bangkok	45
Nakon Si Thammarat	37

5.2 *Colletotrichum gloeosporioides* Complex

Traditional identification of *Colletotrichum* has relied heavily on morphological and cultural characteristics. However, it should be noted that morphology alone does not provide sufficient information for a precise identification, especially for those species in the *C. gloeosporioides* complexes. Species with similar morphological characteristics may have considerable variation at the physiological and pathogenic levels. *C. gloeosporioides* has recently been epitypified so that living cultures and sequence data are, for first time available for comparison with fresh collections (Cannon et al., 2008).

Multi-locus phylogeny is a powerful tool to diagnose *Colletotrichum* species and has been widely employed. Unfortunately, different research groups have utilized different gene regions. In this study we used ITS, CAL, GS, GPDH, ACT and TUB2. To

facilitate future study and comprehensive comparison of *Colletotrichum* species, it is important that an agreement should be established on how many and which genes should be used. An even more important issue is the interpretation of phylogenetic groups in terms of classification. Currently there is no consensus over what constitutes a species, although researchers started to adopt "genealogical concordance" to recognise phylogenetic species. From our study, we suggest that the species rank should be given to well defined phylogenetic lineages that are in conjunction with recognisable phenotypic characters. Taxonomy is presently unsatisfactory and there was a need for a polyphasic approach for identification, which reflects the natural classification of species and subspecific taxa within the genus. In this study, a taxonomic framework for describing *Colletotrichum* epitypes and new species is followed.

The *C. gloeosporioides* species complex is defined genetically, based on a strongly supported clade within the *Colletotrichum* ITS gene tree. Recently, it has been shown that the *C. gloeosporioides* complex comprises of 22 spp. plus sub species based on multi-gene analysis (Cai et al., 2009).

Our recent analysis of sequence data of 25 isolates from eight tropical fruits are compared with the *C. gloeosporioides* epitype. Contrary to previous understanding, none of the 25 *Colletotrichum* isolates from tropical fruits was *C. gloeosporioides*. The five gene regions used in this study resolved *Colletotrichum asianum, C. fructicola, C. horii, C.kahawae* and *C.gloeosporioides* in the 'gloeosporioides' complex as distinct phylogenetic lineages with high statistical support.

Table 5.3 *Colletotrichum gloeosporioides* Complex (modified from Weir et al. (2012)

Colletotrichum gloeosporioides
C. asianum
C. cordylinicola
C. fructicola
C. gloeosporioides
C. horii

Table 5.3 (continued)

Colletotrichum gloeosporioides
C. kahawae subsp. kahawae
C. musae
C. nupharicola
C. psidii
C. siamense
C. theobromicola
C. tropicale
C. xanthorrhoeae
*C. aenigma**
*C. aeschynomenes**
*C. alatae**
*C. alienum**
*C. aotearoa**
*C. clidemiae**
*C. salsolae**
*C. ti**
*C. queenslandicum**

Note. The taxa described new species

5.3 Novel Species of *Colletotrichum*

It has been shown clearly that morphology cannot be used as the key criteria for *C. gloeosporioides* identification. Besides, several studies have recently shown that *C. gloeosporioides* is a broad / complex group consisting of several distinct species. Weir et al. (2012) identified the new species *C. aenigma*, *C. aeschynomenes*, *C. alatae*, *C. alienum*, *C. aotearoa*, *C. clidemiae*, *C. kahawae* sub

sp. *ciggaro*, *C.salsolae*, and *C.ti*. These new species are proposed since they form distinct clades when the multi-gene analysis was used.

Four new species are described in my study. *C. cordylinicola* recorded from *Cordyline fruticosa* is morphologically similar to several species in the *C. gloeosporioides* complex. Species in this complex are difficult to differentiate based solely on morphology. *C.brevisporum* is recorded from *Neoregelia* sp. and *Pandanus pygmaeus* Hook. It should be noted interestingly that this is the fist report of the isolates of *Colletotrichum* spp. on *Citrus maxima* and *Paphiopedilum bellatolum* plants. *C.thailandicum* from *Hibiscus rosa-sinensis* differs from *C.hibisci* and *C. hibiscicola* differ in producing larger conidia.

5.4 Future Work

In my study, we used multi-locus phylogeny as a powerful tool to diagnose *Colletotrichum* species. To increase and substantiate knowledge about *Colletotrichum* and anthracnose disease I suggest that future research should be carried out on the following topics.

5.4.1 Establish phylogenetic relationships between *Colletotrichum* species.

5.4.2 Establish host ranges of *Colletotrichum* species.

5.4.3 Discover other effective genes for sequencing to confirm *Colletotrichum* species.

5.4.4 Establish virulence of *Colletotrichum* species from different geographic areas to apply to plant disease management and control.

5.4.5 Improve plant anthracnose disease control and establish quarantine measurements for the spread of anthracnose disease.

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APPENDICES

APPENDIX A

ABSTRACT PERTAINING THESIS

ABSTRACT

Phoulivong, S., Phan, P. P., Prihastuti, H. & Hyde, K. D. (2008). ***Colletotrichum* and Identification of *Colletotrichum* spp**. Causing Anthracnose of fruit crops in Laos. Abstract The 3th Annual Meeting of Thai Mycological Association and Mycology Conference. KhonKaen, Thailand.

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APPENDIX B

PUBLICATION LIST

Than, P. P., Prihastuti, H., Phoulivong, S., Taylor, P. W. J & Hyde, K. D. (2008). Review: Chili anthracnose disease caused by *Colletotrichum* species. **Journal Zhejiang University, 9**, 764-778.

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