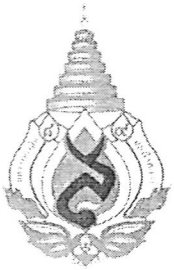

รายงานฉบับสมบูรณ์

การปรับปรุงวิธีการที่เหมาะสมต่อการจัดจำแนกวงศ์วานวิวัฒนาการของเชื้อราในกลุ่ม *Colletotrichum* ที่เป็นสาเหตุที่สาคัญของโรคพืช

A practical phylogeny-based approach for revision of the important pathogenic genus *Colletotrichum*

โดย

Associate Professor Dr. Kevin D. Hyde และคณะ

งานวิจัยนี้ได้รับเงินอุดหนุนการวิจัยจากมหาวิทยาลัยแม่ฟ้าหลวง

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ACKNOWLEDGEMENT

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Associate Professor Dr. Kevin D. Hyde

บทสรุปผู้บริหาร (EXECUTIVE SUMMARY)

1. ความสำคัญและที่มาของปัญหาในการการวิจัย (Rationale and review)

Species concepts in the important pathogenic genus *Colletotrichum* are currently based largely on morphology in culture. Host-specificity is now considered to operate only at the microspecies level. Assumptions in the past that *Colletotrichum* species are host-specific has led to more than 650 species having been named (http://www.ukncc.co.uk/cabipages/Names/NAMES.ASP). This has severely constrained accurate exchange of information on *Colletotrichum* species.

Morphology and culture-based systems are generally appropriate for diagnosis of species aggregates, but are of little use to distinguish the evolutionary units of relevance to plant pathologists. Hence the outcome of the project will be a significant refinement of current *Colletotrichum* taxonomy based on phylogenetic evidence potentially leading to molecular identification systems and diagnostic tools. This in turn will lead to a better understanding of the requirements for plant breeders who require knowledge of species they need to breed plant resistance against.

2. วัตถุประสงค์ของโครงการวิจัย (Objectives of the research)

i. To create a preliminary multigene-based phylogeny of the genus *Colletotrichum* using nuclear and mitochondrial genomes, ribosomal DNA, β-tubulin, TEF1α, and other appropriate genes.

ii. To determine the utility for phylogenetic inferences of single gene sequence data sets as compared to combined data sets at the generic level using *Colletotrichum* species.

iii. To indicated species concepts within the genus by linking molecular result and morphology.

3. ขอบเขตของโครงการวิจัย (Scope of the research)

The genus *Colletotrichum* contains about 50 species, which cause plant diseases often known as anthracnose. We will collect more than 200 isolates in Thailand (mostly northern part) and some specimens from elsewhere in Thailand. In this study we will develop a practical phylogeny-based approach for the identification of *Colletotrichum*, focusing on Thai species and the *C. gloeosporioides* complex. Variations in the mitochondrial genome, ribosomal DNA, β-tubulin, TEF1α and other appropriate genes will be investigated; all are in wide use in other fungal genera to resolve problems in identification. The successful outcome of this project will have important practical implications to the plant pathology, plant breeding and quarantine communities and result in important publications.

4. ระเบียบวิธีวิจัยและผลผลิตจากการวิจัย (Methodology and the research output)

Research Plan from October 2010 to September 2013
Year 2 (October 2011 to September 2012): (12 months).

Anthracnose diseased samples will be collected in Thailand and specimens will be procured from elsewhere as needed for this study. Diseased samples will be observed and any the *Colletotrichum* species associated with the disease tissues will be isolated by single spore isolation techniques. Cultures will be grown in PDA at room temperature for one week and the morphology of selected species will be observed and documented by stereomicroscope and compound microscope. More than 200 isolates will be obtained. Molecular sequence data, initially ITS, will be used to characterize species and decide which strains need further study. All the cultures will be deposited at MFLU culture collection (MFLUCC, Mae Fah Luang University, Thailand). We will also work towards epitypification of other species.

5. ประโยชน์ที่ได้รับ (Benefit)

Colletotrichum species are important pathogens causing disease of crops and ornamental plants and it is essential we can accurately identify species for plant disease control, plant breeding programs, quarantine regulations and important publications. We have surveyed *Colletotrichum* species infecting crops, fruits and other plants in Thailand and isolated endophytes from healthy grasses. By identifying the disease causal agents we were able to establish which species infect which plants in Thailand. Moreover, we published several new species and SCI publications and we have been working towards epitypification of other species. Phylogenetic analyses of ITS and morphology were used to characterize species that need further work and we have been looking towards establishing a single gene(s) that can readily identify species. Ultimately we have been developing a practical phylogeny for the identification of *Colletotrichum* species, by focusing on Thai species. Our research has involved international collaborations so that the techniques used to classify *Colletotrichum* species are globally accepted.

6. แผนการถ่ายทอดเทคโนโลยีหรือผลการวิจัยสู่กลุ่มเป้าหมาย

Systematics, plant pathologists, plant health practitioners, plant breeders and quarantine officers, since they cannot name organisms confidently

บทคัดย่อ

เชื้อราในสกุล *Colletotrichum* (หรือ "Glomerella") ซึ่งโดยส่วนใหญ่เป็นสาเหตุของโรคแอนแทรคโนสโดยเข้าทำลายพืชเศรษฐกิจ อาทิเช่น ธัญพืช พืชผัก พืชตระกูลถั่ว ไม้ดอกไม้ประดับ และ ไม้ผล และทางด้านการควบคุมและกักกัน อันรวมไปถึงการปรับปรุงพันธุ์พืช ซึ่งเชื้อราในสกุล *Colletotrichum* สามารถแพร่ระบาดได้อย่างกว้างขวาง โดยเชื้อราในสกุลนี้ยังเป็นสิ่งมีชีวิตที่ต้องการพืชอาศัยและจะปรากฏร่วมกับบริเวณที่เกิดอาการของโรคคล้ายเชื้อราเอนโดไฟต์ ในอดีตการจัดจำแนกนี้พิจารณาจากลักษณะทางด้านสัญฐานวิทยาและชนิดของพืชอาศัยซึ่งพบว่ามีความซับซ้อนเป็นอย่างมาก จึงได้มีการใช้ ITS ยีนส์ร่วมกับลักษณะทางสัณฐานวิทยา อย่างไรก็ตามก็ยังไม่สามารถจัดจำแนกในระดับสปีชี่ส์ได้อย่างแม่นยำ เมื่อไม่นานมานี้ จึงมีการใช้ความหลากหลายของยีนส์ actin (*act*), calmodulin (*cal*), chitin synthase (*chs1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) และ ITS ร่วมกับการศึกษาทางด้านสัญฐานวิทยารวมไปถึงการทดลองความสามารถในการก่อโรค ทำให้ขณะนี้มีการรายงานจะนวนสปีชีส์กว่า 100 สปีชีส์ โดย 4 สปีชีส์ได้รับการยอมรับว่าเป็นสปีชีส์ที่มีความซับซ้อนโดยเฉพาะอย่างยิ่ง *C. gloeosporioides* ประกอบไปด้วยลักษณะทางสัณฐานวิทยา 22 ลักษณะที่มีความคล้ายคลึงกัน อย่างไรก็ตาม ITS, beta tubulin (*tub2*), DNA lyase และ the intergenic region of *apn2* and *MAT1-2-1* genes (*ApMat*)) ได้ใช้ในการจัด ดังนั้น 28 สปีชีส์ จึงได้รับการยอมรับ ในงานวิจัยฉบับนี้ผู้วิจัยได้ยืนยันผลการวิจัยทางด้านชีวิโมเลกุล มากกว่า 50 สปีชีส์ โดย 15 สปีชี่ส์ค้นพบภายในประเทศไทยและได้สำรวจเชื้อราในกลุ่มดังกล่างที่เข้าทำลายพืชผลและเอนโดไฟต์ในพืชตระกูลหญ้า โดยได้มีการรวบรวมจากตัวอย่างสดในแต่ละสถานที่กว่า 200 ตัวอย่าง โดยผู้วิจัยได้มีการนำเสนอผลงานการวิจัย 5 ฉบับ ตั้งแต่เชื้อราในสกุลนี้ได้เป็นปัญหาที่พบได้ทั่วโลกดังนั้นทางผู้วิจัยจึงได้เลือกการติดต่อประสานงานกับคณะวิจัยในประเทศบลาซิล จีน และ อินเดีย เพื่อให้เกิดการศึกษาอย่างกว้างขวางและครอบคลุม

ABSTRACT

The genus *Colletotrichum* (sexual state "Glomerella") are important pathogens causing serious disease of plants and infected crops are subjected of import control (quarantine) and plant breeding programs. *Colletotrichum* species have a worldwide distribution and are associated with leaf spots, fruit anthracnose and when serious infections occur they are responsible for reducing economic plant yields (e.g. of cereals, vegetables, legumes, ornamental plants and fruits). They are also obligate symbionts and occur in a symptomless parts of plants as endophytes and the relationships between life modes (i.e. can the fungi switch modes) is poorly establish. Previous identification and classification was based on host association and morphological characteristics. Molecular sequence data analysis has become commonplace in classifying plant pathogenic genera like *Colletotrichum*, which have been found to comprise several species complexes. Initially ITS and morphology was used to characterize species, however, they could not resolve species well. Recent multigene phylogenetic analysis have involved actin (*act*), calmodulin (*cal*), chitin synthase (*chs1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and ITS gene regions as well as morphology and pathogenicity testing so at present there are about 100 described species and this is increasing monthly. There are also four accepted species complexes and *C. gloeosporioides* is the most important. Recently multigene phylogenetic analysis confirmed that *C. gloeosporioides* is a species complex that comprises 22 morphologically similar, phylogenetically distinct species. However, ITS, beta tubulin (*tub2*), DNA lyase and the intergenic region of *apn2* and *MAT1-2-1* genes (*ApMat*)) have also been used to identify new lineages and new species within this species-complex and presently, there are 28 accepted species names within the species complex. There is however, no consensus among mycologists as to which gene markers should be used to define and delimit a species within the species complex.

At the beginning of this study (October 2010) there were more 50 confirmed "molecular" species in the genus causing plant diseases often known as anthracnose with 15 species known from Thailand. We therefore initiated a survey of *Colletotrichum* species infecting fruits in Thailand and also those which are

endophytes of healthy grasses. In the first and second year we collected and isolated strains from more than 200 fresh specimens of various disease plants and fruits and asymptomatic grass endophytes from difference places in Thailand. We also started to sequence these isolates and carry out morphological as well as pathogenicity studies. We identified several new species and also worked towards epitypification of other species and five publications (four SCI) appeared in year two of the grant. We are involved in developing a practical phylogeny and morphology based approach for the identification of *Colletotrichum* species, focusing on Thai species. However, since this is a global problem we have also chosen to collaborate with Brazilian, Chinese and Indian colleagues in order to bring greater depth to the research and international agreement to the findings. The latter was achieved with the formation of the International Subcommission on *Colletotrichum* taxonomy (http://www.fungaltaxonomy.org/ subcommissions) of which our group is a founder member.

Keywords: *Colletotrichum*, Phylogeny, Plant pathogen, Taxonomy

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ABBREVIATION AND SYMBOLS

ACT	=	Actin
CTAB	=	Hexadecyltrimethylammonium bromide
DNA	=	Deoxyribonucleic acid
ITS	=	Internal transcribed spacer
LSU	=	Large subunit (28S rDNA)
PDA	=	Potato dextrose agar
RFLP	=	restriction fragment length polymorphism
Sp.	=	Species
TEF 1-α	=	Translation elongation factor 1-alpha
β-tubulin	=	Beta-tubulin
μm	=	micrometer

CHAPTER 1
INTRODUCTION

1.1 Background

Species concepts in the important pathogenic genus *Colletotrichum* are currently based largely on morphology in culture. Host specificity is now considered to operate only at the micro-species level. An assumption in the past that Colletotrichum species are host-specific has led to numerous problems in identification, with more than 650 species having been named (http://www.ukncc.co.uk/cabipages/Names/NAMES.ASP). This has severely constrained accurate exchange of information on *Colletotrichum* species.

Morphology and culture-based systems are generally appropriate for diagnosis of species aggregates, but are of little use to distinguish the evolutionary units of relevance to plant pathologists. Hence the outcome of the project will be a significant refinement of current *Colletotrichum* taxonomy based on phylogenetic evidence potentially leading to molecular identification systems and diagnostic tools. This in turn will lead to a better understanding of the requirements for plant breeders who require knowledge of species they need to breed plant resistance against.

1.2 Objectives

1.2.1 To create a preliminary multigene-based phylogeny of the genus *Colletotrichum* using nuclear and mitochondrial genomes, ribosomal DNA, β-tubulin, TEF1α, and other appropriate genes.

1.2.2 To determine the utility for phylogenetic inferences of single gene sequence data sets as compared to combined data sets at the generic level using *Colletotrichum* species.

1.2.3 To elucidate species concepts within the genus by linking molecular and morphological approaches.

1.3 Scope of research

The genus *Colletotrichum* contains about 50 species, which cause plant diseases often known as anthracnose. *Colletotrichum* species are worldwide in distribution and cause major damage to cereals, vegetables, legumes, ornamental plants and fruit trees. The current naming of *Colletotrichum* species is largely based on a combination of morphological and cultural characteristics. Due to limited numbers of morphological character-suites available in culture coupled with inherent phenotypic plasticity, precise identification of the species has always been difficult. Physiological specialization within species and overlapping host ranges mean that our current classification system is impracticable for users. This causes problems to systematics, plant pathologists, plant health practitioners, plant breeders and quarantine officers, since they cannot name organisms confidently. In our proposal we will develop a practical phylogeny-based approach for identification of *Colletotrichum*, focusing on Thai species and the *C. gloeosporioides* complex. Variations in the mitochondrial genome, ribosomal DNA, β-tubulin, TEF1α and other appropriate genes will be investigated; all are in wide use in other fungal genera to resolve problems in identification and taxonomy. The successful outcome of this project will have important practical implications to the plant pathology, plant breeding and quarantine communities and important publications.

CHAPTER 2

LITERATURE REVIEW

2.1 The need for species recognition

The genus *Colletotrichum* causes various plant diseases often known as anthracnose and is worldwide in distribution (Sutton, 1992). *Colletotrichum* species cause major damage to crops in tropical, subtropical and temperate regions (Than *et al.*, 2008 and Hyde *et al.*, 2009a, 2010). Cereal, vegetables, legumes, ornamentals and fruit trees may be seriously affected by this pathogen (Freeman, 2000). Also, *Colletotrichum* sp. is cosmopolitan and has been shown that multiple species can infect single host or single species can infect multiple hosts (Cai *et al.*, 2009 and Hyde *et al.* 2009). *Colletotrichum* species are also commonly isolated as endophytes, and latent and quiescent infections by these species on several hosts have been reported (Bills, 1996; Brown *et al.*, 1998 and Photita *et al.*, 2004, 2005). Their ability to cause latent infection i.e. infection without visible symptom makes them one of the most successful pathogens causing post-harvest disease in a wide range of crop species (Sutton, 1992).

At least nine different *Colletotrichum* species, *C. capsici*, *C. coccodes*, *C. crassipes*, *C. dematium*, *C. destructivum*, *C. gloeosporioides*, *C. lindemuthianum*, *C. trifolii* and *C. truncatum* for example, have been reported on legumes in tropical and temperate regions (Lenne, 1992). These legume hosts include many important human food sources including grain legumes, root crops and fruits, and pasture plants, medicinal plants, timber trees and ornamentals (Anonymous, 1979). All of these *Colletotrichum* species are reported to infect at least two hosts, and *C. capsici*, *C. gloeosporioides* and *C. lindemuthianum* are reported to have the widest host ranges among these nine. These reports however, are not backed up by voucher specimens and their occurrence cannot be verified. If we want to breed legumes that are resistant

to anthracnose caused by *Colletotrichum* species we must know which species infect which hosts.

Colletotrichum gloeosporioides is a particularly large complex comprising taxa which cause diseases of a wide range of crops. The taxa have been isolated as pathogens, endophytes and saprobes and it is not clear whether these different lifestyles are associated with specific lineages or have evolved many times. It is therefore particularly important that we gain an understanding of the diversity of organisms within this complex.

Colletotrichum sp. is the anamorphic stage of several species of *Glomerella* sp. and has a taxonomic history of about 200 years (Corda, 1837). There are 17 acknowledged generic synonyms for *Colletotrichum* sp. and two further names are dubiously included, and there are about 900 species names assigned to this genus (Sutton, 1980, 1992). The identification and characterization of *Colletotrichum* species are mainly based on morphological and cultural criteria or a combination of both. It has become apparent that the classification system presently used has limited scope since some species names assigned to collection and isolates lack the precision required by users. The numbers of morphological characters derived from growth in culture are limited, and growth conditions have been rarely standardized. Moreover the inherent phenotypic plasticity of individual isolates creates confusion in identification. There are group species or species complexes such as *C. gloeosporioides*, *C. dematium*, *C. lindemuthianum* which are known to be represented by at least nine distinct subtaxa (Sutton, 1992).

Under these circumstances the species name has limited practical significance to the plant pathologist involved in disease management and quarantine, and the breeder involved in resistant breeding. The evolution of different systems for identification of species over time has largely been the result of subtle changes in the concept concerning the importance of different aspects of morphology combined with ideas about host range and host-pathogen relationships for particular taxa. Despite these amendments the current concept of *Colletotrichum* sp. systematics is still very broad, unreliable and unpredictable being based on the combination of classical

criteria such as conidial shape and size, presence, absence and morphology of setae, presence of sclerotia and appressoria and symptom expression on host. Moreover, the current classification system for *Colletotrichum* in general is unsatisfactory because the constituent species are inadequately defined (Cannon *et al.*, 2000; Than *et al.*, 2008a).

Molecular approaches are being used to resolve problems in fungal taxonomy and fungal identification by many workers (Ma *et al.*, 1997; Ranghoo *et al.*, 1999; Rollo *et al.*, 1995; Zhang *et* al., 1997; Than *et al*, 2008a.b). Because of the shortcomings of *Colletotrichum* sp. systematic based on cultural characteristics and morphology there is need for a combined approach including the use of molecular data. Various DNA-based systems have been used to study phylogeny, systematic, genetic diversity and population structure of *Colletotrichum* species. These markers include restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified restriction fragment length polymorphisms (AFLP), rDNA internal transcriber spacer (ITS1 to ITS2) and small subunit ribosomal RNA (18S rDNA). RFLP was found extremely useful to determine genetic relationships within *C. gloeosporioides* from *Stylosanthes* spp (Braithwaite *et al.*, 1990). The same marker was used to study diversity in *C. gloeosporioides* from *Stylosanthes guianensis*, and avocado and almond (Freeman *et al.*, 1996; Kelemu *et al.*, 1999). RAPD was used to study genetic diversity and variability in *C. lindemuthianum*, *C. acuminatum* and *C. gloeosporioides* (Balardin *et al.*, 1997; Gonzalez *et al.*, 1998; Kelemu *et al.*, 1999; Lander *et al.*, 1999; Sicard *et al.*, 1997). Similarly AFLP was used to characterize *C. lindemuthianum* isolates (Gonzalez *et al.*, 1998), however these days these techniques are considered crude.

Cannon *et al.* (2000) stated that data derived from nucleic acid analyses should provide the most reliable framework to build a classification of *Colletotrichum* sp., as DNA characters were not directly influenced by environmental factors. Most fungal phylogenetic studies utilized sequences from the ribosomal gene cluster, since they were present in large numbers as tandem repeats and evolved as a single unit (Mitchell *et al.*, 1995). In particular, sequence analysis of the internal transcribed spacer (ITS) regions which lie between the 18S and 5.8S genes and the 5.8S and 28S

genes, has proved useful in studying phylogenetic relationships of *Colletotrichum* species because of their comparative variability (Sreenivasaprasad *et al.*, 1994; 1996; Moriwaki *et al.*, 2002; Photita *et al.*, 2005; Shenoy *et al.*, 2007; Than *et al.*, 2008a,b). The ITS region was used to analyze *Colletotrichum* species from various fruits, to study intraspecific diversity in *C. acuminatum* and *C. lindemuthianum* and to study phylogeny and systematic of several *Colletotrichum* spp. (Freeman *et al.*, 2000, 2001; Sicard *et al.*, 1997; Sreenivasaprasad *et al.*, 1996). Small subunit rRNA was used to infer phylogenetic placement of *Athelia bambacina*, *Aureobasidium pullunans* and *C. gloeosporioides* (Illingworth *et al.*, 1991). Besides these A+T rich DNA analysis, ß-tubulin genes have been used to study *Colletotrichum* species (Freeman *et al.*, 2000, 2001; Thon and Royse, 1999; Than *et al.*, 2008a,b). Sequence analysis of protein coding genes such as partial β-tubulin gene, has also been applied to resolve phylogenetic relationships among *C. acutatum* species complexes (Sreenivasaprasad and Talhinhas, 2005). Sequences of introns from two genes (glutamine synthase and glyceraldehyde-3-phosphate dehydrogenase) were also used to evaluate a diverse collection of isolates of *C. acutatum* (Guerber *et al.*, 2003; MacKenzie *et al.*, 2008). *C. acutatum* isolates clustered into groups (Guerber *et al.*, 2003; MacKenzie *et al.*, 2008; Peres *et al.*, 2008). These groups might represent phylogenetically distinct species of *C. acutatum sensu lato* (Guerber *et al.*, 2003). Yun *et al.* (1999) stated that, because of the high intra-species variability and the low inter-species variability, MAT1-2 mating type sequences gave strong support for branches, allowing differentiation of closely related *Cochliobolus* spp. whose relationships were not resolved by ITS sequences alone. Consequently, Du *et al.*(2005) confirmed that MAT1-2 mating type was useful in differentiating the groups of isolates from the species complexes (*C. graminicola*, *C. gloeosporioides* and *C. acutatum*).

A combined application of molecular diagnostic tools along with traditional morphological characterization is an appropriate and reliable approach for studying *Colletotrichum* species complexes (Cannon *et al.*, 2000). Than *et al.* (2008a) differentiated isolates of chilli anthracnose from Thailand into three species: *C. acutatum*, *C. capsici* and *C. gloeosporioides*, based on morphological characterization, sequencing based on rDNA-ITS region and partial ß tubulin gene

and pathogenicity testing. Hong and Kim (2007) reported that Korea isolates of *C. acutatum* were phylogenetically separated from the global groups of *C. acutatum* A1 to A8 based on the sequences in partial beta-tubulin 2 (exons 3-6). Restriction fragment length polymorphisms (RFLP) of ITS region resulting from *Alu*I, *Rsa*I and *Bam*HI digestions have also been employed to differentiate *Colletotrichum* species from chilli anthracnose in Taiwan region (Sheu *et al.*, 2007).

The current classification of *Colletotrichum* species is broad and has a limited practical significance. It is well accepted that the systematic of the genus *Colletotrichum* awaits a detailed investigation and refinement. In this proposal we will initiate the work to refine *Colletotrichum* taxonomy focusing on Thailand taxa and in particular the *C. gloeosporioides* complex. Various molecular techniques such as PCR amplification of rDNA; β-tubulin, TEF1α and other appropriate gene sequences will be used in this study. We will also determine the utility for phylogenetic inferences of single gene sequence data sets as compared to combined data sets at the generic level using *Colletotrichum* species.

CHAPTER 3

MATERIAL AND METHODS

3.1 Material

3.1.1 Petri dish

3.1.2 Loop

3.1.3 Needle

3.1.4 Blade

3.1.5 Slide

3.1.6 Cover slide

3.1.7 Forceps

3.1.8 Mortar and pestle

3.1.9 Tubes

3.1.10 Tips

3.1.11 Pipette/Autopipette

3.1.12 Gel Electrophoresis

3.1.13 Autoclave

3.1.14 Hot air oven

3.1.15 Lamina air flow

3.1.16 Microscopes (Optical and Stereo microscope)

3.1.17 Microtome

3.1.18 Media (Potato dextrose agar, Difgo and Agar)

3.1.19 Chemical

3.1.19.1 Dye (Lactophenol cotton blue stain)

3.1.19.2 Ethanol

3.1.19.3 DNA extract kit

3.1.19.4 Agarose gel

3.1.19.5 Distilled water

3.2 Method

3.2.1 Fungal isolates and cultures

Strains of *Colletotrichum* spp. were isolated by single spore isolation method on the water agar (WA) and Potato dextrose agar (PDA, Difgo). After that cultures were grown on PDA at room temperature (25-30°C) for one week. And then they were deposited at MFLU culture collection (MFLUCC, Mae Fah Luang University, Thailand).

3.2.2 Molecular analysis

3.2.2.1 DNA extraction

DNA extracted from the fungal mycelium that were grown on PDA petridis (one week). Re-suspended fungal mycelium in 1-2 ml of sterile water. Suspensions vortexed briefly and left in the dark at 25°C for 24 hrs to encourage spore germination and/or mycelium were softened. Total genomic DNA extracted by the adapted CTAB method used to extract fungal genomic DNA from fungi (Ford *et al.*, 2000).

3.2.2.2 Polymorphisms in nuclear DNAs

A nuclear DNA probe, GcpR1, from the bean anthracnose pathogen *C. lindemuthianum* had been found very useful to differentiate between species of *Colletotrichum* (Rodriguez and Yoder, 1991). This probe was also used to differentiate between *C. gloeosporioides* from Almond and Avocado (Freeman *et al.*, 1996). The same probe used to study inter-specific differences between these *Colletotrichum* species.

3.2.2.3 Analysis of A + T-rich DNA associated with mt DNA

Polymorphisms in A+T rich DNA revealed by restriction enzyme H*ae*III used to characterize and differentiate various *Colletotrichum* isolates (Freeman and Shabi, 1996; Freeman *et al.*, 1996; Hodson *et al.*, 1993; Sreenevasaprasad *et al.*, 1992). The restriction enzyme H*ae*III recognizes and cleaves the DNA sequence GGCC. Hence, most of the nuclear DNA digested to the fragment of less than 2 kb whereas A+T rich DNA is cleaved infrequently by this enzyme (Freeman *et al.*, 1996).

3.2.2.4 DNA fragment amplification (for ITS, β-tubulin and TEF 1-α genes)

Universal PCR primers available for amplification of ITS 1 and ITS 2 regions between the small and large nuclear rDNA including the 5.8S rDNA used as previously described by (White *et al.*, 1990). Similarly conserved regions of β-tubulin and TEF 1-α genes amplified using specific primers. Amplification reactions carried out in an automated thermal cycler using the basic amplification protocol described by Guo *et al.* (2001).

3.2.2.5 DNA sequencing

PCR products purified by Promega Wizard DNA purification kits. The purified DNA sequenced on an automated sequencer using fluorescent dye-labeled sequencing primers (ALFExpress, Pharmacia). The amplification product with multiple bands were appearing by electrophoresis in 1% low-melting temperature agarose gel, visualized with ethidium bromide, excised separately, purified and sequenced. When the two products were too closed for gel elution they cloned into pGEM-T Easy vector with an overnight ligation. Recombinants vector could identified and plasmid DNA did extracted, purified and sequenced. All of the data made available to the scientific community via submission to GenBank.

3.2.2.6 Data analysis

RFLP auto-radiograms of nDNA RFLP and gel photographs of mtDNA were scored for the presence (1) or absence (0) of band separately. A multi-locus genotype based on these two set of data were constructed for each study isolate. Genetic similarity between and within species were computed to construct UPGMA tree using the NTSYS-pc version 2.1 (Rolf, 2000). nDNA RFLP data for *C. lindemuthianum* populations from Hong Kong and Southern China were subjected to similar analysis, and the genetic diversity within and between the study populations were estimated by Gleason and Shannon indices.

PCR sequenced data of each gene was aligned for all isolates and with published sequences from GenBank and EMBL databases with computer program (eg. SeqApp, Clustal X) and then aligned manually. Non-informative sites was omitted or re-coded in the analysis. All characters were treated as unordered. Alignment data was subjected to three methods of phylogenetic analysis; maximum Parsimony (MP), Weighted parsimony (WP) and Maximum-likelihood (ML) using

PAUP* 4.0 (Swofford, 1998). Out group rooting was used to determine polarity. Bootstrapping and decay indices were used to measure branch robustness.

The techniques involved in the approach described are familiar with the investigators. Dr. Hyde has extensive knowledge in collection, isolation, identification and growth of fungal cultures. Drs Crous and Rampai had hand-on experience in the specific molecular and physiological evaluation techniques. Dr Hyde had many years experience in identification of *Colletotrichum* species and in relating molecular and morphological species concepts.

CHAPTER 4

RESULTS

4.1 Morphological study

Basic morphological characters of *Colletotrichum* species on various plant are the fruiting bodies called acervuli which may have dark setae. Orange masses of conidia may from on the acervuli. Examples of some species we have documented are illustrated in Figs 1-5. Conidia are unicellular, hyaline and differently shaped.

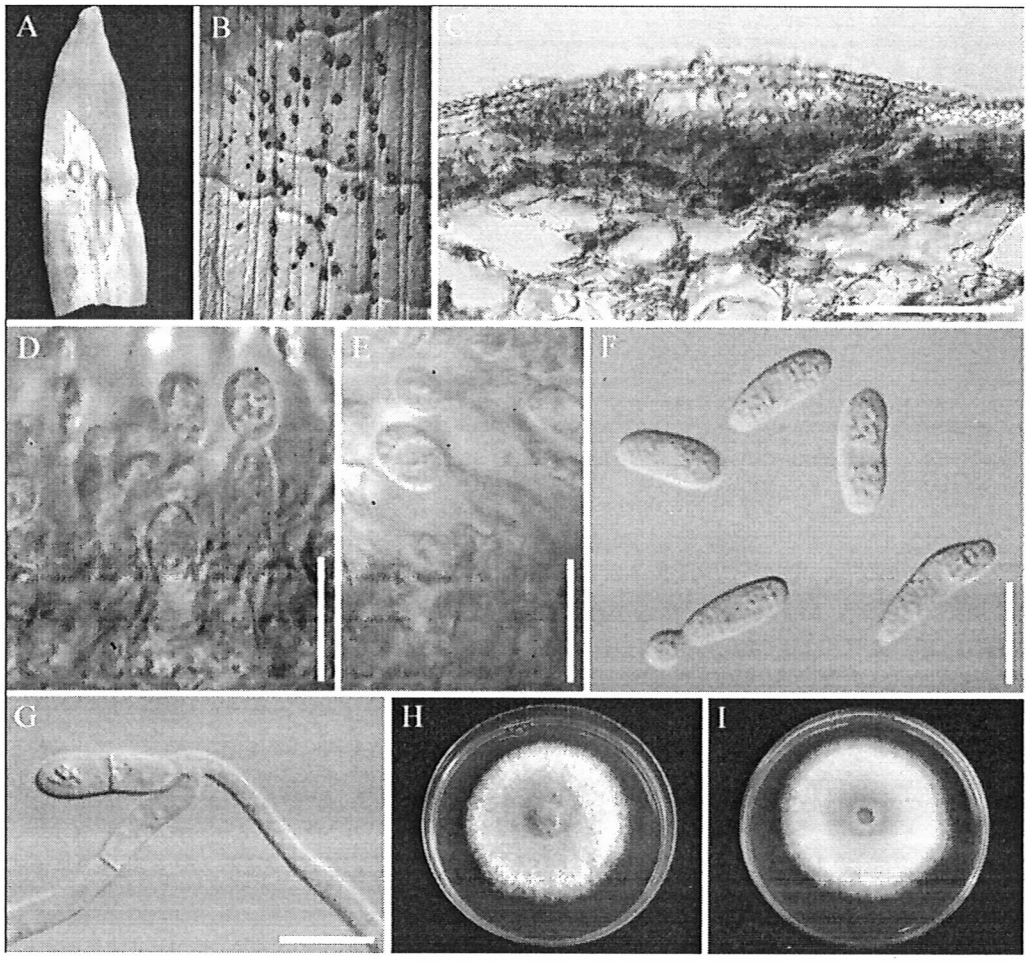

Fig 1. ***Colletotrichum*** **sp.** (MFLUCC12-0205, NTCL045): **A.** Specimen on living leaf. **B.** Conidiomata on the host surface. **C.** Longitudinal section of a conidioma. **D-E.** Conidiogenous cells with developing conidia. **F.** Conidia. **G.** Germinating conidium. **H-I.** Colonies on PDA; **H.** From top; **I.** From reverse. **Scale bar: C** = 100 μm; **D-G** = 10 μm.

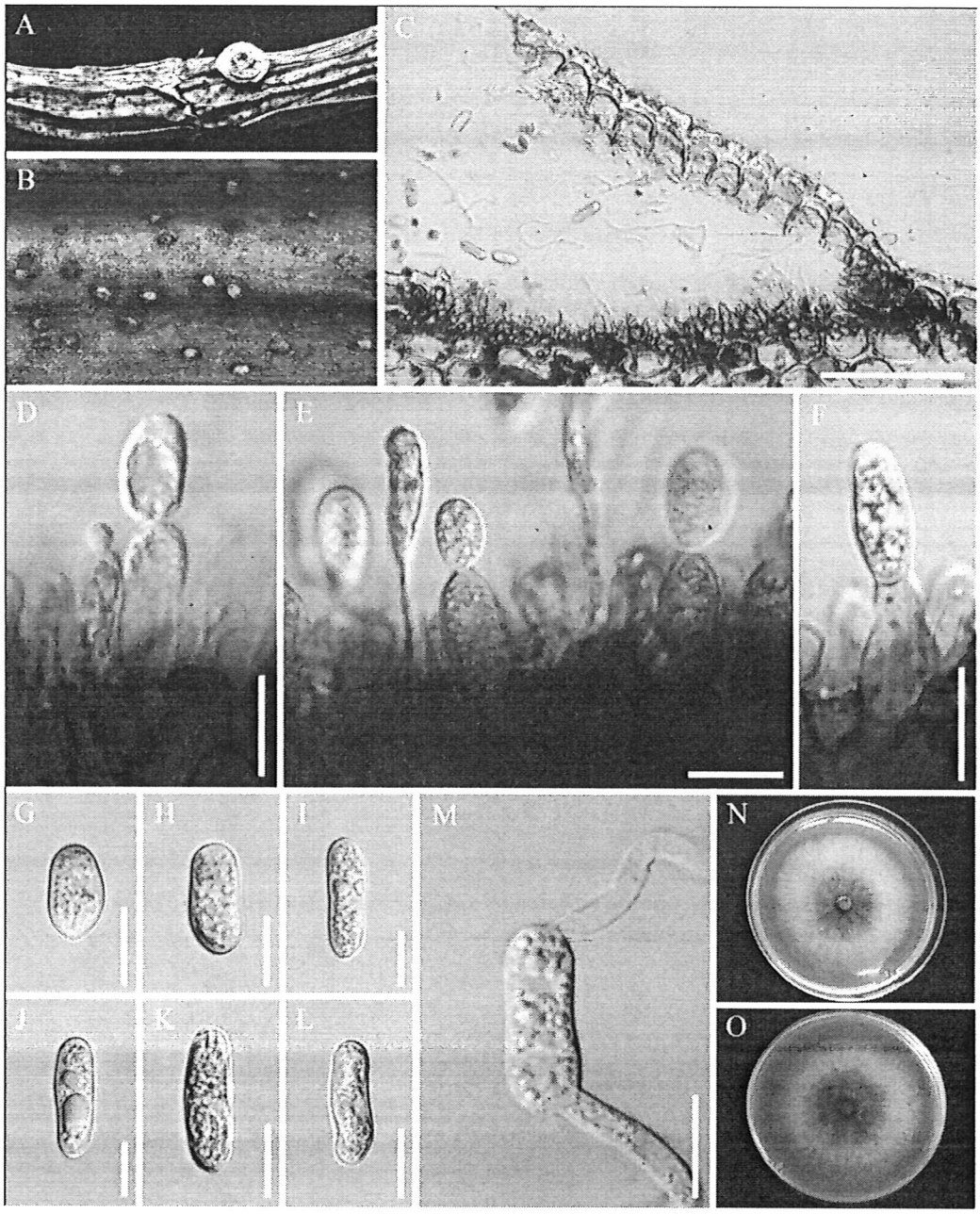

Fig 2. ***Colletotrichum*** **sp.** (MFLUCC12-0138, NTCB001): **A.** Specimen on dead bark. **B.** Conidiomata on the host surface. **C.** Longitudinal section of a conidioma. **D-F.** Conidiogenous cells with developing conidia. **G-L.** Conidia. **M.** Germinating conidium. **N-O.** Colonies on PDA; **N.** From top; **O**. From reverse. **Scale bar: C** = 100 μm; **D-N** = 10 μm.

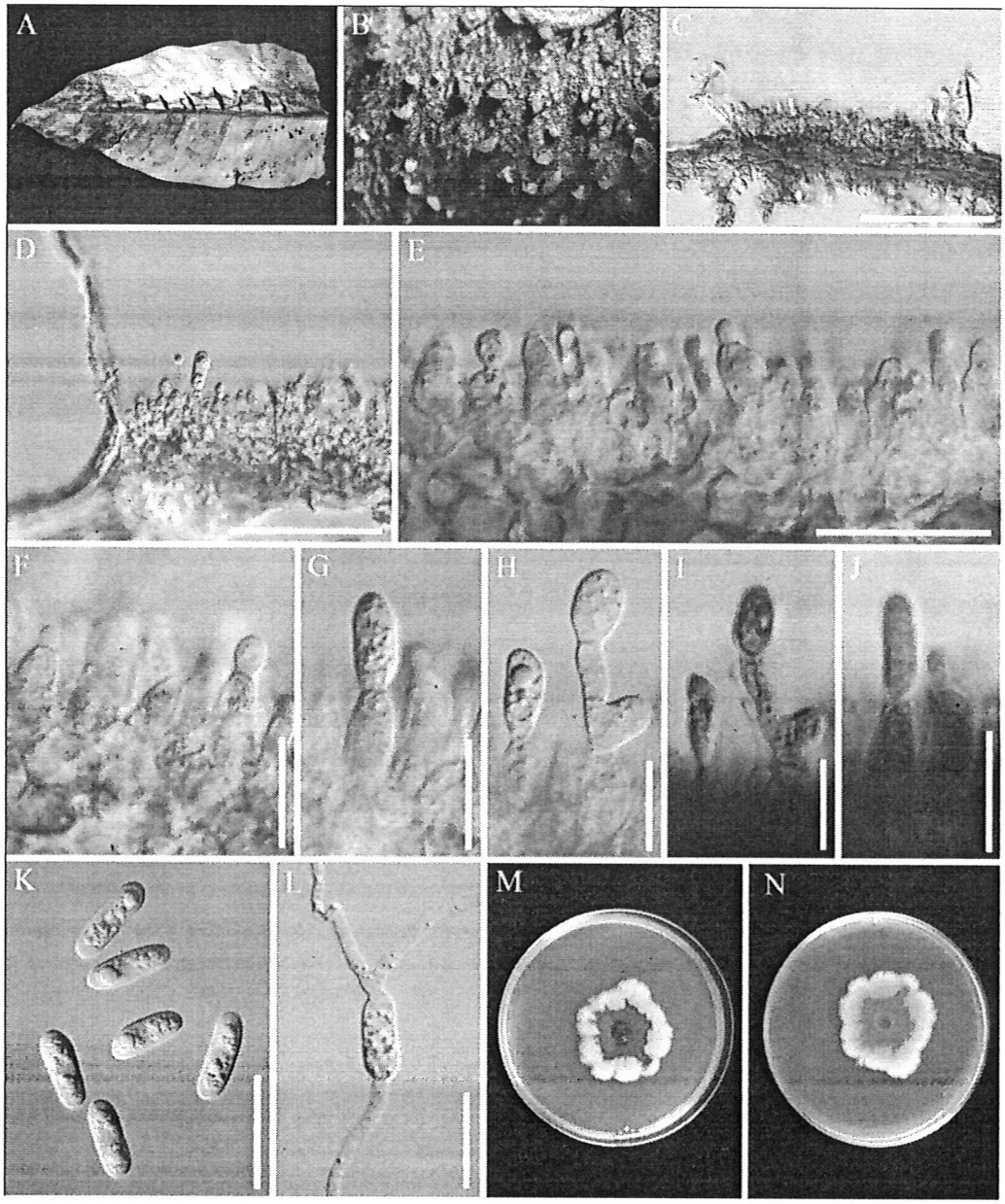

Fig 3. ***Colletotrichum*** **sp.** (MFLUCC12-0148, NTCL031): **A.** Specimen on living leaf. **B.** Conidiomata on the host surface. **C.** Longitudinal section of a conidioma. **D.** Longitudinal section of a conidioma wall. **E-H.** Conidiogenous cells with developing conidia. **I-J.** Conidiogenous cells stained with lactophenol cotton blue. **K.** Conidia. **L.** Germinating conidium. **M-N.** Colonies on PDA; **M.** From top; **N.** From reverse. **Scale bar: C** = 100 μm; **D** =50 μm; **E, K-L** = 20 μm; **F-J** = 10 μm.

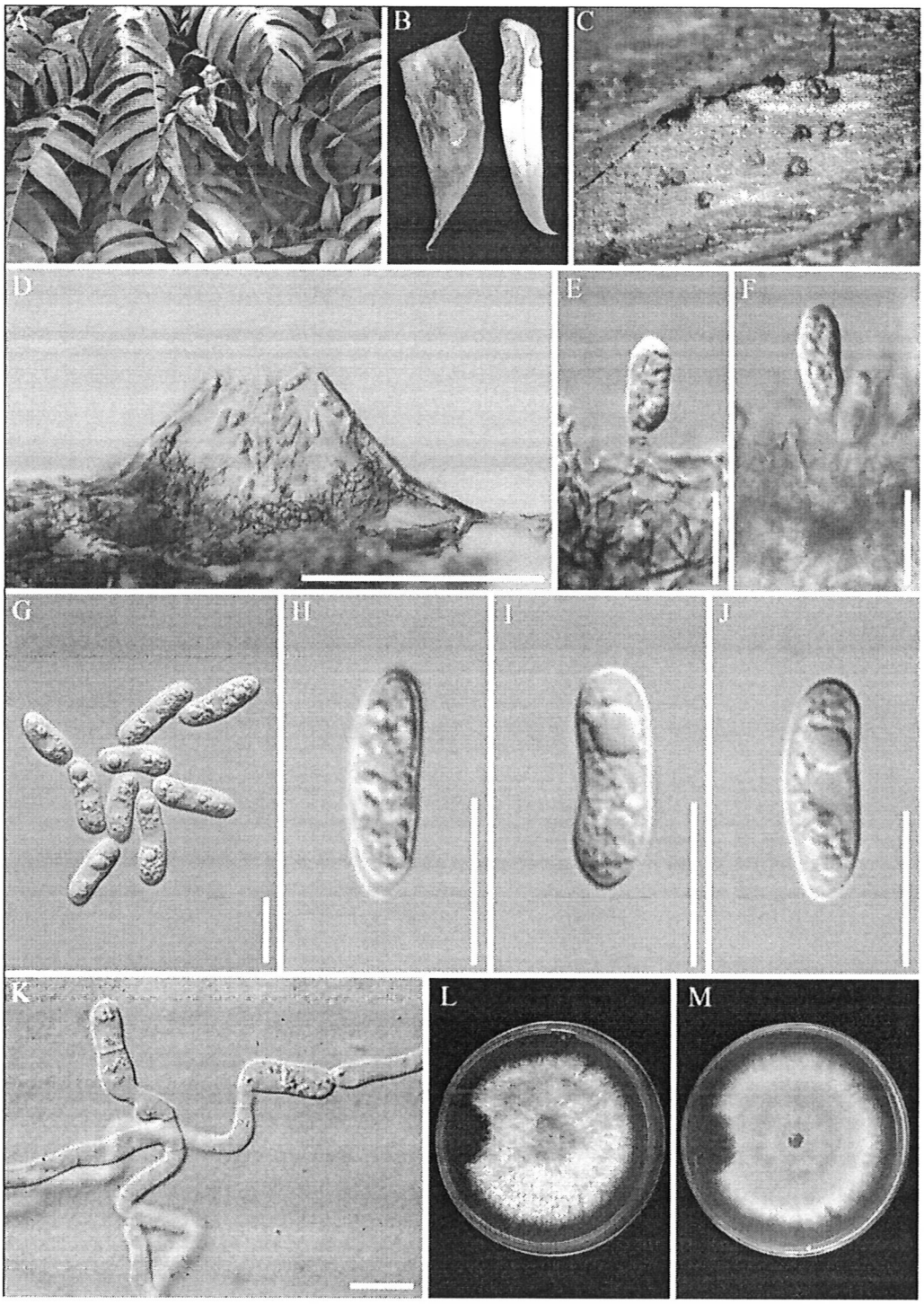

Fig 4. ***Colletotrichum*** **sp.** (MFLUCC12-0203, NTCL042): **A-B.** Specimen on living leaf. **C.** Conidiomata on the host surface. **D.** Longitudinal section of a conidioma. **E-F.** Conidiogenous cells with developing conidia. **G-J.** Conidia. **K.** Germinating conidium. **L-M.** Colonies on PDA; **L.** From top; **M.** From reverse. **Scale bar: D** = 100 μm; **E-K** = 10 μm.

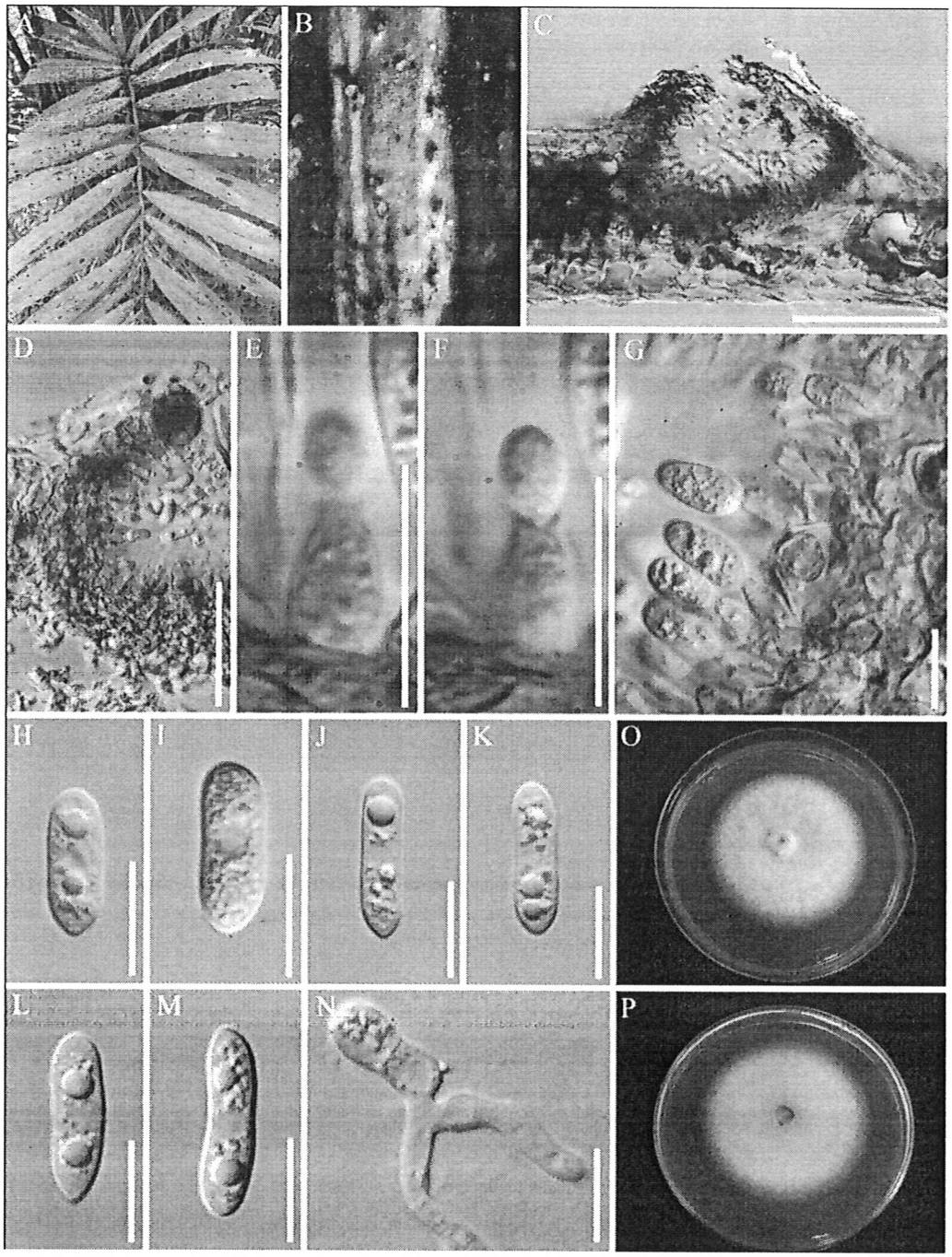

Fig 5. ***Colletotrichum*** **sp.** (MFLUCC12-0204, NTCL043): **A.** Specimen on living leaf. **B.** Conidiomata on the host surface. **C.** Longitudinal section of a conidioma. **D.** Longitudinal section of a conidioma wall. **E-G.** Conidiogenous cells with developing conidia. **H-M.** Conidia. **N.** Germinating conidium. **O-P.** Colonies on PDA; **O.** From top; **P.** From reverse. **Scale bar: C** = 100 μm; **D** = 50 μm; **E-N** = 10 μm.

4.2 Fungal isolates and cultures

In year two, we obtained more than 200 strains of *Colletotrichum* species (Table 4.1) from difference hosts and host parts and difference geographical regions. We have preserved these in MFLU herbaria and MFLUCC culture collection and have carried out DNA sequencing for both.

Table 4.2 Some strains of *Colletotrichum* species collected in Thailand.

MFLUCC	Host	Collection Site
10-0610	Jujube	Chiang Rai
10-0611	Strawberry	Chiang Rai
10-0612	Strawberry	Chiang Rai
10-0613	Strawberry	Chiang Rai
10-0614	Mango	Chiang Rai
10-0615	Strawberry	Chiang Rai
10-0616	Jujube	Chiang Rai
10-0617	*Mangifera* sp. small	Chiang Rai
10-0618	Mango	Chiang Rai
10-0619	Rose apple	Chiang Rai
10-0620	Jujube	Chiang Rai
10-0621	Citrus	Chiang Rai
10-0622	Strawberry	Chiang Rai
10-0623	Jujube	Chiang Rai
10-0624	Green jambu	Chiang Rai
10-0625	Rose apple	Chiang Rai
10-0626	Caryota urens	Chiang Rai

Table 4.2 Some strains of *Colletotrichum* species collected in Thailand (Continued)

MFLUCC	Host	Collection Site
10-0627	Dragon fruit	Chiang Rai
10-0628	Banana	Chiang Rai
10-0629	Citrus	Chiang Rai
10-0630	Rose apple	Chiang Rai
10-0631	Banana	Chiang Rai
10-0632	Banana	Chiang Rai
10-0633	Pine apple	Chiang Rai
10-0634	Strawberry	Chiang Rai
10-0635	Cassowary plum	Chiang Rai
10-0636	Cassowary plum	Chiang Rai
10-0637	Banana	Chiang Rai
10-0638	Banana	Chiang Rai
10-0639	Banana	Chiang Rai
10-0640	Banana	Chiang Rai
10-0641	Strawberry	Chiang Rai
10-0642	Strawberry	Chiang Rai
10-0643	Banana	Chiang Rai
10-0644	Unknown	Chiang Rai
10-0645	Strawberry	Chiang Rai
10-0646	Banana	Chiang Rai
10-0647	Banana	Chiang Rai
10-0648	Banana	Chiang Rai
10-0649	Banana	Chiang Rai
10-0650	Neem	Chiang Rai
10-0651	Neem	Chiang Rai
10-0652	Rose apple	Chiang Rai
10-0653	*Citrus* sp.	Chiang Rai
10-0654	*Citrus* sp.	Chiang Rai

Table 4.2 Some strains of *Colletotrichum* species collected in Thailand (Continued)

MFLUCC	Host	Collection Site
10-0655	Papaya	Chiang Rai
10-0656	Dragon fruit	Chiang Rai
10-0657	Papaya	Chiang Rai
10-0658	Dragon fruit	Chiang Rai
10-0659	*Amaranthus* sp.	Chiang Rai
10-0660	*Annona* sp.	Chiang Mai
10-0661	*Annona* sp.	Chiang Mai
10-0662	Wild Pepper	Mae Kathong
10-0663	Wild Pepper	Mae Kathong
10-0664	*Hibiscus* sp.	Chiang Rai
10-0665	*Schlegera* sp.	Chiang Rai
10-0666	Wild Legume	Chiang Rai
10-0667	*Mirabilis* sp.	Chiang Rai
10-0668	Banana	Chiang Rai
10-0669	Aeschynanthus radicans	Southern Thailand
10-0670	Long kong Banan	Southern Thailand
10-0671	Boa mango	Southern Thailand
10-0672	Mango	Southern Thailand
10-0673	Mango	Southern Thailand
10-0674	Banana	Southern Thailand
10-0675	Nongyang Banana	Southern Thailand
10-0676	*Ficus* sp.	Chiang Mai
10-0677	Cassawarry plum	Chiang Rai
10-0678	Coffee berries	Chiang Mai
10-0679	Coffee berries	Chiang Mai
10-0680	Coffee berries	Chiang Mai
10-0681	Coffee berries	Chiang Mai
10-0682	Coffee berries	Chiang Mai

Table 4.2 Some strains of *Colletotrichum* species collected in Thailand (Continued)

MFLUCC	Host	Collection Site
10-0684	*Ficus* sp.	Chiang Rai
11-0456	-	France
11-0460	Oak, Quercus	France
11-0534	*M. indica*	Chiang Rai
11-0535	Monocot plant	Chiang Rai
11-0536		Chiang Rai
11-0538		Chiang Rai
11-0539		
11-0541		
11-0542	*M. indica*	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	Lemmon grass	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	*Pennisetum* sp.	Chiang Rai
-	Lemmon grass	Chiang Rai

Table 4.2 Some strains of *Colletotrichum* species collected in Thailand (Continued)

MFLUCC	Host	Collection Site
	Pennisetum sp.	Chiang Rai
	Pennisetum sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai
-	*Panicum* sp.	Chiang Rai

4.3 Molecular analysis

For years 1 and 2, we did PCR and sequenced the ITS, Actin, CAL, GPDH, GS and TUB gene regions of most strains (Table 4.3) and are in the process of analyzing this data. All sequence data has been deposited in NCBI and is listed reported in subsequent publications (see publications in attachment)

Table 4.3 Some of the PCR results for *Colletotrichum* strains collected.

MFLUCC	Region							
	ITS	SSU	LSU	Actin	CAL	GAPDH	GS	TUB
10-0610	/	x	x	x	x	x	x	x
10-0611	/	x	x	x	x	x	x	x
10-0612	/	x	x	x	x	x	x	x
10-0613	/	x	x	x	x	x	x	x
10-0614	/	x	x	x	x	x	x	x
10-0615	/	x	x	x	x	x	x	x
10-0616	/	x	x	/	/	/	/	/
10-0617	/	x	x	x	x	x	x	x
10-0618	/	x	x	x	x	x	x	x
10-0619	/	x	x	x	x	x	x	x
10-0620	/	x	x	x	x	x	x	x
10-0621	/	x	x	/	/	/	/	/
10-0622	/	x	x	x	x	x	x	x
10-0623	/	x	x	x	x	x	x	x
10-0624	/	x	x	/	/	/	/	/
10-0625	/	x	x	/	/	/	/	/
10-0626	/	x	x	x	x	x	x	x
10-0627	/	x	x	x	x	x	x	x
10-0628	/	x	x	x	x	x	x	x
10-0629	/	x	x	x	x	x	x	x
10-0630	/	x	x	/	/	/	/	/
10-0631	/	x	x	x	x	x	x	x

Table 4.3 Some of the PCR results for *Colletotrichum* strains collected (Continued)

MFLUCC	Region							
	ITS	SSU	LSU	Actin	CAL	GAPDH	GS	TUB
10-0632	/	x	x	x	x	x	x	x
10-0633	/	x	x	x	x	x	x	x
10-0634	/	x	x	x	x	x	x	x
10-0635	/	x	x	/	/	/	/	/
10-0636	/	x	x	/	/	/	/	/
10-0637	/	x	x	x	x	x	x	x
10-0638	/	x	x	x	x	x	x	x
10-0639	/	x	x	x	x	x	x	x
10-0640	/	x	x	/	/	/	/	/
10-0641	/	x	x	x	x	x	x	x
10-0642	/	x	x	x	x	x	x	x
10-0643	/	x	x	x	x	x	x	x
10-0644	/	x	x	x	x	x	x	x
10-0645	/	x	x	x	x	x	x	x
10-0646	/	x	x	x	x	x	x	x
10-0647	/	x	x	x	x	x	x	x
10-0648	/	x	x	x	x	x	x	x
10-0649	/	x	x	x	x	x	x	x
10-0650	/	x	x	/	/	/	/	/
10-0651	/	x	x	/	/	/	/	/
10-0652	/	x	x	/	/	/	/	/
10-0653	/	x	x	/	/	/	/	/
10-0654	/	x	x	/	/	/	/	/
10-0655	/	x	x	/	/	/	/	/
10-0656	/	x	x	/	/	/	/	/
10-0657	/	x	x	/	/	/	/	/
10-0658	/	x	x	x	x	x	x	x

Table 4.3 Some of the PCR results for *Colletotrichum* strains collected (Continued)

MFLUCC	Region							
	ITS	SSU	LSU	Actin	CAL	GAPDH	GS	TUB
10-0659	/	x	x	x	x	x	x	x
10-0660	/	x	x	/	/	/	/	/
10-0661	/	x	x	x	x	x	x	x
10-0662	/	x	x	x	x	x	x	x
10-0663	/	x	x	x	x	x	x	x
10-0664	/	x	x	x	x	x	x	x
10-0665	/	x	x	x	x	x	x	x
10-0666	/	x	x	x	x	x	x	x
10-0667	/	x	x	x	x	x	x	x
10-0668	/	x	x	/	/	/	/	/
10-0669	/	x	x	/	/	/	/	/
10-0670	/	x	x	x	x	x	x	x
10-0671	/	x	x	x	x	x	x	x
10-0672	/	x	x	x	x	x	x	x
10-0673	/	x	x	/	/	/	/	/
10-0674	/	x	x	/	/	/	/	/
10-0675	/	x	x	x	x	x	x	x
10-0676	/	x	x	/	/	/	/	/
10-0677	/	x	x	/	/	/	/	/
10-0678	/	x	x	/	/	/	/	/
10-0679	/	x	x	x	x	x	x	x
10-0680	/	x	x	x	x	x	x	x
10-0681	/	x	x	/	/	/	/	/
10-0682	/	x	x	/	/	/	/	/
10-0684	/	x	x	x	x	x	x	x
11-0456	/	x	x	x	x	x	x	x
11-0460	/	x	x	x	x	x	x	x

Table 4.3 Some of the PCR results for *Colletotrichum* strains collected (Continued)

MFLUCC	Region							
	ITS	**SSU**	**LSU**	**Actin**	**CAL**	**GAPDH**	**GS**	**TUB**
11-0534	/	x	x	x	x	x	x	x
11-0535	/	x	x	x	x	x	x	x
11-0536	/	x	x	x	x	x	x	x
11-0538	/	x	x	x	x	x	x	x
11-0539	/	x	x	x	x	x	x	x
11-0541	/	x	x	x	x	x	x	x
11-0542	/	x	x	x	x	x	x	x

* /: yes and x: No.

CHAPTER 5
CONCLUSION

Colletotrichum species have a worldwide distribution and are important pathogens subjected to import control and plant breeding programs. Previous identification and classification was based on host-association and morphological characteristics. Molecular sequence data, initially ITS and morphology was used to characterize species, however, they could not resolve species well. Recent multigene phylogenetic analysis as well as morphology and pathogenicity testing have resolved about 100 *Colletotrichum* species. There are four important species complexes. *C. gloeosporioides* species complex is the most important and multigene phylogenetic analysis have confirmed that *C. gloeosporioides* comprises 22 morphologically similar, phylogenetically distinct species. However, ITS, beta tubulin (*tub2*), DNA lyase and the intergenic region of *apn2* and *MAT1-2-1* genes (*ApMat*)) have also used shown new lineages within this species-complex and presently, there are 28 accepted species names within the complex. No consensus among mycologists as to which gene markers should be used to define and delimit a species within the species complex exists. We have collected more than 200 strains of *Colletotrichum* from various diseased plants and also grasses as asymptomatic endophytes. We have sequence and characterised any new species and are now in the process of preparing these results for publication.. Between October 2011 and September 2012 we published five international manuscripts of which four were SCI (Table 5.1). We are also now preparing six publications for 2013. Since this is a global problem we have chosen to collaborate with Brazilian, Chinese and Indian colleagues in order to bring greater depth to the research and international agreement to the findings. The latter was achieved with the formation of the International Subcommission on *Colletotrichum* taxonomy (http://www.fungaltaxonomy.org/ subcommissions) of which our group is a founder member.

Table 5.1 Publication list in 2012

Year	Publications
2012	Ko Ko TW, Moslem MA, Abdelsalam K, Chamyuang S, CheewangkoonR, Chukeatirote E, Jonglaekha N, Kobsueb R, McKenzie EHC,Promputtha I, Soytong K, To-anun C, Wikee S, Wulandari NF, HydeKD (2011) The need for re-inventory of Thai phytopathogens. ChiangMai Journal of Science 38(4): 625-637. Peng LJ, Yang YL, Hyde KD,Bahkali AH, Liu ZY (2012) Colletotrichum species on Citrus leavesin Guizhou and Yunnan provinces, China. Cryptogamie Mycologie33(3): 267-283. Noireung P, Phoulivong S, Liu F, Cai L, Mckenzie EHC, Chukeatirote E,Jones EBG, Bahkali AH, Hyde KD (2012) Novel species ofColletotrichum revealed by morphology and molecular analysis.Cryptogamie, Mycologie 33(3): 347-362. Phoulivong S, McKenzie EHC, Hyde KD (2012) Cross infection ofColletotrichum species; a case study with tropical fruits. CurrentResearch in Environmental & Applied Mycology 2(2): 99–111 DOI10.5943/cream/2/2/2. Yang Y, Liu Z, Cai L, Hyde KD (2012) New species and notes ofColletotrichum on daylilies (Hemerocallis spp.). Tropical PlantPathology 37 (3): 165-174.