รายงานวิจัยฉบับสมบูรณ์

การใช้เทคนิค **polyphasic approach** เพื่อปรับปรุงการจัดกลุ่มของเชื้อราสกุล ***Phyllosticta*** ซึ่งเป็นเชื้อก่อโรคที่สำคัญ

A polyphasic approach to the revision of the important pathogenic genus *Phyllosticta*

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บทสรุปผู้บริหาร (EXECUTIVE SUMMARY)

1. ความสำคัญและที่มาของปัญหาในการการวิจัย (Rationale and review)

The genus *Phyllosticta* and its *Guignardia* sexual morph causes economically significant diseases of important crops and horticultural plants such as banana, citrus, grape, orchids and palms. Species concepts in *Phyllosticta* and *Guignardia* are however ambiguous as there are more than 3000 names (more than 100 accepted species in *Phyllosticta*) and very few characters to differentiate species. It is therefore important that species concepts are clarified so that plant pathologists can readily identify species, thus they can implement disease control management strategies. This project is important for *Phyllosticta* taxonomy and will study pathogenic species but also include saprobes, and endophytes. We will investigate the morphological and cultural characters as well as the phylogenetic relationships of *Phyllosticta* species on various hosts (e.g., on banana, citrus, grapes, orchids, palms) and attempt to link the taxa to their *Guignardia* sexual morph. Relationships will be elucidated using morphological and cultural characters and phylogenetic interpretation of gene sequences. This project will therefore provide a clear understanding of the taxonomy of *Phyllosticta* species in Thailand, and worldwide. We will also establish which species cause disease and reduce yield and quality of plant products.

2. วัตถุประสงค์ของโครงการวิจัย (Objective of the research)

i. To clarify the species of *Phyllosticta* associated with disease in a range of hosts in northern Thailand.
ii. To understand the relationship between morphology and phylogeny characters of *Phyllosticta* species and their relationships with hosts.
iii. To elucidate species concepts within the genus by linking molecular and morphological approaches.

3. ขอบเขตของโครงการวิจัย (Scope of the research)

The genus *Phyllosticta* contains more than 3000 names and presently there are more than 50 estimated species. *Phyllosticta* species are worldwide in distribution and cause major damage to cereals, vegetables, legumes, ornamental plants and fruit trees. The current naming of *Phyllosticta* species is largely based on a combination of morphological and cultural characteristics. These are however, 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 systematists, 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 *Phyllosticta*, focusing on Thai species. 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. This is a hot topic and will result in several highly cited papers and bring Mae Fah University and Thailand as one of the world leaders in Plant Pathology research.

4. ระเบียบวิธีวิจัยและผลผลิตจากการวิจัย (Methodology and the research output)

Research Plan from October 2010 to September 2013

***Year 3*:** (start 1 October 2011 to 30 September 2013) Continue to collect diseased plants in Thailand and isolate taxa, and maintain cultures. Continue to carry out detailed study on the morphology and cultural characters of species. Investigate infraspecific variation within *Phyllosticta* using nuclear DNA and mtDNA. Publish first papers.

Probably the most significant finding of this study is the new species described from *Citrus maxima* (Pomelo) which causes tan spot on fruits. This finding will mean that Pomelo will no longer be subject to quarantine control in the European Union since Pomelo does not grow in these countries. *Phyllosticta* species are common on many hosts in Thailand and some species are host-specific. They are also endophytic in plants. In years 1 and 2 more than 200 collections of *Phyllosticta* were made in northern Thailand. There will be about 10 new species resulting from this study.

Year 3: The recent studies based on molecular data have resolved some cryptic species and some novel taxa have been discovered. We sequenced the ITS, LSU, ACT, TEF and GPDH gene regions of most of our isolates However, compared to the wide species diversity and taxonomic records, there is a lack of molecular studies to resolve current names in the genus. The study has resulted in eight publications to date and another three publications are in preparation. We have also shown that *Phyllosticta* species have anti-microbial activity.

5. ประโยชน์ที่ได้รับ (Benefit)

We will develop a practical phylogeny-based approach for the identification of species of *Phyllosticta*, focusing on Thai species. Molecular and genetic identification will be used to define species. Analysis of ITS gene region and other gene regions will be used as genetic evidence to define species and resolve problems in identification and taxonomy. The successful outcome of this project have important practical implications to the plant pathology, plant breeding and quarantine communities and we will publish several important SCI papers resulting from this work which will be frequently cited. We have also shown antimicrobial activity in *Phyllosticta* spp. against gram positive and gram negative bacteria that can be applied for medicine and industry in the future.

บทคัดย่อ

เชื้อราสายพันธุ์ *Phyllosticta* ส่วนใหญ่เป็นเชื้อราสาเหตุโรคพืชซึ่งแพร่กระจายตัวไปทั่วโลก สามารถก่อให้เกิดโรคต่าง ๆมากมายรวมทั้งโรคใบจุดและใบจุดดำบนผลไม้ หลายสายพันธุ์ถูกรายงานว่าเป็นเชื้อราแซบโพรบและบางส่วน โดยเฉพาะอย่างยิ่ง *Phyllosticta capitalensis* เป็นเอนโดไฟท์ซึ่งแพร่กระจายไปทั่ว การศึกษาถึงลักษณะของเชื้อราชนิดนี้มีมาอย่างยาวนาน ทั้งทางด้านสัณฐานวิทยา ลักษณะของเชื้อบนอาหารเลี้ยงเชื้อ รวมถึงการอยู่ร่วมกับพืชอาศัย ถึง แม้ว่ามีการศึกษาทางด้านอนุกรมวิธานอย่างต่อเนื่องและมีการปรับปรุงแก้ไขการจัดหมวดหมู่และศึกษาจำนวนเชื้อราชนิดนี้หลายครั้ง แต่ก็ยังคงมีความสับสนมากในการกำหนดชื่อ *Phyllosticta* การวิเคราะห์ทางด้านชีวโมเลกุลจึงเข้ามามีบทบาทในการจัดจำแนกเชื้อราเช่น *Phyllosticta* โดยเบื้องต้น การใช้ส่วนของยีนITS สามารถใช้ในการจัดจำ แนกได้ แต่ยังคงจำ แนกในระดับสายพันธ์ได้ไม่ดีเท่าที่ควรในปัจจุบัน การเข้ามามีบทบาทของ multigene phylogenetic analysis โดยการวิเคราะห์เชื้อราในสกุลนี้จะใช้ 2 ยีน (ITS และ ACT) และ 5 ยีน (ITS, LSU, ACT, TEF และ GPDH) ในการแปลผลออกมาให้อยู่ในรูปของแผนผังวงศ์วานวิวัฒนาการควบคู่ไปกับการศึกษาด้านสัณฐานวิทยาและการศึกษาด้านความสามารถในการก่อโรค โดยในการศึกษาครั้งนี้ได้ทำการอธิบายรูปร่างลักษณะของเชื้อราสกุลนี้ประมาณ 10 ตัวอย่าง

ตั้งแต่เริ่มต้นการศึกษา (ตุลาคม 2010) มีเชื้อมากกว่า 10 ตัวอย่าง ได้ถูกยืนยันแล้วว่าเป็นเชื้อก่อโรค ซึ่ง เป็นสายพันธุ์ที่พบในประเทศไทย ในปีแรกของงานวิจัย ได้มีการเก็บเชื้อได้มากว่า 200 ตัวอย่างจากพืชและผลไม้ที่เป็นโรคในหลายพื้นที่ในประเทศไทยและมากกว่า 72 ตัวอย่างที่ถูกจำแนกให้อยู่ในสถานะ Endophyte ในปีที่สองได้ศึกษาถึง *Phyllosticta capitalensis* ซึ่งเป็นราที่อยู่ใน สถานะ Endophyte และ เชื้อก่อโรคที่ไม่รุนแรง โดยมีการกระจายตัวของโรคไปอย่างกว้างขวาง โดยพบเชื้อสายพันธ์นี้ในกว่า 70 สายพันธุ์ของพืชอาศัย รวมถึง ทางคณะวิจัยได้ทำการศึกษาลำดับพันธุกรรมของยีน 5 ยีน ของเชื้อ 28 ตัวอย่างรวมทั้งการศึกษาทางด้านความสามารถในการก่อโรค ทั้งนี้ในปีที่สามทางผู้วัจัยได้ทำการหาลำดับพันธุกรรมของเชื้อ 160 ตัวอย่าง จากคลังเก็บเชื้อหลายแห่งและยังคงทำการวิเคราะห์ดังกล่าวอย่างต่อเนื่อง จากข้อมูลเบื้องต้น ทางคณะวิจัยได้เชื้อพบเชื้อราสายพันธุ์ใหม่ 9 สายพันธุ์ เนื่องจากปัญหาด้านการจำแนกและระบุเชื้อเป็นปัญหาระดับโลก ทั้งนี้ทางผู้วิจัยมีความร่วมมือกับต่างประเทศทั้งคณะวิจัยจากประเทศจีน และ ความร่วมมือกับนักวิจัยจากประเทศแถบยุโรป เพื่อจัดการแก้ไขปัญหาดังกล่าวในเชิงลึกต่อไป ในการศึกษาวิจัย ตลอดโครงการทางคณะผู้จัดทำ ได้ตีพิมพ์ผลงานจำนวน 8 เรื่อง โดย 5 เรื่องถูกตีพิมพ์เผยแพร่ลงในวารสารทางวิทยาศาสตร์ที่ได้รับการยอมรับ (SCI) โดยมีตัว

วัดความถี่ของบทความในวารสารโดยเฉลี่ยที่ถูกนำไปอ้างอิง(impact facter) อยู่ที่ 5.03 และได้ถูกนำไปอ้างอิงถึง 19 ครั้งนับตั้งแต่บทความนั้นได้ถูกเผยแพร่ลงในวารสาร

คำสำคัญ: เอนโดไฟท์/ *Guignardia*/ ลักษณะทางสัณฐานวิทยา/ วงศ์วานวิวัฒนาการระดับโมเลกุล/ เชื้อราโรคพืช

ABSTRACT

Phyllosticta species are predominantly plant pathogens with a worldwide distribution. They are responsible for numerous diseases including leaf spots and black spots on fruits. Several species have been reported as saprobes and some, in particular *Phyllosticta capitalensis* as endophytes with a worldwide distribution. Species recognition in *Phyllosticta* has historically been based on morphology, culture characters and host-association. Although there have been several taxonomic revisions and enumerations of species, there is still considerable confusion in the determination of *Phyllosticta* species. Molecular sequence data analysis has become commonplace in classifying plant pathogenic genera like *Phyllosticta*. Initially ITS and morphology was used to characterize species, however, they could not resolve species well. Recent multigene phylogenetic analysis in the genus have involved multi-loci combined genes with two (ITS and ACT) and five genes (ITS, LSU, ACT, TEF and GPDH) trees, as well as morphology and pathogenicity testing, so at present there are about 10 described species in the genus

At the beginning of this study (October 2010) there more than ten confirmed “molecular” species in the genus causing plant diseases worldwide and only two were known from Thailand. We therefore initiated a survey of *Phyllosticta* species infecting plants in Thailand. In the first and second years of this study we collected more than 200 fresh specimens of various disease plants and fruits from different places in Thailand. From these we successfully isolated 72 strains from fresh diseased samples or as endophytes from asymptomatic samples. The major finding of year 2 is that *Phyllosticta capitalensis* is an endophyte and weak plant pathogen with a worldwide distribution presently known from 70 plant families. We also sequenced the five genes of 28 isolates and carried out morphological as well as pathogenicity studies. Year 3 we sequenced 160 strains of other species from various culture collections and are in the process of analysing this data. Initial results indicate we have nine potential new species. We have developed collaboration with China and Netherlands and are involved in developing a practical phylogeny and morphology based approach for the identification of *Phyllosticta* species. Our collaboration with Chinese and European colleagues will bring greater depth to the research and international agreement to the findings. This project we published eight papers of which five were SCI. One of these was a review paper on *Phyllosticta* and was published in an SCI journal of 5.03 and has been cited 19 times since being published.

Keywords: endophytes / *Guignardia* /morphology / molecular phylogeny / plant pathogenic fungi

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ABBREVIATION AND SYMBOLS

ACT = Actin

% = Percent

cm = centimeter

DNA = Deoxyribonucleic acid

GPDH = Glyceraldehyde-3- phosphate dehydrogenase

ITS = Internal transcribed spacer

LSU = Large subunit (28S rDNA)

μm = micrometer

No. = Number

PDA = Potato Dextrose Agar

sp. = species

TEF1α = Translation elongation factor 1-alpha

USA = United States of America

USDA = United States Department Of Agriculture

CHAPTER 1

INTRODUCTION

The genus *Phyllosticta* contains more than 3,000 names and presently there are more than 50 estimated species. *Phyllosticta* species are worldwide in distribution and cause major damage to cereals, vegetables, legumes, ornamental plants and fruit trees. The current naming of *Phyllosticta* species is largely based on a combination of morphological and cultural characteristics. These are however, 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 *Phyllosticta*, focusing on Thai species. Variations in the mitochondrial genome, ribosomal DNA, 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. This is a hot topic and will result in several highly cited papers and bring Mae Fah University and Thailand as one of the world leaders in Plant Pathology research.

CHAPTER 2

REVIEW OF RELATED LITERATURE

The genus *Phyllosticta* and its *Guignardia* sexual morph cause economically significant diseases of banana, citrus, coffee, grape, orchids, palms and mango (Van der Aa and Vaney, 2002; Wulanderi *et al*., 2009). *Phyllosticta* species cause losses by damaging the fruits; or affecting leaves, thereby reducing yield and quality of plant products (Van der Aa and Vaney, 2002).

The diseases caused by *Phyllosticta* species are usually leaf spots which reduce the yield of the crop or make the leafy vegetables valueless. *Phyllosticta* species may cause black or tan spots on fruits such as orange or pomello; this makes the product both valueless, but also has important quarantine implications. For instance, in yam, *Phyllosticta dioscorae* appears as a leaf spot that spreads and develops rapidly and kills leaves, and sometimes entire yam plants. Citrus Black spot caused by *Phyllosticta citrocarpa* is a quarantine pest in Europe and the USA (Wulanderi *et al*., 2009).

Many species of *Phyllosticta* are relatively unspecialized in their host range and disease symptoms (Van der Aa and Vaney, 2002), while other are thought to be specific in their host range. However, knowledge of host occurrence of most species is relatively poor and should be researched. The taxonomy of *Phyllosticta* species is complicated by the fact that there are few morphological characters to differentiate species and by the practices of some earlier mycologists, who defined new species based on fungus/host relationships with little or no consideration of morphology of previously described species (Van der Aa and Vaney, 2002).

Of the diseases caused by *Phyllosticta*, those on Citrus have been relatively well researched (Wulanderi *et al*., 2009), however few other species have been well researched and our knowledge of the genus *Phyllosticta* in Thailand is poor. A few species of *Phyllosticta* causing leaf spot diseases have been reported in Thailand. such as *Phyllosticta* sp. on pear (Visarathanonth, http://www.actahort.org/members/showpdf?book nrarnr= 279_67) and a *Phyllosticta* sp causing spots on Soybean leaves

(Nachaiwiang *et al*., 2001). *Phyllosticta* endophytes have been isolated from banana and *Amomum* leaves (Photita et al. 2001; Bussaban et al. 2001) and a *Phyllosticta* sp. is known to cause post harvest disease of Durian (Poeltz, 2003). However a search on the topic reveals that very little is known concerning *Phyllosticta* species in Thailand and most taxa are named as *Phyllosticta* sp. There is obviously much work required to establish the diversity and importance of the genus in Thailand.

The clarification of species concepts in *Phyllosticta* is a matter of considerable practical importance for identifying taxa as well as establishing host range and geographic distribution data (Bailey *et al*., 1992). This is essential for the work of quarantine and trade, and plant pathologists who need to diagnose and control diseases using appropriate disease management strategies. It is important that we develop new methods to identify *Phyllosticta* species easily using morphology or cultural data, but which can be confirmed by molecular data. This project thus will clarify the understanding of the taxonomy of *Phyllosticta* species, particularly for taxa which cause disease of a range of hosts using morphological characters and sequence data. It will also look for new methods to identify taxa.

Molecular approaches are being used to resolve problems in fungal taxonomy and fungal identification by many workers (Lee and Taylor 1990; Rollo et al. 1995; Ranghoo and Hyde 1998; Guo et al. 2000; Liew et al. 2002;). Because of the shortcomings of *Phyllosticta* systematics based on cultural characteristics and morphology there is need for a combined approach including the use of molecular data. The current classification of *Phyllosticta* species is broad and has a limited practical significance. It is well accepted that the systematic of the genus *Phyllosticta* awaits a detailed investigation and refinement.

CHAPTER 3

RESEARCH METHODOLOGY

Fungal isolates

(1) Collection of the samples

Phyllosticta isolates were collected from leaf spots and diseased fruits of various hosts, such as agaves, banana, coffee, palms, mango and Yams from the Provinces of Chiang Mai and Chiang Rai in northern Thailand.

(2) Morphological examination

Morphological characters of selected isolates collected, such as characters of culture colony, conidia, appressoria, setae and sclerotia were examined from pure culture.

(3) Phylogenetic study

Strains were grown on Malt Extract Agar at room temperature for 2-3 days, after which the mycelium was harvested. DNA were isolated using Ultraclean ^{TM} Microbial DNA kit (Mo Bio, Calsbad, CA, USA) according to manufacturer's protocol. Transcribed spacer-polymerase chain reaction (ITS-PCR) was performed with primers V9G (5′-TTAAGTCCCTGCCCTTTGTA-3′; De Hoog & Gerrits van den Ende 1998) and ITS4 (5′ TCCTCCGCTTATTGATATGC-3′) as described by white et al 1990, the primer LROR (5′-GTACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTACCACCAAGATCT-3′) were used for amplify part of 28S large subunit snRNA (LSU) were described by Vilgalys and Hester, 1990. Part of elongation factor 1-α gene (TEF-1) were amplified with forward primers EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and reverse primer EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) (O'Donnell et al 1998). The primers ACT-512 F and ACT – 783R were used for amplify the part of actin gene (ACT) (Carbone and Kohn 1999). The partial glyceraldehyde-3- phosphate dehydrogenase (GPDH) were amplified by primers Gpd1-LM (5′-

ATTGGCCGCATCGTCTTCCGCAA-3′) and Gpd2-LM (5′-CCCACTCGTTGTCGTACCA-3′) for forward and reverse primers (Myllys *et al.* 2002). For *P. citricarpa* isolated were amplified by specific primer Gpd1 (Guerber et al. 2003) and GPDHR2 (5′- CTCRGMRGCRGCCTTGATGG-3′) was developed by Glienke et al. (2011). Cycle sequencing of PCR products was performed in PCR condition. PCR products were separated by gel electopholesis at 130 volt for 20 min in 1% agarose gel in 1x TAE running buffer and visualized under UV light by using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK). Purified PCR sequenced using both PCR primers with a BigDay Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, Foster City, CA, USA) containing AmpliTag DNA Polymerase. The amplify product were analyzed on an automatic DNA sequence (Perkin-Elmer, Norwalk, CN). Sequences generated were automatically aligned using MAFFT v.6 (http://mafft.cbrc.jp/alignment/server/). And the sequences were corrected manually aligned using MEGA v5.05 software (Tamura et al. 2011). Phylogenetic analyzing were executed by Phylogenetic analyses Using Parsimony; PAUP version 4.0b10 (Swafford 2003). For parsimony analysis, alignment gaps were treated as a fifth character state and all character were unordered and equal weight. *Botryosphaeria obtusa* was represented as outgroup for the phylogenetic tree. The confidence limit of the resulting tree was estimated by bootstrap analysis with 1000 replication (Hillis and Bull 1993). Tree length (TL), consistency (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting tree were done in Adobe Illustrator CS3. Novel and representative sequences were deposited in GenBank

CHAPTER 4

RESULTS

Phyllosticta spp. was collected throughout Northern of Thailand from agricultural fields, waterfalls, national parks and house gardens (Tables 4-1). To date we have collected more than 100 specimens belonging to at least 25 species. *Phyllosticta* species cause spots on living leaves and are also saprobes on dead leaves, but the pathogenic species are generally different from those on fallen leaves. Normally, pycnidia develop as black spots and black hyphae on leaf lesions. The *Guignardia* teleomorph and *Phyllosticta* asexual state are often found in the same leaf lesion. Fresh material of plant infected by *Phyllosticta* or *Guignardia* was isolated by endophyte technique, hyphal tip and single spore isolation. Conidia are typically small to medium sized, 5–10 μm in diam, hyaline, one–celled, have a thin and flexible sheath, are smooth-walled with an apical appendage. Often in dried specimens the appendage could not be observed. Characteristics and morphology have been examined in pure culture, where colonies form irregularly folded crusts and have dark mycelium. Some species produce white tendril of mycelium on the upper surface of the colony, which after 2 weeks on PDA is 2-3 cm in diam.

Table 4-1 ***Phyllosticta*** **and "Guignardia" strains in Mae Fah Luang Culture Collection (MFLUCC)**

No	MFLUCC code	Original code	Species
1	**10-0306**	NCC 001	*Guignardia* sp.
2	**10-0307**	NCC 002	*Guignardia* sp.
3	**10-0308**	NCC 003	*Guignardia* sp.
4	**10-0309**	NCC 004	*Guignardia* sp.
5	**10-0310**	NCC 005	*Guignardia* sp.
6	**10-0311**	NCC 006	*Guignardia* sp.
7	**10-0312**	NCC 007	*Guignardia* sp.
8	**10-0313**	NCC 008	*Guignardia* sp.
9	**10-0314**	NCC 009	*Guignardia* sp.
10	**10-0315**	NCC 010	*Guignardia* sp.
11	**10-0316**	NCC 011	*Guignardia* sp.
12	**10-0317**	NCC 012	*Guignardia* sp.
13	**10-0318**	NCC 013	*Guignardia* sp.
14	**10-0319**	NCC 014	*Guignardia* sp.
15	**10-0320**	NCC 015	*Guignardia* sp.
16	**10-0321**	NCC 016	*Guignardia* sp.
17	**10-0322**	NCC 017	*Guignardia* sp.
18	**10-0323**	NCC 018	*Guignardia* sp.
19	**10-0324**	NCC 019	*Guignardia* sp.
20	**10-0325**	NCC 020	*Guignardia* sp.
21	**10-0326**	NCC 021	*Guignardia* sp.
22	**10-0327**	NCC 022	*Guignardia* sp.
23	**10-0328**	NCC 023	*Guignardia* sp.
24	**10-0329**	NCC 024	*Guignardia* sp.
25	**10-0330**	NCC 025	*Guignardia* sp.

26	**10-0331**	NCC 026	*Guignardia* sp.
27	**10-0332**	NCC 027	*Guignardia* sp.
28	**10-0333**	NCC 028	*Guignardia* sp.
29	**10-0334**	NCC 029	*Guignardia* sp.
30	**10-0335**	NCC 030	*Guignardia* sp.
31	**10-0336**	NCC 031	*Guignardia* sp.
32	**10-0337**	NCC 032	*Guignardia* sp.
33	**10-0338**	NCC 033	*Guignardia* sp.
34	**10-0339**	NCC 034	*Guignardia* sp.
35	**10-0340**	NCC 035	*Guignardia* sp.
36	**10-0341**	NCC 036	*Guignardia* sp.
37	**10-0342**	NCC 037	*Guignardia* sp.
38	**11-0051**	WK002	*Phyllosticta* sp.
39	**11-0053**	WK004	*Phyllosticta* sp.
40	**11-0054**	WK005	*Phyllosticta* sp.
41	**11-0055**	WK006	*Phyllosticta* sp.
42	**11-0056**	WK007	*Phyllosticta* sp.
43	**11-0057**	WK010	*Phyllosticta* sp.
44	**11-0058**	WK011	*Phyllosticta* sp.
45	**11-0059**	WK012	*Phyllosticta sp.*
46	**11-0060**	WK013	*Guignardia* sp.
47	**11-0062**	WK016	*Phyllosticta* sp.
48	**11-0063**	WK017	*Phyllosticta* sp.
49	**11-0064**	WK018	*Phyllosticta* sp.
50	**11-0066**	WK020	*Phyllosticta* sp.
51	**11-0067**	WK021	*Phyllosticta* sp.
52	**11-0068**	WK022	*Phyllosticta* sp.
53	**11-0069**	WK023	*Guignardia* sp.
54	**10-0016**	WK024	*Phyllosticta* sp.
55	**10-0017**	WK026	*Phyllosticta* sp.

56	**10-0019**	WK031	*Phyllosticta* sp.
57	**10-0020**	WK032	*Phyllosticta* sp.
58	**10-0021**	WK033	*Phyllosticta* sp.
59	**10-0022**	WK034	*Phyllosticta* sp.
60	**10-0023**	WK035	*Phyllosticta* sp.
61	**10-0025**	WK036	*Phyllosticta* sp.
62	**10-0025**	WK037	*Phyllosticta* sp.
63	**10-0026**	WK038	*Phyllosticta* sp.
64	**10-0028**	WK039	*Phyllosticta* sp.
65	**10-0132**	WK040	*Phyllosticta* sp.
66	**10-0133**	WK041	*Phyllosticta* sp.
67	**10-0134**	WK042	*Phyllosticta* sp.
68	**10-0135**	WK043	*Phyllosticta* sp.
69	**10-0029**	WK044	*Phyllosticta* sp.
70	**10-0136**	WK045	*Phyllosticta* sp.
71	**10-0137**	WK046	*Phyllosticta* sp.
72	**10-0138**	WK047	*Phyllosticta* sp.
74	**12-0014**	WK048	*Phyllosticta* sp.
75	**12-0015**	WK049	*Phyllosticta* sp.
76	**12-0232**	WK050	*Phyllosticta* sp.

***Phyllosticta* collected during this study.**

1. ***Phyllosticta* sp.**

Teleomorph:

Host: *Chlorophylum bichetii* (Karrer) Backer

Symptom: Black spot

Habitat: Living leaf

Collecting Site: PAYAO, Mueang district, Jam Pha Tong Waterfall, 12 March 2012

Pycnidia 100–110 µm diameter, 90–105 µm high, black, globose to subglobose, immersed in host tissues, solitary to gregarious, uniloculate, with or without a central ostiole. *Pycnidia wall* composed of 2-3 layers of *textura angularis*, brown to dark brown. *Conidiogenous cells* holoblastic, phialidic, cylindrical, thin-walled, smooth. *Conidia* 10–13 × 7–9 µm (x = 12 × 8 µm, n = 20), hyaline, 1-celled, coarse-guttulate, smooth to verruculose walled, globose, ellipsoidal, with an round apex, sometimes truncate at the base, bearing single apical appendage, usually 8–13 µm long, straight to flexible, unbranched, thick mucilaginous sheath which lacking at maturity and in some conidia.

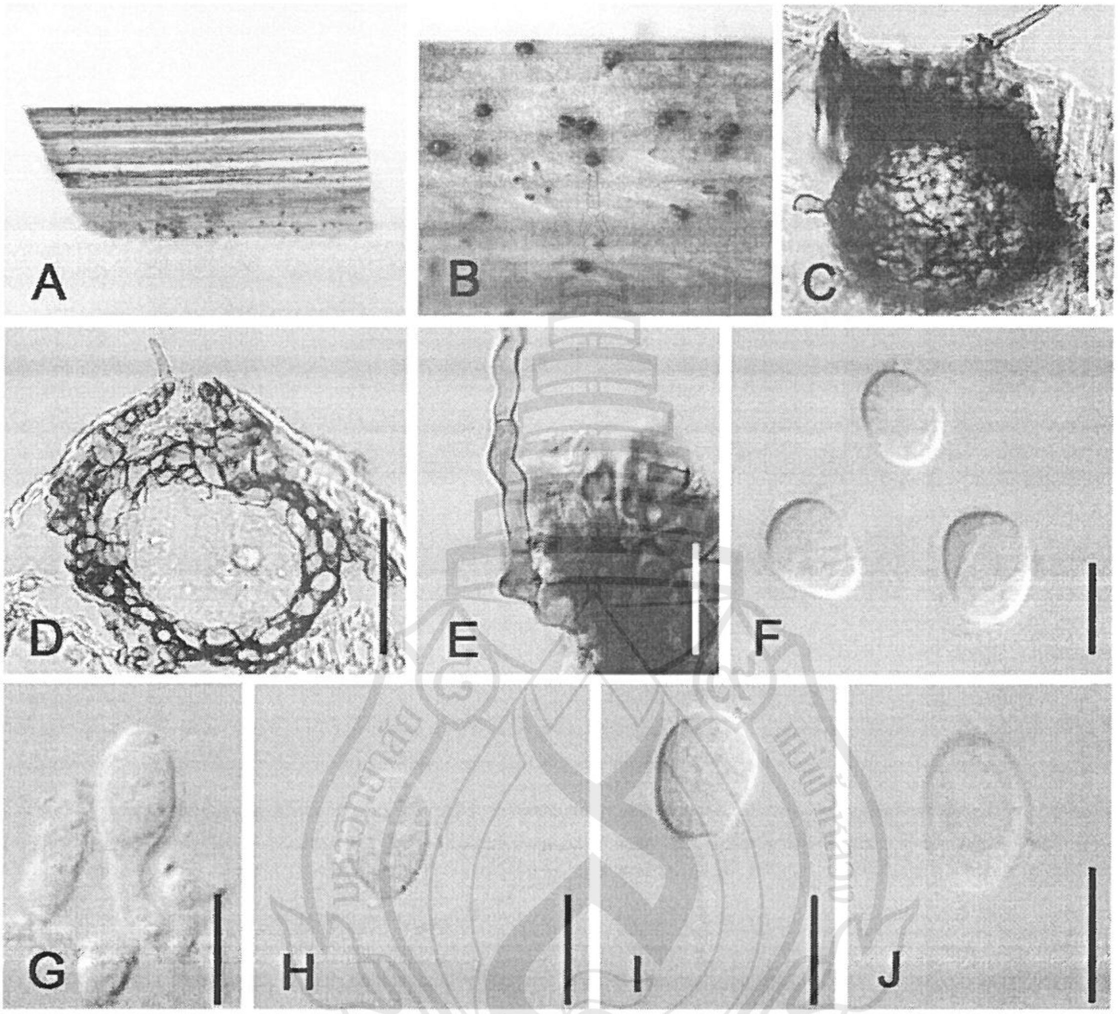

Fig 1. ***Phyllosticta* sp.** A-B. Symptom on *Chlorophylum bichetii* leaf. C. Exterior of pycnidia on host. D. Vertical section through pycnidia on host. E. Apendex. G. Conidiogenous cell. F, H-J. Conidia. Scale bars: C, D= 50 µm. E=20µm. F-J= 10 µm.

2. *Phyllosticta* sp.

Teleomorph:

Host: Unkonwn

Symptom: Black spot

Habitat: Living leaf

Collecting Site: PAYAO, Mueang district, 12 March 2012

Pycnidia (120-)150–170(-185) µm diameter, (105-)120–150 µm high, black, globose to subglobose, immersed in host tissues, solitary to gregarious, uniloculate. *Pycnidia wall* composed of 3-4 layers of *textura angularis*, dark brown to black. *Conidiogenous cells* holoblastic, phialidic, cylindrical, thin-walled, smooth. *Conidia* 8–11 × 4–6 µm (x = 10 × 5 µm, n = 20), hyaline, 1-celled, coarse-guttulate, verruculose walled, globose, ellipsoidal, with an round apex, sometimes truncate at the base, surrounded by mucilaginous sheath, bearing single apical appendage, usually 4–6 µm long, unbranched, straight to flexible.

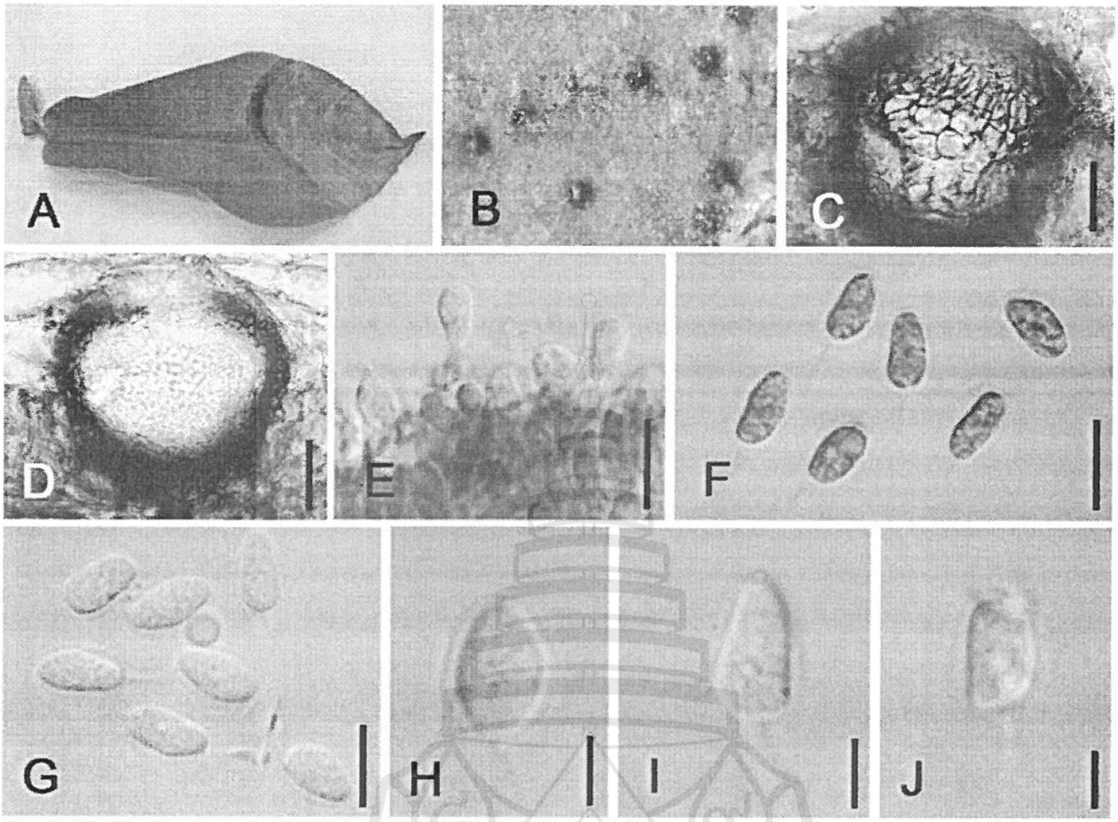

Fig 2 ***Phyllosticta* sp.** A. Symptom on leaf plant. B. Pycnidia on host. C. Exterior of pycnidial wall. D. Vertical section through pycnidia on host. E. Coniodigenous cell. F. Conidia with lacto phenol cotton-blue. G-J. conidia. Scale bars: C, D=50μm. E-G=10 μm. H-J=5 μm.

3. ***Phyllosticta*** **sp.**

Teleomorph:

Host:

Symptom: Black spot

Habitat: Living leaf

Collecting Site: CHIANG RAI, Mueang District, Mae Fah Luang University campus grounds, 29 July 2011

Pycnidia 80–110 µm long µm, 70–100 wide, circular, black, solitary to gregarious, slightly globose, immersed in host tissues. *Conidiogenous cells* lining wall of pycnidia, phialidic, cylindrical, hyaline. *Pycnidia wall* composed of 3-4 layers of *textura angularis*, dark brown to black. *Conidia* 7–9 × 5–6 µm (x = 8 × 5 µm, n = 10), ellipsoidal, hyaline, 1-celled, smooth to verruculose -walled, surrounded by mucilaginous sheath, bearing single apical appendage, usually 4–5 µm long, unbranched, straight to flexible.

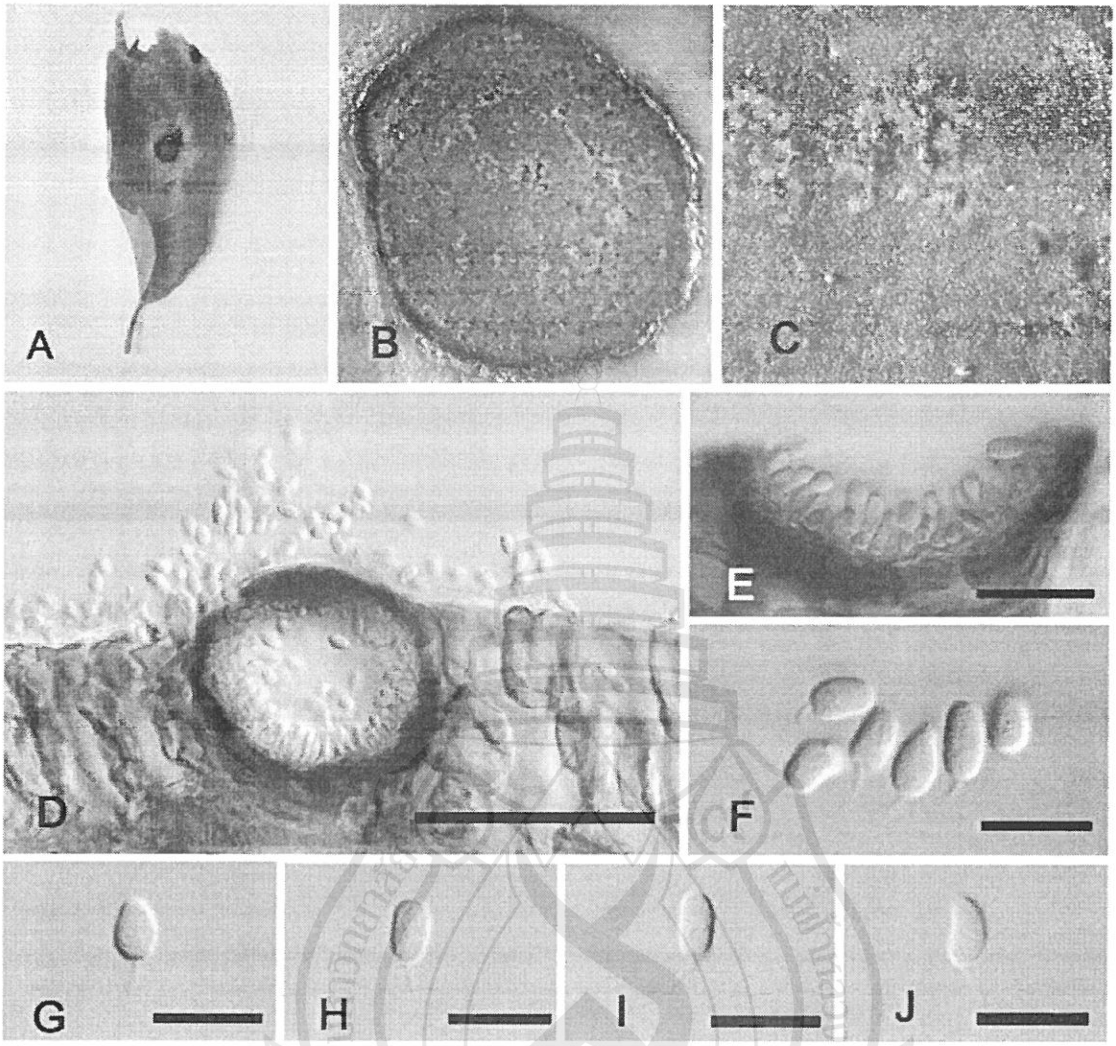

Fig 3. ***Phyllosticta*** **sp.** A-C. Symptom on leaves. C. Close-up of Conidiomata. D. section through pycnidia on host. E. Conidiogenous cells. F-J. Conidia. Scale bars: D= 100 µm. E= 20µm. F-J=10µm.

The species of *Phyllosticta* are associated with disease in a broad range of hosts. However, morphological character is inadequate for separating cryptic species in *Phyllosticta*. Therefore, a molecular approach was then introduced to further confirm the species identity. For this, the ITS, LSU, ACT, TEF and GPDH gene region sequences of these fungal strains were sequenced and used in a phylogenetic analysis including the known sequences of *Phyllosticta* strains.

The phylogenetic relationship was determined of the internal transcribed spacer (ITS) and actin gene (ACT) sequences of 160 *Phyllosticta* strains (including one outgroup). The combined partial dataset of *Phyllosticta* comprised 883 characters (including gaps), of which 341 characters are constant, and 150 characters are variable and parsimony-uninformative. Parsimony analysis generated 1,000 trees, one of the trees with similar with bootstrap values was chosen (number of bootstrap replicated = 1,000), as shown in Fig. 4.4 (TL = 2099, CI = 0.481, RI = 0.898, RC = 0.432, HI = 0.519). The phylogenetic tree of the ITS and ACT region resolved 46 clades (see Table 4-2 for details).

Fungi 129 strains including the outgroup was run based on the internal transcribed spacer (ITS), 28s rRNA gene region (LSU), actin gene (ACT), translation elongation factor 1-α gene (TEF1) and glyceraldehyde-3- phosphate dehydrogenase (GPDH) (Table 4-2). The combined partial dataset of *Phyllosticta* comprised 2,577 characters (including gaps), of which 1,547 characters are constant, 296 characters are variable and parsimony-uninformative. Parsimony analysis generated 1,000 trees, of which one tree with similar bootstrap values was chosen (number of bootstrap replicates = 1,000) and is shown in Fig. 4-4 (TL = 3173, CI = 0.517, RI = 0.906, RC = 0.468, HI = 0.483). The phylogenetic tree using combined multi-gene data resolved 33 clades (see Table 4-2 for details).

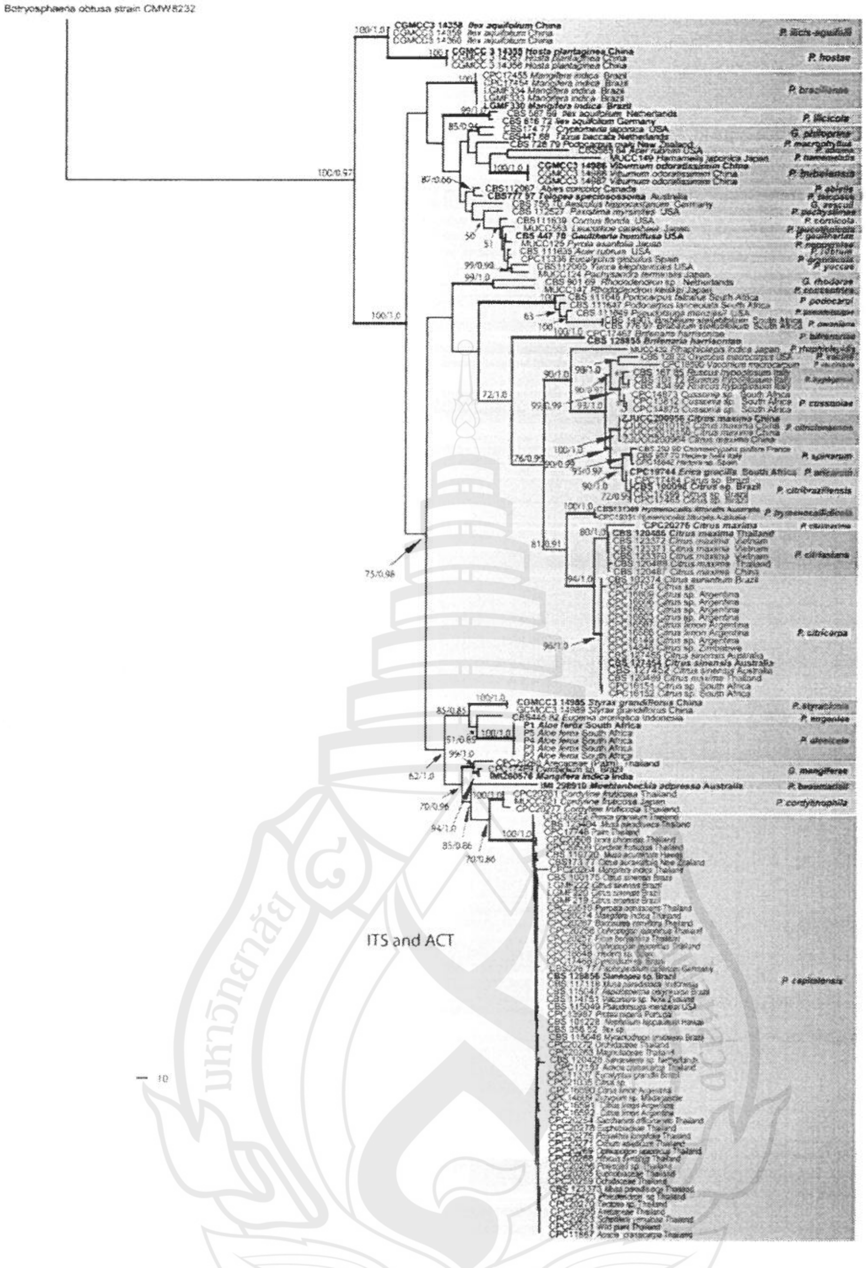

Fig. 4.1 Phylogenetic tree of *Phyllosticta* generated from a maximum parsimony analysis based on the ITS and ACT sequence alignment. Names in bold are represented types and ex-types. Values on the branches represent parsimony bootstrap support value (> 50%). Thickend branches represent significant Bayesian analysis value (≥ 90%) and the scale bar indicates 10 changes. *Botryosphaeria obtusa* represent out group.

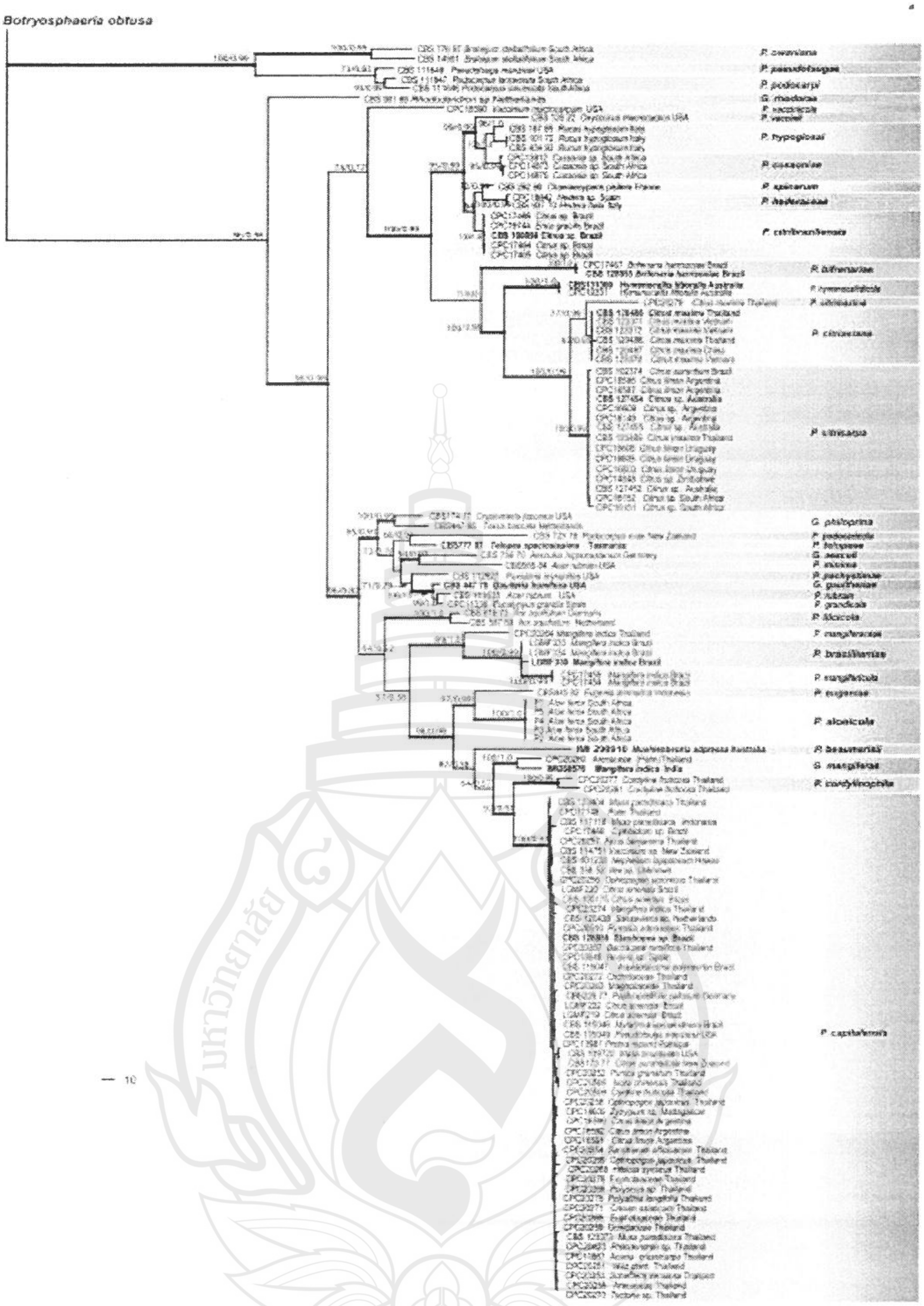

Fig. 4.2 Phylogenetic tree of *Phyllosticta* generated from a maximum parsimony analysis based on the ITS, LSU, ACT, TEF and GPDH sequence alignment. Names in bold are represented types and ex-types. Values on the branches represent parsimony bootstrap support value (>50%). Thickened branches represent significant Bayesian analysis value (≥ 90%) and the scale bar indicates 10 changes. *Botryosphaeria obtusa* represent out group.

Table 4-2 Table of isolates *Phyllosticta* and *Guignardia* in this study with GenBank number

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CBS 756.70	*Aesculus hippocastanum*	Germany	AY042934	KF206294	KF289202	KF289253	KF289133
CBS 447.70	*Gaultheria humifusa*	USA	JN692543	KF206298	JN692531	KF289248	JN692508
CPC 17469	*Cymbidium sp.*	Brazil	KF206189	-	-	KF289285	-
IMI 260576	*Mangifera indica*	India	JF261459	KF206222	JF261501	JF343641	JF343748
CPC 20260	*Arecaceae*	Thailand	KF206193	KF206243	KF289187	KF289294	KF289114
CBS 112067	*Abies concolor*	Canada	KF206208	EU754193	-	KF289238	-
CBS 535.87;IMI 298910	*Muehlenbekia appressa*	Australia	AY042927	KF306229	KF289170	KF306232	KF289074
CBS 123370	*Citrus maxima*	Vietnam	FJ538355	KF206310	FJ538413	FJ538471	JF343689
CBS 120487	*Citrus maxima*	China	FJ538361	KF206313	FJ538419	FJ538477	JF343687
CBS 128855; VIC30556	*Bifrenaria harrisoniae*	Brazil	JF343565	KF206209	JF343586	JF343649	JF343744
CPC 17467	*Bifrenaria harrisoniae*	Brazil	KF170299	KF206260	KF289207	KF289283	KF289138
CBS 126270; LGMF330	*Mangifera indica*	Brazil	JF343572	KF206217	JF343593	JF343656	JF343758
LGMF 333	*Mangifera indica*	Brazil	JF343574	KF206216	JF343595	JF343658	JF343760
LGMF 334	*Mangifera indica*	Brazil	JF343566	KF206215	JF343587	JF343650	JF343752
CPC 17454	*Mangifera indica*	Brazil	KF206206	KF206265	KF289192	KF289278	KF289123
CPC 17455	*Mangifera indica*	Brazil	KF206207	KF206264	KF289191	KF289279	KF289122
CPC20251	*Wild plant*	Thailand	KC291333	KF206252	KC342553	KC342530	KF289101
CPC20252	*Punica granatum*	Thailand	KC291334	KF206251	KC342554	KC342531	KF289097
CPC20254	*Saccharum officinarum*	Thailand	KC291335	KF206249	KC342555	KC342532	KF289103
CPC20255	*Arecaceae*	Thailand	KC291336	KF206248	KC342556	KC342533	KF289115

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CPC20256	*Ophiopogon japonicus*	Thailand	KC291337	KF206247	KC342557	KC342534	KF289089
CPC20257	*Ficus benjamina*	Thailand	KC291338	KF206246	KC342558	KC342535	KF289099
CPC20258	*Ophiopogon japonicus*	Thailand	KC291339	KF206245	KC342559	KC342536	KF289094
CPC20259	*Orchidaceae*	Thailand	KC291340	KF206244	KC342560	KC342537	KF289104
CPC20263	*Magnoliaceae*	Thailand	KC291341	KF206241	KC342561	KC342538	KF289085
CPC20266	*Polyscias sp.*	Thailand	KC291342	KF206238	KC342562	KC342539	KF289109
CPC20267	*Baccaurea ramiflora*	Thailand	KF206195	KF206237	KF289173	KF306233	KF289078
CPC20268	*Hibiscus syriacus*	Thailand	KC291343	KF206236	KC342563	KC342540	KF289117
CPC20269	*Ophiopogon japonicus*	Thailand	KC291344	KF206235	KC342564	KC342541	KF289118
CPC20270	*Tectona grandis*	Thailand	KC291345	KF206234	KC342565	KC342542	KF289110
CPC20272	*Orchidaceae*	Thailand	KC291346	KF206232	KC342566	KC342543	KF289079
CPC20275	*Polyalthia longifolia*	Thailand	KC291347	KF206230	KC342567	KC342544	KF289107
CPC20278	*Euphorbia milii*	Thailand	KC291348	KF206227	KC342568	KC342545	KF289113
CPC20423	*Philodendron sp.*	Thailand	KC291349	KF206226	KC342569	KC342546	KF289116
CBS 100175	*Citrus sp.*	Brazil	FJ538320	KF206327	FJ538378	FJ538436	JF343699
CBS 114751	*Vaccinium sp.*	New Zealand	EU167584	EU167584	FJ538407	FJ538465	KF289088
CBS 117118	*Musa acuminata*	Indonesia	FJ538339	JQ743603	FJ538455	FJ538397	KF289090
CBS 115046	*Myracrodruon urundeuva*	Brazil	FJ538322	KF206319	FJ538380	FJ538438	KF289082
CBS 115047	*Aspidosperma polyneuron*	Brazil	FJ538323	KF206318	FJ538381	FJ538439	KF289077

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CPC 20510	*Pyrrosia adnascens*	Thailand	KF206200	KF206223	KF289174	KF289304	KF289080
CBS 120428	*Sensevieria sp.*	Netherlands	JN692544	KF206315	JN692532	JN692520	JN692509
CBS 356.52	*Ilex sp.*	Unknown	FJ538342	KF206300	FJ538400	FJ538458	KF289087
CBS 101228	*Naphelium lappaceum*	Hawaii	FJ538319	KF206325	FJ538377	FJ538435	KF289086
CPC 13987	*Protea repens*	Portugal	KF206183	KF206281	KF289176	KF289263	KF289083
CPC 14609	*Zyzygium sp.*	Madagascar	KF206184	KF206280	KF289175	KF289264	KF289081
CBS 128856	*Stanhopea sp.*	Brazil	JF261465	KF206304	JF261507	JF343647	JF343776
CBS 115049	*Bowdichia nitida*	Brazil	FJ538324	KF206317	FJ538382	FJ538440	KF289084
CBS 123373	*Musa paradisiaca*	Thailand	FJ538341	JQ743604	FJ538399	FJ538457	JF343703
CBS 123404	*Musa paradisiaca*	Thailand	FJ538333	JQ743601	FJ538391	FJ538449	KF289095
CBS 292.90	*Chamaecyparis pisifera*	France	JF343585	KF206301	JF343606	JF343669	JF343773
CBS 937.70	*Hedera helix*	Spain	FJ538350	KF206291	FJ538408	KF289257	JF411745
CPC 18842	*Hedera sp.*	Italy	KF170310	KF206256	KF289228	KF289288	KF289163
CBS 226.77	*Paphiopedilum callosum*	Germany	FJ538336	KF206289	FJ538394	FJ538452	JF343718
CPC 18848	*Stanhopea graveolens*	Brazil	JF261465	KF206255	JF261507	KF289289	JF343776
CBS 120486	*Citrus maxima*	Thailand	FJ538360	KF206314	FJ538418	FJ538476	JF343686
CBS 120488	*Citrus maxima*	Thailand	JN692545	KF206312	JN692533	JN692521	KF289144
CBS 123371	*Citrus maxima*	Vietnam	FJ538356	KF206309	FJ538414	FJ538472	JF343690
CBS 123372	*Citrus maxima*	Vietnam	FJ538357	KF206308	FJ538415	FJ538473	KF289145

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CBS100098	*Citrus sp.*	Brazil	FJ538352	KF206221	FJ538410	FJ538468	JF343691
CPC 17464	*Citrus sp.*	Brazil	KF170300	KF206263	KF289224	KF289280	KF289159
CPC 17465	*Citrus sp.*	Brazil	KF170301	KF206262	KF289225	KF289281	KF289160
CPC 17466	*Citrus sp.*	Brazil	KF170302	KF206261	KF289226	KF289282	KF289161
CPC 19744	*Erica gracilis*	Brazil	KF206170	KF206253	KF289227	KF28291	KF289162
CBS 102374	*Citrus aurantium*	Brazil	FJ538313	KF206324	GU349053	FJ538429	JF343679
CBS 120489	*Citrus sinensis*	Thailand	FJ538315	KF206311	FJ538373	FJ538431	KF289150
CBS127454	*Citrus limon*	Australia	JF343583	KF206306	JF343604	JF343667	JF343771
CBS 127452	*Citrus sp.*	Australia	JF343581	KF206307	JF343602	KF289241	JF343769
CBS 127455	*Citrus sinensis*	Australia	JF343584	KF206305	JF343605	JF343668	JF343772
CPC 16586	*Citrus limon*	Argentina	KF170293	KF206274	KF289220	KF289269	KF289155
CPC 16587	*Citrus limon*	Argentina	KF170294	KF206273	KF289219	KF289270	KF289154
CPC 16603	*Citrus limon*	Uruguay	KF170295	KF206269	KF289213	KF289274	KF289147
CPC 16605	*Citrus limon*	Uruguay	KF170296	KF206268	KF289214	KF289275	KF289148
CPC 16606	*Citrus limon*	Uruguay	KF170297	KF206267	KF289215	KF289276	KF289149
CPC 16609	*Citrus sp.*	Argentina	KF170298	KF206266	KF289217	KF289277	KF289152
CPC 14848	*Citrus sp.*	Zimbabwe	FJ538317	KF306230	FJ538375	KF289265	KF289146
CPC 16149	*Citrus sp.*	Argentina	KF170290	KF206277	KF289216	KF289266	KF289151
CPC 16151	*Citrus sp.*	South Africa	KF170291	KF206276	KF289221	KF289267	KF289156
CPC 16152	*Citrus sp.*	South Africa	KF170292	KF206275	KF289218	KF289268	KF289153

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CPC 20134	*Citrus sp.*	Unknown	KF170298	-	-	KF289292	-
ZJUCC 200956	*Citrus reticulata*	China	JN791664	-	JN791515	JN791589	-
ZJUCC 200964	*Citrus maxima*	China	JN791662	-	JN791514	JN791582	-
ZJUCC 2010150	*Citrus maxima*	China	JN791620	-	JN791459	JN791533	-
ZJUCC 2010152	*Citrus sinensis*	China	JN791611	-	JN791461	JN791535	-
MUCC 521	*Cordyline fruticosa*	Japan	AB454357	-	-	AB704244	-
CPC 20261	*Cordyline fruticosa*	Thailand	KF170287	KF206242	KF289172	KF289295	KF289076
CPC 20277	*Cordyline fruticosa*	Thailand	KF170288	KF206228	KF289171	KF289301	KF289075
CPC 13812	*Cussonia sp.*	South Africa	KF170311	KF206282	KF289223	KF289262	KF289158
CPC 14873	*Cussonia sp.*	South Africa	JF343579	KF206279	JF343600	JF343663	JF343764
CPC 14875	*Cussonia sp*	South Africa	JF343578	KF206278	JF343599	JF343662	JF343765
CBS 434.92	*Ruscus aculeatus*	Italy	FJ538367	KF206299	FJ538425	FJ538483	JF343695
CBS 101.72	*Ruscus aculeatus*	Italy	FJ538365	KF206326	FJ538423	FJ538481	JF343694
CBS 167.85	*Ruscus hypoglossum*	Italy	FJ538366	KF206302	FJ538424	FJ538482	JF343696
CGMCC 3.14358	*Ilex aquifolium*	China	JN692538	-	JN692526	JN692514	-
CGMCC 3.14359	*Ilex aquifolium*	China	JN692539	-	JN692527	JN692515	-
CGMCC 3.14360	*Ilex aquifolium*	China	JN692540	-	JN692528	JN692516	-
CGMCC 3.14355	*Hosta plantaginea*	China	JN692535	-	JN692523	JN692511	JN692503
CGMCC 3.14356	*Hosta plantaginea*	China	JN692536	-	JN692524	JN692512	JN692504
CGMCC 3.14357	*Hosta plantaginea*	China	JN692537	-	JN692525	JN692513	JN692505

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CBS 776.97	*Brabejum stellatifolium*	South Africa	FJ538368	KF206293	FJ538426	KF289254	JF343767
CPC 14901	*Brabejum stellatifolium*	South Africa	JF261462	KF206303	JF261504	KF289243	JF343766
CBS 111646	*Podocarpus falcatus*	South Africa	AF312013	KF206323	KC357671	KC357670	KF289169
CBS 445.82	*Eugenia aromatica*	Indonesia	AY042926	KF206288	KF289208	KF289246	KF289139
CGMCC 3.14985	*Styrax gradiflorus*	China	JX052040	-	JX025045	JX025035	JX025030
CGMCC 3.14989	*Styrax gradiflorus*	China	JX052041	-	JX025046	JX025036	JX025031
CPC 18590	*Vaccinium macrocarpum*	USA	KF170312	KF206257	KF289229	KF289287	KF289165
CBS 126.22	*Oxycoccus macrocarpos*	USA	FJ538353	AB095508	FJ538411	FJ538469	KF289164
CBS 901.69	*Rhododendron sp.*	Netherlands	KF206174	KF206292	KF289230	KF289256	KF289166
CBS 111647	*Podocarpus lanceolata*	South Africa	KF154276	KF206322	KF289232	KF289235	KF268168
CBS 111649	*Pseudotsuga manziesii*	USA	KF154277	KF206321	KF289231	KF289236	KF289167
CBS 131309	*Hymenocallis littoralis*	Australia	JQ044423	JQ044443	KF289211	KF289242	KF289142
CPC 19331	*Hymenocallis littoralis*	Australia	KF170303	KF206254	KF289212	KF289290	KF289143
CPC 20276	*Citrus maxima*	Thailand	KF170304	KF206229	KF289222	KF289300	KF289157
CBS 777.97	*Telopea speciosissima*	Australia	KF206205	KF206285	KF289210	KF289255	KF289141
CBS 112527	*Paxistima mysinites*	USA	KF206172	KF206320	KF289209	KF289239	KF289140
CBS 587.69	*Ilex aquifolium*	Netherlands	KF154278	KF206297	KF289206	KF289250	KF289137
CBS 616.72	*Ilex aquifolium*	Netherlands	KF154279	KF206296	KF289205	KF289251	KF289136
CBS 174.77	*Cryptomeria japonica*	USA	KF170308	KF206290	KF289200	KF289245	KF289131

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CBS 447.68	*Taxus baccata*	Netherlands	KF170309	KF206287	KF289201	KF289247	KF289132
CBS 728.79	*Podocarpus maki*	New Zealand	KF206173	KF206295	KF289203	KF289252	KF289134
CBS 585.84	*Acer rubrum*	USA	KF206176	KF206286	KF289204	KF289249	KF289135
CPC 11336	*Eucalyptus grandis*	Spain	KF206177	KF206284	KF289199	KF289258	KF289130
CPC20264	*Mangifera indica*	Thailand	KF170305	KF206240	KF289190	KF289296	KF289121
CPC 21020	*Aloe ferox*	South Africa	KF154280	KF206210	KF289193	KF289311	KF289124
CPC 21021	*Aloe ferox*	South Africa	KF154281	KF206211	KF289194	KF289312	KF289125
CPC 21022	*Aloe ferox*	South Africa	KF154282	KF206212	KF289195	KF289313	KF289126
CPC 21023	*Aloe ferox*	South Africa	KF154283	KF206213	KF289196	KF289314	KF289127
CPC 21024	*Aloe ferox*	South Africa	KF154284	KF206214	KF289197	KF289315	KF289128
CPC 17748	*Palm*	Thailand	KF206190	KF206258	KF289180	KF289286	KF289096
CPC 17468	*Cymbidium sp.*	Brazil	KF206188	KF206259	KF289189	KF289284	KF289120
LGMF 220	*Citrus sinensis*	Brazil	KF206203	KF206219	JF261488	KF289307	JF343735
CPC 20274	*Mangifera indica*	Thailand	KF206197	KF206231	KF289188	KF289299	KF289119
LGMF 222	*Citrus sinensis*	Brazil	KF206204	KF206218	JF343632	KF289308	JF343739
LGMF 219	*Citrus sinensis*	Brazil	KF206202	KF206220	JF261490	KF289306	JF343737
CBS 119720	*Musa acuminata*	USA	KF206178	KF206316	FJ538398	KF289240	KF289098
CBS 173.77	*Citrus aurantiifolia*	New Zealand	KF206179	KF306231	FJ538393	KF289244	KF289100
CPC 20508	*Ixora chinensis*	Thailand	KF206198	KF206225	KF289185	KF289301	KF289111
CPC 20509	*Cordyline fruticosa*	Thailand	KF206199	KF206224	KF289186	KF289302	KF289112

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
CPC 16590	*Citrus limon*	Argentina	KF206185	KF206272	KF289177	KF289271	KF289091
CPC 16592	*Citrus limon*	Argentina	KF206187	KF206270	KF289178	KF289273	KF289092
CPC 16591	*Citrus limon*	Argentina	KF206186	KF206271	KF289179	KF289272	KF289093
CPC 20271	*Crinum asiaticum*	Thailand	KF206196	KF206233	KF289183	KF289298	KF289106
CPC 20265	*Euphobiaceae*	Thailand	KF206194	KF206239	KF289182	KF289297	KF289105
CPC 11867	*Acacia crassicarpa*	Thailand	KF206181	KF206283	KF289184	KF289260	KF289108
CPC 20253	*Scheffera venulosa*	Thailand	KF206192	KF206250	KF289181	KF289293	KF289102
MUCC 149	*Hamamelis japonica*	Japan	KF170289	-	-	KF289309	-
CGMCC3.14986	*Viburnum odoratissimim*	China	JX025037	-	JX025042	JX025032	JX025027
CGMCC3.14987	*Viburnum odoratissimim*	China	JX025038	-	JX025043	JX025033	JX025028
CGMCC3.14988	*Viburnum odoratissimim*	China	JX025039	-	JX025044	JX025034	JX025029
CBS 111639	*Coemus florida*	USA	KF170307	-	-	KF289234	-
MUCC553	*Leucothoe catesbaei*	Japan	AB454370	-	-	KF289310	-
MUCC125	*Pyrola asarifolia*	Japan	AB454318	-	-	AB704233	-
CBS 111635	*Acer rubrum*	USA	KF206171	EU754194	KF289198	KF289233	KF289129
CBS 112065	*Yucca elephantipes*	USA	KF206175	-	-	KF289237	-
MUCC 124	*Pachysandra leminalis*	Japan	AB454317	-	-	AB704232	-
MUCC 147	*Rhododendron keiskei*	Japan	AB454319	-	-	AB704234	-

Code	Host name	Country	Gene and GenBank No.				
			ITS	LSU	TEF1	ACT	GPDH
MUCC 432	*Rhaphiolepis indica*	Japan	DQ632660	-	DQ632724	AB704242	-
CPC 12157	*Acacia crassicarpa*	Thailand	KF206182	-	-	KF289261	-
CPC 11337	*Eucalyptus gradis*	Brazil	KF206180	-	-	KF289259	-
CPC 21035	*Citrus sp.*		KF206201	-	-	KF289305	-
CMW 8232	*conifers*	South Africa	AY972105	-	DQ280419	AY972111	-

The multigene DNA sequence analysis to discriminate among all species of *Phyllosticta* that were available to us from the CBS culture collection, supplemented by our own working collections, which resulted in a total of 160 strains. Other than dealing with old synonymies that represented names that now again had to be resurrected, a further challenge has been to also merge *Phyllosticta* and *Guignardia* epithets, to derive the best possible unit nomenclature for these species (Wingfield et al., 2012).

Obviously a multi-gene approach works well for distinguishing these taxa. In this study the intron dominated genes (ITS, ACT, TEF), and highly conserved gene coding regions (LSU, GPDH) were used. However, the result from the two gene analysis and the five gene analysis tended to be the similar (Figure 4.4, 4.5), suggesting that for *Phyllosticta* a phylogeny derived from the ITS and ACT gene loci is sufficiently robust to distinguish most taxa. The biggest challenge, however, will still be to recollect specimens representative of the more than 3000 names that exist in this complex.

CHAPTER 5

CONCLUSION

Phyllosticta species are predominantly plant pathogens with a worldwide distribution. They are responsible for numerous diseases including leaf spots and black spots on fruits. Several species have been reported as saprobes and some, in particular *P. capitalensis* are endophytes with a worldwide distribution. Species recognition in *Phyllosticta* has historically been based on morphology, culture characters and host-association. Accuracy in identifying species is important in identifying plant disease, in understanding disease epidemiology, in developing protocols import and export of crops and in developing disease resistant plants. Although there have been several taxonomic revisions and enumerations of *Phyllosticta* species, there is still considerable confusion in the determination of species. Molecular sequence data analysis has become commonplace in classifying plant pathogenic genera such as *Phyllosticta*. Initially ITS and morphology was used to characterize species, however, the ITS gene cannot resolve species well. Recent multigene phylogenetic analysis in the genus have involved multi-loci combined genes with two (ITS and ACT) and five genes (ITS, LSU, ACT, TEF and GPDH) trees, as well as morphology and pathogenicity testing, so at present there are about 10 described species in the genus

At the beginning of this study (October 2010) there more than ten confirmed “molecular” species in the genus causing plant diseases worldwide and only two were known from Thailand. We therefore initiated a survey of *Phyllosticta* species infecting plants in Thailand. In the first and second years of this study we collected more than 200 fresh specimens of various disease plants and fruits from different places in Thailand. From these we successfully isolated 72 strains from fresh diseased samples or as endophytes from asymptomatic samples. We also started to sequence these isolates and carry out morphological as well as pathogenicity studies. We identified several new potential species. We are involved in developing a practical phylogeny and morphology based approach for the identification of *Phyllosticta* species, focusing on Thai species. However, since this is a global problem we have also chose to collaborate with Chinese and European colleagues in order to bring greater depth to the research and international agreement to the findings. In year two we will continue to isolate more strains of *Phyllosticta* from Thailand, sequence multigenes and carry out multigene analysis. We will also study worldwide strains of the genus and develop a practical phylogeny and morphology based approach for the identification of *Phyllosticta* species. Our first publications from the grant appeared in year two, which included a review paper on the problems in identification of the genus *Phyllosticta* and their biology, use in biological control and novel compound discovery from the genus.

The major finding of Year 2 is that *Phyllosticta capitalensis* is an endophyte and weak plant pathogen with a worldwide distribution presently known about 70 plant families. We isolated *P. capitalensis* from different host plants in northern Thailand, and in the process establish their different life modes. Twenty-one strains of *P. capitalensis* isolated as endophytes from 20 hosts, were subjected to phylogenetic analysis. An additional 14 strains of *P. capitalensis* from other hosts and geographic locations were also obtained from established culture collections. In all cases there was no infection of the healthy plant leaves, suggesting that this endophyte does not cause disease on healthy, unstressed host plants. The fact that *P. capitalensis* is often isolated as an endophyte has important implications for studies in fungal biology and plant health. Due to its endophytic nature, *P. capitalensis* is commonly found associated with lesions of plants, and frequently incorrectly identified as a species of serious quarantine importance, which again has serious implications for trade in agricultural and forestry produce. We have also multigene sequence data which has been analyzed for 129 isolates and resulted in 35 taxa of which nine are new species. The study has resulted in five publications up to the end of year two. Future work will investigate more species at the molecular level and result in a comprehensive understanding of the genus.

Table 5-1 List of publications resulting from the grant

Years	**Publications**
2011	Wulandari N, To-Anun C, McKenzie E, Hyde KD (2011) ***Guignardia bispora* and *G. ellipsoidea* spp. nov. and other *Guignardia* species from palms (Arecaceae).** Mycosphere 2(2):115–128
2011	Ko Ko TW, McKenzie EHC, Bahkali AH, To-anun C , Chukeatirote E, Promputtha I, Abd-Elsalam KA, Soytong K, Wulandari NF, Sanoamuang N, Jonglaekha N, Rampai Kodsueb R, Cheewangkoon R, Wikee S, Chamyuang S, Hyde KD (2011) **The need for re-inventory of Thai phytopathogens.** Chiang Mai Journal of Science 38(4) : 625-637
2011	Wikee S, Udayanga D, Crous PW, Chukeatirote E, McKenzie EHC, Bahkali AH, Dai DQ, Hyde KD (2011) ***Phyllosticta*—an overview of current status of species recognition.** Fungal Diversity 51:43–61
2011	Wikee S, Wulandari NF, McKenzie EHC, Hyde KD (2011b) ***Phyllosticta ophiopogonis* sp. nov. from *Ophiopogon japonicas* (Liliaceae).** Saudi Journal of Biological Science 19(2):13–16
2012	Wang X, Chen G, Huang F, Zhang J, Hyde KD, Li H (2012) ***Phyllosticta* species associated with citrus diseases in China.** Fungal Diversity 52:209–224

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