รายงานวิจัยฉบับสมบูรณ์

วงศ์วานวิวัฒนาการและความหลากหลายทางชีวโมเลกุลของราในสกุล Pestalotiopsis ในประเทศไทย

Biochemistry and Phylogeny of *Pestalotiopsis*

By

Associate Professor Kevin David Hyde
Assistant Professor Ekachai Chukeatirote
et al. 2011

This research was made possible by support of

Mae Fah Luang University 2011

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บทสรุปผู้บริหาร (EXECUTIVE SUMMARY)

1. ความสำคัญและที่มาของปัญหาในการการวิจัย (Rationale and review)

The species in the genus *Pestalotiopsis* have received much attention in recent years, not only because of their role as plant pathogens, but also as commonly isolated endophytes which have been shown to produce a wide range of chemically novel diverse metabolites. There are numerous reports in the literature that various species produce taxol, while others produce newly discovered compounds with medicinal potential and still others cause disease. The names assigned to these novel compound-producing taxa lack an accurate taxonomic basis, since the taxonomy of the genus is markedly confused. This confusion arises mainly due to the fact that *Pestalotiopsis* species are generally not host-specific, conidial characters vary and thereby species limits overlap and many of the sequences available in the GenBank for a species are not based on type. This calls for critical re-examination of the type material of the large number of species described under *Pestalotiopsis* using multi-gene analysis and establishment of epitypes, wherever necessary, with the living cultures. Only such combined molecular and morphological studies of the type would provide a reliable species base for taxonomic treatment of the genus *Pestalotiopsis.* So the current research mainly focuses on resolving the taxonomic confusion and harvesting natural chemical substances from economically important genus *Pestalotiopsis.*

2. วัตถุประสงค์ของโครงการวิจัย (Objective)

2.1 To document the diversity of *Pestalotiopsis* species mainly in northern Thailand and to isolate strains for conservation in the BIOTEC Culture Collection.

2.2 To construct phylogenetic trees to examine the inter-relationships of *Pestalotiopsis* species and to determine their relationships

2.3 To use a polyphasic approach and epitypification to stabilize the nomenclature of *Pestalotiopsis* species.

2.4 To establish chemical profiles for species and discover new bioactive compounds.

3. ขอบเขตของโครงการวิจัย (Scope of the research)

The genus *Pestalotiopsis* contains more than 230 names and presently there are no exact estimated species. The genus has received much attention from the scientific community. However, this not only because of its pathogenic nature, but rather because its species have been shown to produce many important secondary metabolites. *Pestalotiopsis* species are cosmopolitan in distribution and traditionally naming according to the host association. However, resent research showed that *Pestalotiopsis* are generally not host specific and may inhibit in range of hosts. Thus the actual number of species in *Pestalotiopsis* is likely to be much lower than presently recorded in the literature. Furthermore the species identification is challenging due to 1) conidial characters vary and species limits overlap and 2) species arrangements in literature are problematic.

In our study we will develop a polyphasic approach for identification of *Pestalotiopsis*, focusing on global species. The taxonomically important morphological characters will be identified and multi-locus DNA data will use to develop a strong taxonomic base to the genus. Re-examination of the type material of the large number of species described under *Pestalotiopsis* using multi gene analysis will be done with establishment of epitypes, with the living cultures. As a part of study species in Thailand will be documentation and at the later stages cultures will be used to screen novel secondary metabolites. The successful outcome of this project will have important practical implications to the plant pathology, plant breeding and quarantine communities and secondary metabolites profiling. One doctoral student will be train as an expert in the field. As an outcome several highly cited papers will be published and bring Mae Fah University and Thailand as one of the world leaders in mycological research.

4. ระเบียบวิธีวิจัยและผลผลิตจากการวิจัย (Methodology and the research output)

Research Plan from October 2011 to September 2014

Year 1 (October 2011 to September 2012): This study will commence with the isolation of a number of *Pestalotiopsis* species from different substrates in Thailand and recovery of cultures from various culture collections such as CBS, CGMCC, ICMP and CABI. Isolated *Pestalotiopsis* spp. in different substrata around Thailand will be compared with *Pestalotiopsis* species available in different international culture collections. The morphology and cultural characters of species will be examined and phylogenetic study will be carried out including representatives of many species as possible, initially using rDNA ITS sequence data followed by beta-tubulin, TEF 1α and other gene loci. The outcome of the research will undoubtedly be

useful to provide strong taxonomic foundation to this economically important genus *Pestalotiopsis* using polyphasic approach and epitypification. There is no doubt that many new species of the genus will emerge out in this molecular re-examination. Such a study will always aid in conservation of the biodiversity of *Pestalotiopsis* of Thailand and documentation of diversity.

5. ประโยชน์ที่ได้รับ (Benefit)

5.1 Addition of 50 distinct *Pestalotiopsis* strains per year to the BIOTEC and MFUCC Culture Collection.

5.2 Production of one scientific paper in an international refereed journal or oral papers at an international conference each year.

5.3 Training one researcher in taxonomy, plant pathology, molecular biology and metabolite profiling.

5.4 Production of new records for the checklist of Thai fungi.

5.5 Description of any new taxa collected during the research program.

5.6 Descriptions of new bioactive chemicals

6.แผนการถ่ายทอดเทคโนโลยีหรือผลการวิจัยสู่กลุ่มเป้าหมาย (A plan to transfer technology or research to target group)

The results will be published and be useful to systematists, plant pathologists, plant health practitioners, plant breeders, and quarantine officers. Any new chemical may have medicinal significance.

บทคัดย่อ

ในปัจจุบันนี้ เชื้อราสกุล *Pestalotiopsis* ได้รับความสนใจศึกษาอย่างแพร่หลาย โดยเชื้อราในสกุลนี้สามารถพบได้ทั้งลักษณะที่เป็นเอนโดไฟท์ และเชื้อสาเหตุก่อโรคในพืช นอกจากนี้เชื้อราในกลุ่มที่เป็นเอนโดไฟท์ยังมีความสามารถในการผลิตสารชีวภาพชนิดใหม่อย่างหลากหลาย ดังนั้น เหตุผลในการศึกษาเชื้อราสกุล *Pestalotiopsis* ได้แก่ 1) เป็นเชื้อราในกลุ่ม non-host specific ที่พบได้ทั่วไป 2) ลักษณะของโคนิเดียมีความหลายหลาย และในบางสปีชีส์มีข้อจำกัดของการใช้ลักษณะทางสัณฐานวิทยาในการจัดจำแนก 3) และในงานวิจัยของ Steyaert และ Guba ยังไม่สามารถแก้ปัญหาการจัดจำแนกในระดับสปีชีส์ได้อย่างชัดเจน รวมถึงจำนวนสปีชีส์ที่แท้จริงของเชื้อราในสกุล *Pestalotiopsis* มีจำนวนน้อยกว่าที่ถูกบันทึกไว้ในเอกสารตีพิมพ์ทางวิชาการในปัจจุบัน ในงานวิจัยนี้ การศึกษาตัวอย่างต้นแบบ (type material) และการจัดตั้ง epitype ด้วยผลของเชื้อที่แยกเพาะเลี้ยงได้ (living culture) นั้นเป็นสิ่งจำเป็นต่อความก้าวหน้าในการวิเคราะห์ข้อมูลระดับพันธุกรรมด้วยหลายยีน (multi gene analysis) ต่อการแยกความแตกต่างทางลักษณะทางสัณฐานวิทยาที่ชัดเจน และเป็นพื้นฐานการพัฒนาระบบอนุกรมวิธานระดับสปีชีส์ที่แข็งแกร่งของเชื้อราในสกุล *Pestalotiopsis* ดังนั้น โครงการวิจัยนี้จึงมุ่งเน้นความสำคัญของการแยกเพาะเลี้ยง (culture isolation) เชื้อราในสกุล *Pestalotiopsis* จากตัวอย่างพืชที่มีความแตกต่างกันในเขตพื้นที่ภาคเหนือของประเทศไทย โดยเฉพาะในกลุ่มที่เป็นเชื้อสาเหตุโรคพืชและกลุ่มเอนโดไฟท์ที่สามารถผลิตสารชีวภาพทุติยภูมิ (secondary metabolites) ที่มีคุณสมบัติทางเคมีได้ เชื้อที่แยกเพาะได้เลี้ยงได้ทั้งหมดจะฝากเก็บไว้ที่ MFLU และ BIOTEC Culture Collections ซึ่งไอโซเลทเหล่านี้ยังนำมาใช้ในการศึกษาความสัมพันธ์ในเชิงวิวัฒนาการด้วยการวิเคราะห์ข้อมูลทางชีวโมเลกุลและลักษณะทางสัณฐานวิทยา เพื่อช่วยในการตั้งชื่ออย่างถูกต้องและแก้ไขความสับสนในระดับอนุกรมวิธานของเชื้อราในสกุล *Pestalotiopsis* และอีกส่วนหนึ่งของไอโซเลทได้นำมาใช้หาสารชีวภาพทุติยภูมิชนิดใหม่ รวมถึงการตีพิมพ์ผลศึกษาลักษณะทางสัณฐานวิทยา และความสัมพันธ์วงศ์วานวิวัฒนาการเชิงโมเลกุล (molecular phylogeny) ของเชื้อราสกุล *Pestalotiopsis*

คำสำคัญ: เอนโดไฟท์, โรคพืช, สารทุติยภูมิ, อนุกรมวิธาน

ABSTRACT

The genus *Pestalotiopsis* has received much attention in recent years, not only because of its role as a plant pathogen, but also as a commonly isolated endophyte which has been shown to produce a wide range of chemically novel diverse metabolites. However due to the fact that 1) *Pestalotiopsis* are generally not host-specific 2) conidial characters vary and species limits overlap and 3) species arrangements in Steyaert and Guba are problematic, then the actual number of species in *Pestalotiopsis* is likely to be much lower than presently recorded in the literature. Re-examination of type materials and establishment of epitypes with living cultures is essential for progress and multi gene analysis with distinct morphological characters are needed to develop a strong species base taxonomic system for the genus *Pestalotiopsis*. The present study mainly focuses on the isolation of *Pestalotiopsis* found in different substrata around Northern Thailand especially important plant pathogens and chemically important endophytes. In the first year of this project we collected 200 specimens throughout Northern of Thailand and isolated 53 *Pestalotiopsis* strains. We also examined 300 isolates from CGMCC in China. These isolates were deposited in MFLU culture collection. We described a new species isolated from *Camellia sinensis* (tea) as species *Pestalotiopsis furcata*; one species was epitypified as a *P. theae*; and one species causing serious fruit rot disease of *Syzygium samarangense* fruit was described as a new species, *P. samarangensis*. Morphologically most *Pestalotiopsis* species clusters in three main clades and this is highly supported by analysis of ITS, β-tubulin and *tef1* sequence data . As a result of this study we have published four SCI papers including one important review paper and one large backbone tree for identifying species in the genus. At the later stages cultures will be used to screen novel secondary metabolites.

Keywords: Endophytes, Plant pathogen, Secondary metabolites, Taxonomy

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ABBREVIATION AND SYMBOLS

%	=	Percent
°C	=	Degree Celsius
µm	=	micrometer
µg	=	Microgram
cm	=	centimeter
g	=	gram
g/l	=	gram per liter
hr	=	hour
ml	=	milliliter
mm	=	millimeter
No.	=	Number
PDA	=	Potato Dextrose Agar
sp.	=	species
Temp.	=	Temperature
$\bar{x}$	=	Average
GPDH	=	Glyceraldehyde-3- phosphate dehydrogenase
ITS	=	Internal transcribed spacer
LSU	=	Large subunit (28S rDNA)
TEF1α	=	Translation elongation factor 1-alpha
USA	=	United States of America
USDA	=	United States Department of Agriculture

CHAPTER 1

INTRODUCTION

The genus *Pestalotiopsis* has received considerable attention in recent years, not only because of its role as a plant pathogen but also as a commonly isolated endophyte which has been shown to produce a wide range of chemically novel diverse metabolites. Classification in the genus has been previously based on morphology, with conidial characters being considered as important in distinguishing species and closely related genera. However it is concluded that the large number of described species has resulted from introductions based on host association. We suspect that many of these are probably not good biological species. Recent molecular data have shown that conidial characters can be used to distinguish taxa; however, host association and geographical location is less informative. The taxonomy of the genera complex remains confused. There are only a few type cultures and, therefore, it is impossible to use gene sequences in GenBank to clarify species names reliably. There are numerous reports in the literature that various species produce taxol, while others produce newly discovered compounds with medicinal potential and still others cause disease. The names assigned to these novel compound-producing taxa lack an accurate taxonomic basis, since the taxonomy of the genus is markedly confused. Until the important species have been epitypified with living strains that have been sequenced and deposited in public databases, researchers should refrain from providing the exact name of species. Species of *Pestalotiopsis* have been well-studied because of the diverse array of novel compounds that they have been shown to produce. As such, they are thought to be a rich source for bioprospecting when compared to those of other fungal genera. Moreover, species of *Pestalotiopsis* have been found to produce an enormous number of secondary metabolites that may have medicinal, agricultural and industrial applications.

CHAPTER 2

LITERATURE REVIEW

Pestalotoiopsis Steyaert is an appendage-bearing conidial anamorphic form (coelomycetes) in the family Amphisphaeriaceae (Barr 1975), and molecular studies have shown that *Pestalotiopsis* is monophyletic (Jeewon et al. 2002, 2004). The genus has received much attention from the scientific community. However, this not because of its pathogenic nature (Rivera and Wright 2000; Yasuda et al. 2003), but rather because its species have been shown to produce many important secondary metabolites (Strobel et al. 2002; Aly et al. 2010; Xu et al. 2010). De Notaris (1839) introduced the genus *Pestalotia* De Not. based on the generic type *Pestalotia* pezizoides De Not.. However Steyaert (1949) revised *Pestalotia* and divided the genus into three main groups based on the conidial forms. Steyaert (1949) also introduced two new genera, *Truncatella* Steyaert for 4-celled conidial forms and *Pestalotiopsis* Steyaert for the 5-celled forms, while the 6- celled forms remained in *Pestalotia*. Until 1990, phylogenetic understanding of the taxonomy associated with *Pestalotiopsis* and allied genera was based mainly on conidial characters (Steyaert 1949; Guba 1961; Nag Rag 1993), conidiogenesis (Sutton 1980) and teleomorph association (Barr 1975, 1990; Metz et al. 2000; Zhu et al. 1991).

Morphological characters used to differentiate species of *Pestalotiopsis* and similar genera are limited (Hu et al. 2007); the morphological characters are plastid and morphological markers vary between host and environment (Egger 1995). Hu et al. (2007) showed that colony morphology (colour, growth rate and texture) is highly variable within single isolates of *Pestalotiopsis*; this phenomenon can be easily observed through repeated subculturing. Also within a single species, conidial morphology (shape and colour of the median cells), growth rate and fruiting structure, may vary (Jeewon et al. 2003). Jeewon et al. (2003) evaluated the morphological characters that could be used to differentiate species of *Pestalotiopsis*. He suggested that pigmentation of median cells has taxonomic value. Liu et al. (2010a) proposed that instead of using "concolorous" and "versicolor" as proposed by Steyaert (1949) and Guba (1961), "brown to olivaceous" and "umber to fuliginous" median cells can

be a key character in distinguishing species in *Pestalotiopsis*. Conidial morphology is the most widely used taxonomic character for the genus *Pestalotiopsis*.

Most species are divided into different groups based on the size of the conidia. Colour of the median cells is still a widely used character, and all species separate into three groups based on this- concolorous, versicolorous umber olivaceous and versicolorous fuliginous olivaceous. The length of the apical appendages and the number of the apical appendages are also widely used characters for species identification. Some species can also be identified by the presence of knobbed apical appendages. The apical appendages can arise from the top, middle, bottom or different positions in the apical hyaline cells and such characters are widely used in species identification. Furthermore the apical appendages can be divided into branches; in some species presence or absence of the basal appendages is another character for species diagnosis (Maharachchikumbura et al. 2011). Species of *Pestalotiopsis* commonly cause disease in a variety of plants (Hopkins and McQuilken 2000; Tagne and Mathur 2001), are commonly isolated as endophytes (Liu et al. 2006; Tejesvi et al. 2009; Watanabe et al. 2010) and some species likely have endophytic and pathogenic stages in their life cycle (Wei et al. 2007: Tejesvi et al. 2009). Species have also been recorded as saprobes (Agarwal and Chauhan 1988; Yanna et al. 2002) where they are recyclers of dead plant material (Osono and Takeda 1999; Tokumasu and Aoiki 2002) and even rarely cause disease in humans (Sutton 1999). According to Index Fungorum (http://www.indexfungorum.org, 2011) there are 235 *Pestalotiopsis* names, while in MycoBank (www. mycobank.org, 2011) there are 232 names. The reason for the large number of names is historical and may not reflect the actual number of species (Jeewon et al. 2004) and actual number of species may be fewer than 50 (Maharachchikumbura et al. 2011).

Pestalotiopsis are thought to be a rich source for bioprospecting when compared to those of other fungal genera (Aly et al. 2010; Xu et al. 2010). Strobel and Long (1998) described *Pestalotiopsis* as the 'E. coli of the temperate and tropical rainforest systems'. The majority of compounds have been discovered from endophytic strains of *Pestalotiopsis* (Lee et al. 1996; Li and Strobel 2001). Species of *Pestalotiopsis* have been shown to produce bioactive alkaloids, terpenoids, isocoumarin derivatives, coumarins, chromones, quinones, semiquinones, peptides,

xanthones, xanthone derivatives, phenols, phenolic acids, and lactones with a range of antifungal, antimicrobial, and antitumor activities (Xu et al. 2010). Xu et al. (2010) reviewed 130 different compounds isolated from species of *Pestalotiopsis*.

CHAPTER 3

RESEARCH METHODOLOGY

(1) Collection of the samples

Pestalotiopsis isolates will be collected from the leaf spots and diseased fruits of various hosts, Provinces of Chiang Mai and Chiang Rai in northern Thailand. The cultures will be obtained from CGMCC, CBS and all other collaborating institutes. Most important species, type herbarium material will be loaned and re-examined.

(2) Morphological examination

The obtained pure colony will be transferred onto PDA medium. The plates will be incubated at room temperature (25°C) and to induce sporulation, sterilized Carnation leaves were put on to the medium. Morphology of fungal colonies will be recorded. Fungal mycelium and spores will be observed under light microscope and photographed.

(3) Phylogenetic study

DNA will be extracted from selected isolates and PCR will be carried out to amplify rDNA ITS region by using primers ITS 4 and ITS 5 and partial β-tubulin gene by using Bt2A and Bt2B or other gene loci (Calmodulin, Actin, RPB2 and *tef1*). Sequencing will be carried out for the respective region. Phylogenetic analysis of the sequences will be carried out by using PAUP * and Mega4.

3.1 Molecular analysis

DNA extraction

Total genomic DNA will be extracted from fresh cultures using a modified protocol of Doyle & Doyle (1987) and Lee & Taylor (1990). Fresh fungal mycelia (500 mg) will be scraped from the margin of the PDA plate incubated at 25°C for 7 to 10 days and transferred into a 1.5 ml centrifuge tube with 100 ml of preheated (60°C) 2X CTAB extraction buffer (2% (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0), and 200 mg sterilized quartz sand. Mycelia will be ground using a glass pestle for 5 min and add extra 500 ml preheated (60°C) 2X CTAB and,

incubated in a 65°C water bath for 30 min with occasional shaking. 500 ml of phenol:chloroform (1:1) will be added into each tube and shake thoroughly to form an emulsion. The mixture will be spun 11900 *g* for 15 min at 25°C in microcentrifuge and decants the supernatant phase into a fresh 1.5 ml tube. Supernatant containing DNA will be reextracted with phenol: chloroform (1:1) at 4°C until no interface was visible. 50 ml of 5M KOAc will be added into the supernatant followed by 400 ml of isopropanol and inverted gently to mix. The genomic DNA will be precipitated at 9200 *g* for 2 min at 4°C in a microcentrifuge. The DNA pellet will be washed with 70% ethanol twice and dried using SpeedVac® (AES 1010; Savant, Holbrook, NY, USA) until dry. The DNA pellet will be then resuspended in 100 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA).

3.2 PCR amplification

The ITS and 5.8S region of rDNA molecule will be amplified using primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al 1990; Worapong et al 2002), β-tubulin gene region will be amplified with primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5' ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997) and *tef1* will be amplified using the primer pairs EF1-526F (5'-GTCGTYGTYATY GGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRATACCACCRATCTT-3'). All other regions will be amplification by their respective primers. The PCR will be performed with the 25 μL reaction system consisting of 19.5 μL of double distilled water, 2.5 μL of 10× Taq buffer with MgCl2, 0.5 μL of dNTP (10 mM each), 0.5 μL of each primer (10 μM), 0.25 μL Taq DNA polymerase (5 U/μL), 1.00 μL of DNA template. The thermal cycling program will be as follows:

For ITS initial denaturing step of 95°C for 3 min, followed by 35 amplification cycles of 95°C for 30 sec, 52°C for 45 sec, and 72°C for 90 sec and a final extension step of 72°C for 10 min. For β-tubulin PCR conditions will be an initial step of 3 min at 95°C, 35 cycles of 1 min at 94°C, 50 s at 55°C, and 1 min at 72°C, followed by 10 min at 72°C. For *tef1,* initial step of 5 min at 94°C, 10 cycles of 30 s at 94°C, 55 s at 63°C or 66°C (decreasing 1°C per cycle), 90 s at 72°C, plus 36

cycles of 30 s at 94°C, 55 s at 53°C or 56°C, 90 s at 72°C, followed by 7 min at 72°C. The PCR products will be verified by staining with Goldview (Guangzhou Geneshun Biotech Ltd., China) on 1% agarose electrophoresis gels.

(4) Phylogenetic analysis

DNAStar, SeqMan will be used to obtain consensus sequences from sequences generated from forward and reverse primers. Combination sequence obtained from three gene regions will be aligned using CLUSTALX (1.83) (Thompson et al., 1997). The sequence will be manually adjusted using BioEdit (Hall, 1999), to allow maximum alignment and maximum sequence similarity. A maximum parsimony analysis (MP) will be performed using PAUP * (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2002). Ambiguously aligned regions will be excluded and gaps were treated as missing data. Trees will be inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees will be unlimited, branches of zero length will be collapsed and all multiple parsimonious trees will be saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) will be calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees will be evaluated by 1000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stemwise addition of taxa (Felsenstein 1985). The Kishino–Hasegawa tests (Kishino and Hasegawa 1989) will be performed in order to determine whether the trees inferred under different optimality criteria were significantly different. Trees will be viewed in Treeview (Page, 1996).

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 Ekachai has hand-on experience in the specific molecular and physiological evaluation techniques.

Secondary metabolite profiling

Secondary metabolite will be extraction from selected species of *Pestalotiopsis*, using methods describe by Li et al, 2007 and Xu et al. 2009.

CHAPTER 4

RESULTS AND DISCUSSION

In the first 10 months of this project 53 *Pestalotiopsis* isolates were collected throughout Northern of Thailand from agricultural fields, waterfalls, national parks and house gardens. Species of *Pestalotiopsis* cause a variety of disease in plants, including canker lesions, shoot dieback, leaf spots, needle blight, tip blight, grey blight, scabby canker, severe chlorosis, fruit rots and leaf spots. Fresh plant material infected by *Pestalotiopsis* was isolated by endophyte technique, hyphal tip and single spore isolation. Conidiomata in the genus as variable, ranging from acervuli to pycnidia. Conidiomata can be immersed to erumpent, unilocular to irregularly plurilocular with the locules occasionally incompletely divided and dehiscence by irregular splitting of the apical wall or overlying host tissue. Conidiophores partly or entirely develop inside the conidiomata, and they can be reduced to conidiogenesis cells which are discrete or integrated, cylindrical, smooth, colourless and invested in mucus. Pycnidia can mostly be seen with the unaided eye as a black or brown spore masses with copious conidia. Conidia fusiform to ellipsoid, straight to slightly curved, 4-septate, basal cell colourless or slightly colour; median cells 3, concolourous or versicolourous; apical cell colourless; apical appendages, tubular, 2 to 6 in number, arising from the upper portion of the apical cell, knob or knot. Basal appendages are usually present. Characteristics and morphology have been examined in pure culture, most species produce white colony, which after 1 weeks on PDA is 5-7 cm in diam.

Table 4.1 Some *Pestalotiopsis* from northern Thailand

ORIGINAL Code	SPECIES Name	HOST Name	SUBSTRATE	COLLECTION SITE
SAJ-0008	*Pestalotiopsis adusta*	*Sysygium* sp.	Leaf	Chiang Rai, Thailand
SAJ-0009	*Pestalotiopsis* sp.	*Phylanthus emblica*	fruit	Market infront of Mae Fah Luang University, Chiang Rai, Thailand
SAJ-0010	*Pestalotiopsis samarangensis*	*Syzygium samarangense*	fruit	Market infront of Mae Fah Luang University, Chiang Rai, Thailand
SAJ-0011	*Pestalotiopsis* sp.	*Dracontomelon mangifera*	Leaf	Nam Tak Huey Mesak Forest Park, Chiang Rai, Thailand
SAJ-0012	*Pestalotiopsis* sp.	*Rododendron* sp.	Leaf	Mae Fah Luang University, Chiang Rai, Thailand
SAJ-0013	*Pestalotiopsis* sp.	*Chisocheton siamensis*	Leaf	Huay Mesak Waterfall, Tool Kwan, Chiang Rai, Thailand
SAJ-0014	*Pestalotiopsis* sp.	*Unknown sp.*	Leaf	Nursery orchids,Ching Mai province.Thailand
SAJ-0015	*Pestalotiopsis* sp.	*Artocarpus heterophyllus*	Decaying leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand
SAJ-0016	*Pestalotiopsis* sp.	*Musa paradisiaca*	Leaf	Rachana Dormitory, Mae Fah Luang University, Ching Rai, Thailand
SAJ-0017	*Pestalotiopsis furcata*	*Camellia sinensis*	Leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand
SAJ-0018	*Pestalotiopsis* sp.	*Unknown* sp.	Leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand
SAJ-0019	*Pestalotiopsis* sp.	*Areca* sp.	Leaf	Rachana Dormitory, Mae Fah Luang University, Ching Rai, Thailand
SAJ-0020	*Pestalotiopsis* sp.	*Saccharum* sp.	Leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand
SAJ-0021	*Pestalotiopsis* sp.	Unidentified sp.	Leaf	Vientiane Capital, Dove Makkhai Village,Laos
JKC-0001	*Pestalotiopsis* sp.	Palm	Leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand
JKC-0006	*Pestalotiopsis* sp.	Palm	Rachis	Khow waterfall, chian grai, Thailand
SAJ027	*Pestalotiopsis theae*	*Camellia sinensis*	Leaf	Mushroom Research Centre (MRC), Bahn Pha Deng, Chiang Mai Province, Thailand

4.1 New species/epitype of *Pestalotiopsis* collected during this study

1. *Pestalotiopsis furcata* Maharachchikumbura & K.D. Hyde, sp. nov. Fig. 4.1

MycoBank: MB564563

Etymology: The specific epithet is based on the branching nature of the apical appendages of the species.

Associated with grey blight on leaves of *Camellia sinensis*, small, rounded, yellow-green spots on the leaves become brown to grey, with concentric rings bearing black, scattered conidiomata. *Conidiomata* acervuli scattered or gregarious, rarely confluent, subepidermal in origin, erumpent when mature, round to oval in outline, conical to oval in longitudinal section, 180–300 µm wide, 70–160 µm high, unilocular, glabrous; wall tissue (stroma and parietal cells) only a few cells thick (14–22 µm), forming a textura angularis, cell walls thick, outermost layer hyaline, inner layers pale brown to brown, encrusted. *Conidiophores* reduced to conidiogenous cells lining the inner wall of the conidiomatal cavity. *Conidiogenous* cells discrete, lageniform, smooth, thin-walled, hyaline, with 2–3 proliferations. *Conidia* fusoid to ellipsoid, straight to slightly curved, 4–septate, 29–39 × 8.5–10.5 µm ($\bar{x}$ = 35.5 × 9.7 µm), basal cell obconic, hyaline or slightly olivaceous, thin- and smooth-walled, 4.9–6.4 µm long ($\bar{x}$ = 5.8 µm), with 3 median cells, doliiform to subcylindrical, with thick verruculose walls, constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, wall rugose, together 20.7–25 µm long ($\bar{x}$ = 23.4 µm) (second cell from base 7–9 µm ($\bar{x}$ = 7.9 µm); third cell 7.5–9.1 µm ($\bar{x}$ = 8.2 µm); fourth cell 7.2–9.2 µm ($\bar{x}$ = 8.0 µm); apical cell hyaline, conic to cylindrical 6.3-8.44 µm long ($\bar{x}$ = 7.48 µm); 5–9 tubular apical appendages, some appendages branched, arising from the upper portion of the apical cell, 20–35 µm long ($\bar{x}$ = 27.7 µm), unequal; basal appendages absent.

Colonies on PDA reaching 7 cm after 7 days at 25°C, edge entire, whitish, with dense, aerial mycelium on surface, fruiting bodies black, gregarious; reverse of culture white.

Habitat/Distribution: Known to inhabit living leaves of *Camellia sinensis*, Thailand.

Material examined: THAILAND, Chiang Mai Prov., Mae Taeng Distr., Ban Pha Deng, Mushroom Research Centre, N 19°17.123' E 98°44. 009', elevation 900 m, rainforest, on living leaves of *Camellia sinensis*, January 20, 2010, S.S.N. Maharachchikumbura S200110 (MFLU12-0112, holotype) - ex-type culture MFLUCC 12-0054; ibid., July 10, 2010, S.C. Karunarathna S100710 (MFLU12-0113); ibid., September 9, 2011, S.S.N. Maharachchikumbura S110911 (MFLU12-0114); ibid., December 9, 2011, S.S.N. Maharachchikumbura S91211 (MFLU12-0115).

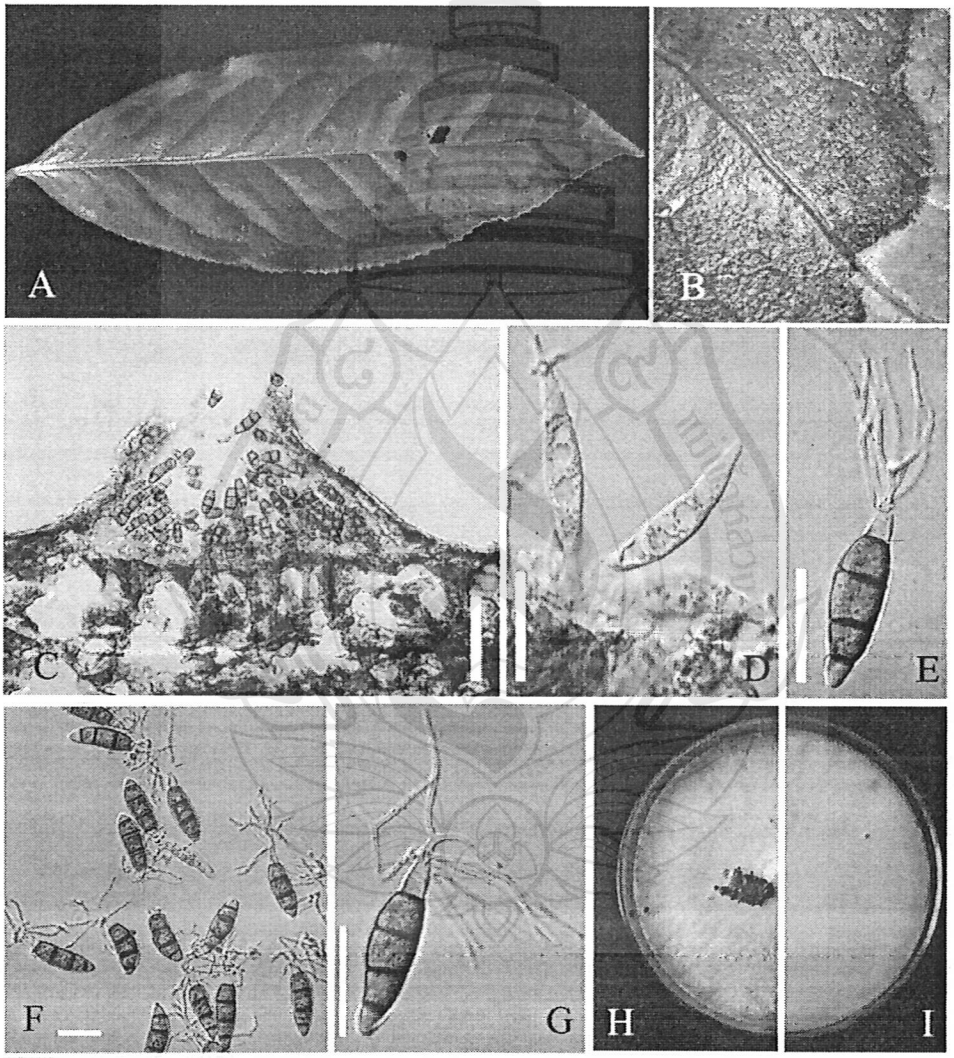

Figure 4.1 *Pestalotiopsis furcata* sp. nov. (holotype). A. Blight on leaf of *Camellia sinensis*. B. Conidiomata, split irregularly. C. Section of conidiomata. D. Conidiophores/ conidiogenous cells. E–G. Conidia with branched appendages. H, I. Colony on PDA, H from above, I from below. Scale Bars: C = 50 μm, D– G= 20 μm.

2. *Pestalotiopsis theae* (Sawada) Steyaert (EPITYPE) **Fig. 4.2**

Conidiophores growing in clusters, simple, short, filiform, fugacious, smooth, thin-walled, hyaline, 4–8 × 1–2 µm ($\bar{x}$ = 6 × 1.5 µm). *Conidia* fusiform to ellipsoid, straight to slightly curved, 4-septate 22.5–28 × 6.7–8.2 µm ($\bar{x}$ = 25.5 × 7.6 µm), basal cell conic or obconic, hyaline, thin and smooth walled, 3.9–5.3 µm long ($\bar{x}$ = 4.55 µm), with 3 median cells, thick verruculose walls, constricted at the septa, concolorous, dark brown, septa and periclinal walls darker than the rest of the cell, together 14.5–18.5 µm long ($\bar{x}$ = 16.7 µm) (second cell from base 5–7.2 µm ($\bar{x}$ = 6.3 µm); third cell 4.8–6 µm ($\bar{x}$ = 5.4 µm); fourth cell 5–6.8 µm ($\bar{x}$ = 5.7 µm)); apical cell hyaline, cylindrical 4.2–5.9 µm long ($\bar{x}$ = 5.2 µm); 3–4 apical appendages, tubular, arising from the upper portion of the apical cell, 22.5–31 µm long ($\bar{x}$ = 26.5 µm), slightly swollen at the apex; basal appendages, filiform, 4–7 µm. Colonies growing relatively fast on PDA, reaching 7 cm after 5 days at 25°C, fimbriate, whitish, dense, aerial mycelium on surface, fruiting bodies black; reverse of the culture yellowish white.

Material examined: TAIWAN, Taipei, on living leaves of *Camellia sinensis*, 13 July 1908, Y. Fujikiro, determined by K. Sawada (BPI 406804, ex-holotype); THAILAND, Chiang Mai PROV., Mae Taeng Distr., Ban Pha Deng, Mushroom Research Centre, N 19°17.123' E 98°44. 009', elevation 900 m, rainforest, on living leaves of Camellia sinensis, January 20, 2010, S.S.N. Maharachchikumbura St200110 (MFLU12-0116, epitype designated here) – extype culture MFLUCC 12-0055

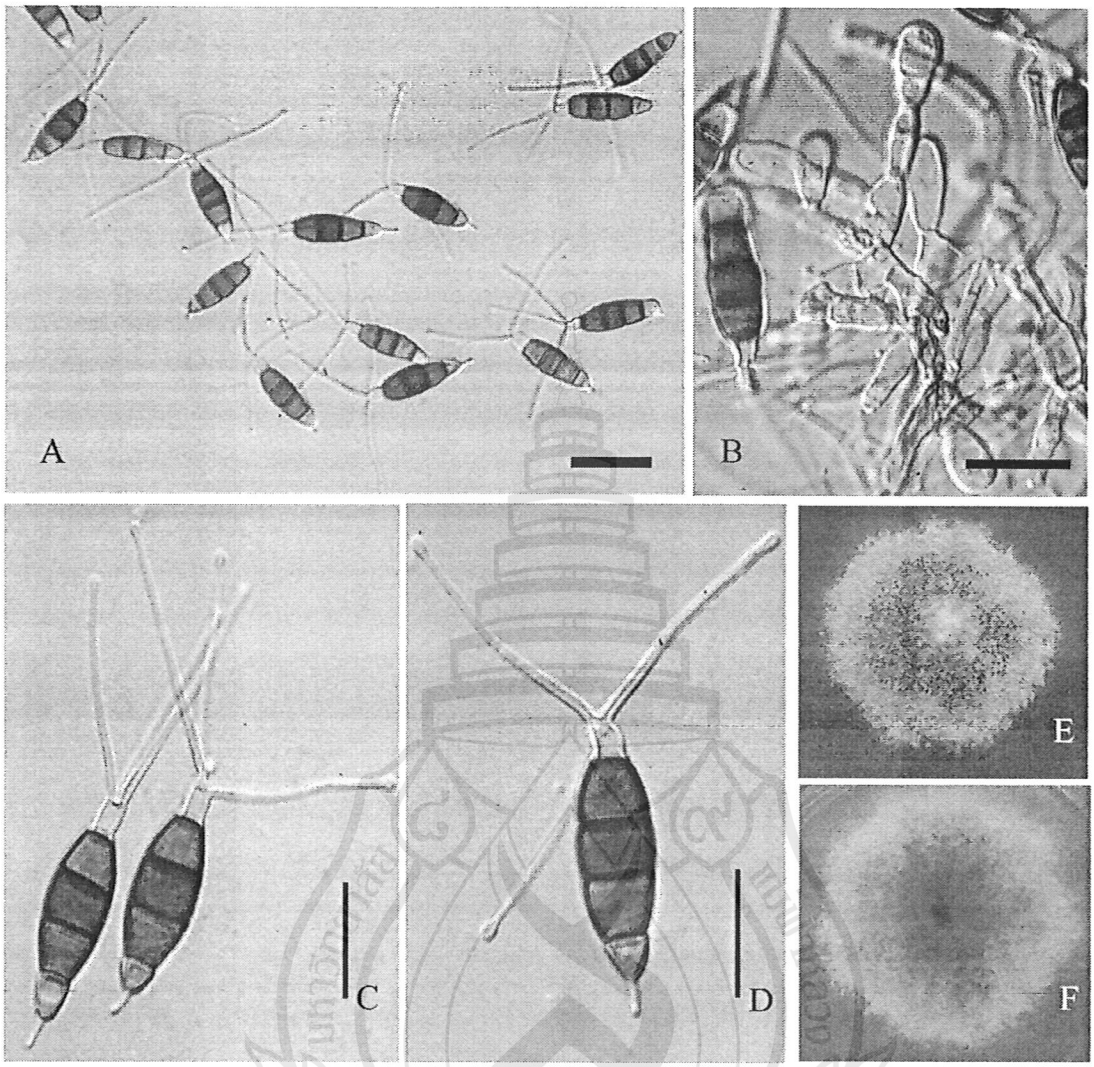

Figure 4.2 *Pestalotiopsis theae* (epitype) A. Conidia in culture. B. Conidiogenous cells. C, D. Conidia. E, F. Colony in culture, E. from above, F. from below. Scale Bars: A-B = 20 µm, C-D=15 µm.

3. *Pestalotiopsis samarangensis* Maharachchikumbura & K.D. Hyde, sp. nov. Fig. 4.3

MycoBank: MB 800178

Etymology: The specific epithet is based on the host species, from which the fungus was isolated.

Conidiomata acervuli, in concentric bands, confluent, erumpent when mature, rounded to oval in outline, epidermal to superficial in origin, basal stroma and lateral wall 2–4 cells thick; cells hyaline to pale brown, textura angularis 100–350 µm wide,

80–150 deep. *Conidiophores* reduced to conidiogenous cells arising within the acervuli. *Conidiogenous* cells discrete, simple, short, filiform. *Conidia* 18–21 × 6.5–7.5 µm ($\bar{x}$ = 20 × 7 µm), fusiform to ellipsoid, broadly clavate, straight to slightly curved, 4-septate, versicoloured; basal cell conical, hyaline, thin and smooth-walled, 3.5–4.8 µm long ($\bar{x}$ = 4 µm); apical cell 2.5–4.6 µm long ($\bar{x}$ = 3.4 µm), conical, hyaline, thin and smooth-walled; three median cells 12.8–13.8 µm long ($\bar{x}$ = 13.5 µm), with thick verruculose walls, dark brown, central cell darker than the cells on either side, the second cell from base pale brown, 4.3–5.3 µm long ($\bar{x}$ = 4.8 µm); third cell darker brown, 3.7–5 µm long ($\bar{x}$ = 4.1 µm; the fourth cell darkest, 4.5–5.3 µm ($\bar{x}$ = 4.9 µm); three apical appendages 12–18 µm long ($\bar{x}$ = 15 µm), tubular, arising from the upper portion of the apical cell; single basal appendage, 3.5–5.2 µm long, filiform.

Colonies on PDA reaching 7 cm diam after 6 days at 25°C, edge entire, whitish aerial mycelium, fruiting-bodies black, gregarious; reverse of culture white.

Habitat/Distribution: Known to cause fruits rots on *Syzygium samarangense*-in Thailand.

Material examined: THAILAND, Chiang Mai Prov., Chiang Mai, on fruits of *Syzygium samarangense*, 20 January 2010, S.S.N. Maharachchikumbura S200110b (MFLU 12-0133; holotype) - ex-type culture MFLUCC 12-0233; ibid., 15 May 2011, S.S.N. Maharachchikumbura S200511 (MFLU 12-0134); Chiang Rai province, Chiang Rai, 15 September 2011, S.S.N. Maharachchikumbura S150911 (MFLU 12-0135).

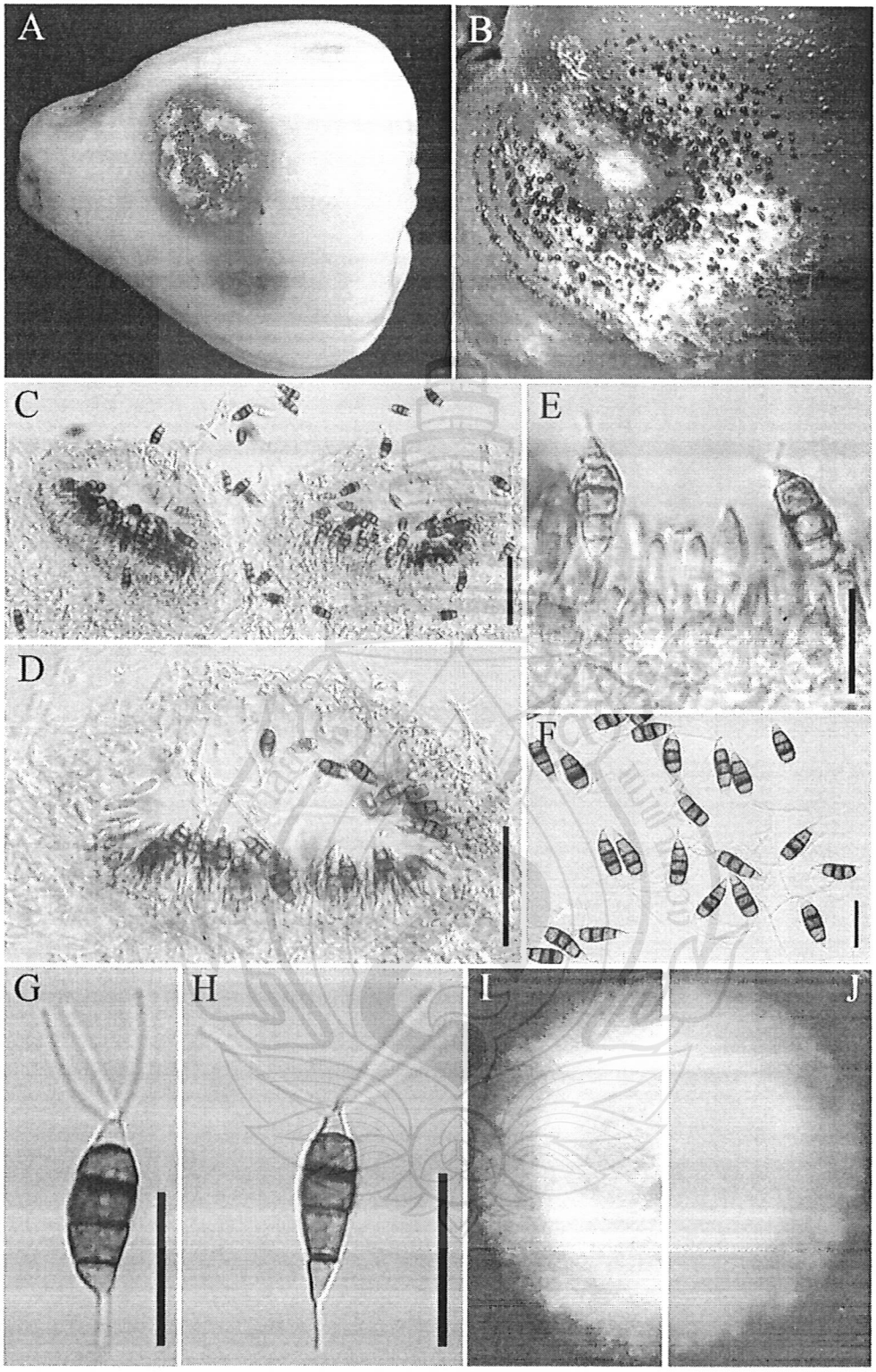

Figure 4.3 *Pestalotiopsis samarangensis* (holotype). A, B. Fruit rot of wax apple C, D. Acervular conidiomata, epidermal to superficial in origin E. Conidiogenous cells, F-H. Versicoloured conidia, I, J. Colony on PDA top (I) reverse (J). Scale bars: C, D = 50 µm, E–H = 20 µm.

4.2 Phylogenetic studies

Of the 10 gene regions (ACT, β-tubulin, CAL, GPDH, GS, ITS, LSU, RPB 1, SSU and tef1) utilized to resolve cryptic *Pestalotiopsis* species, ITS, β–tubulin and tef1 proved to be the better markers. The other gene regions were less useful due to poor success in PCR amplification and/or in their ability to resolve species boundaries. As a single gene tef1 met the requirements for an ideal candidate and functions well for species delimitation due to its better species resolution and PCR success. Although β-tubulin showed fairly good differences among species, a combination of ITS, β-tubulin and tef1 gene data gave the best resolution as compared to single gene analysis.

Table 4.2 Comparison of gene regions used in our study

Region	ITS	β-tub	*tef1*	Combined
PCR success/sequencing success	100%	90%	95%	-
Characters in aligned dataset	546	487	1005	2038
Parsimony-informative characters	78 (14.3 %)	154 (31.6 %)	195 (13 %)	427 (21 %)
Number of bootstrap support >50%	16	24	28	34

Table 4.3 Comparison of gene regions tested but not used in the final phylogenetic studies

Region	Primer/s	PCR success (%)	Sequence success (%)	Species resolution
LSU	LROR /5	100	100	Very low
SSU	NS 1/4	100	100	Very low
Actin	ACT 512F/783R	95	100	Low
GS	GS F1/R1	0	-	-
GPDH	GDF1/GPD2LM	95	100	Low
RPB 1	RPB1 Af/Ac/Cr	60	50	High
CAL	CL 1/2; CAL 228F/737R	70	90	High

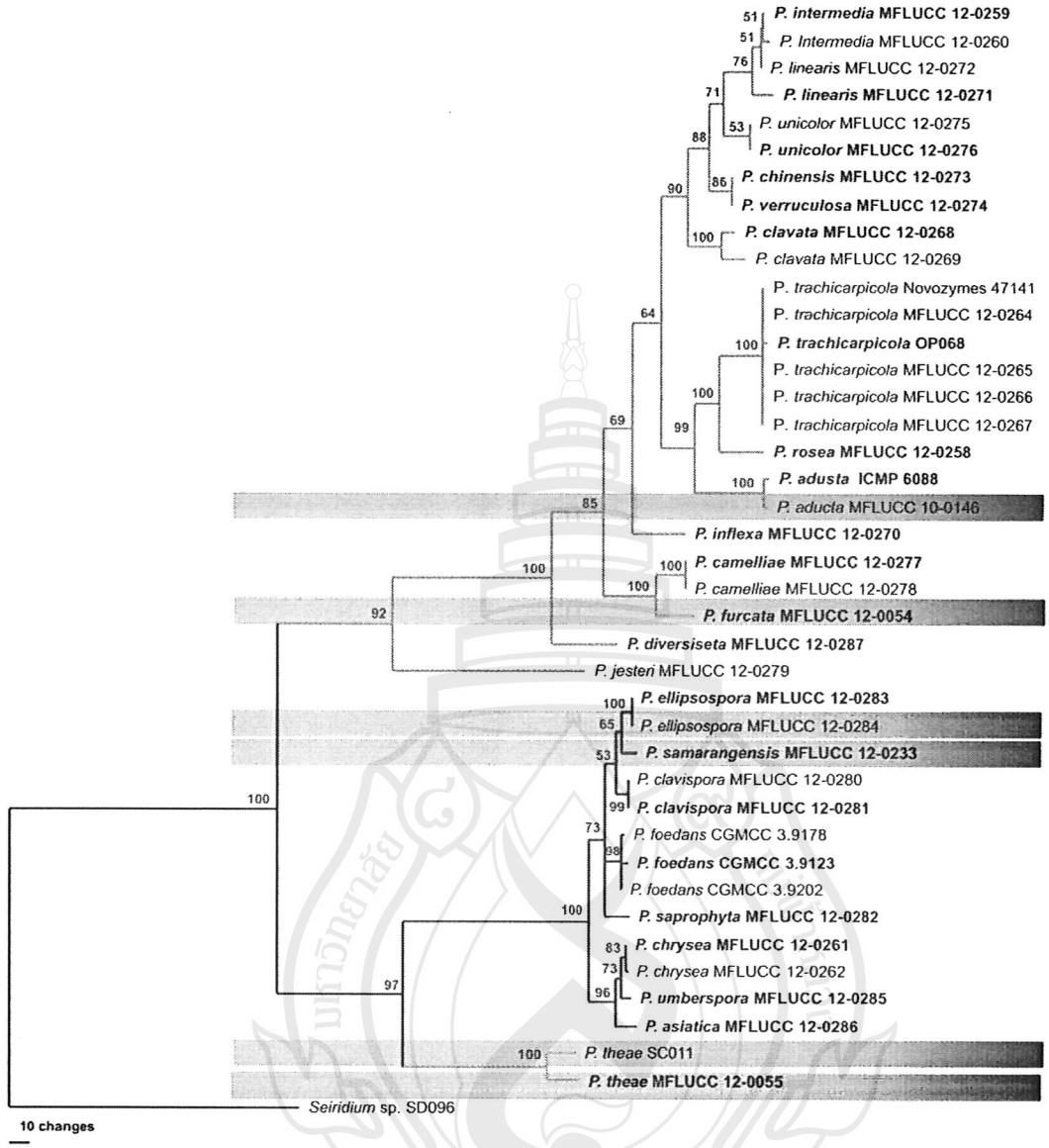

Figure 4.4 Maximum parsimony phylogram generated from combine ITS, β-tubulin and tef1 analysis. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. *Seiridium* spp. was used as the outgroup. Ex-type and ex-epitype sequences are in bold. Thai isolates are in darker.

CHAPTER 5

CONCLUSION

In the present study, we have collected 53 *Pestalotiopsis* isolates throughout Northern of Thailand from different habitats and hosts. Most of the *Pestalotiopsis* isolates were obtained from economically important cash crops such as *Artocarpus heterophyllus*, *Camellia sinensis*, *Musa paradisiaca*, *Phylanthus emblica*, *Saccharum* sp., and *Syzygium samarangense*. Of these important hosts, species isolated from *Camellia sinensis* (tea) are described as the new species *Pestalotiopsis furcata* and one species is epitypified as a *P. theae*. The species causing serious fruit rot disease to the *Syzygium samarangense* is described as a new species *Syzygium samarangensis*.

The morphological characterization of *Pestalotiopsis* isolates clearly show that isolates cluster in to different morphological groups. These morphological groups are highly supported by the sequence data and belong to three distinct clades. These clades corresponded to three conidial types: one clade with pale brown or olivaceous concolorous median conidial cells, the next with versicolorous median conidial cells and the last with dark-coloured concolorous median conidial cells and with knobbed apical appendages. Current research clearly shows that species of *Pestalotiopsis* comprise three species complexes. There are few morphological characters available to distinguish species belonging to these groups. Those morphological important characters include colour of the median cells, the size (length and width) of conidia and the characters within the apical appendages. However the morphological characters are not varied enough to identify species within these complexes. Therefore different gene regions were tested to separate the species within these complexes. Out of tested locus, *tef1* gave the highest species resolution as a single gene and combination of ITS, β-tubulin and *tef1* gave the best resolution. As a result of this study we have published four SCI papers describing 15 new species with three epitypes. Two papers are also in prep and one candidate is training as a PhD student.

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