# FULL REPORT

# Phylogenetics and biochemical diversity of *Pestalotiopsis* in Thailand

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Asst. Prof. Dr. Ekachai Chukeatirote
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# EXECUTIVE SUMMARY

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 i) *Pestalotiopsis* are generally not host-specific; ii) conidial characters vary and species limits overlap; and iii) species arrangements in Steyaert and Guba are problematic. Therefore, 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.* So the present project is mainly focus on the isolation of *Pestalotiopsis* found in different substrata around Northern Thailand especially important plant pathogens and endophytes. These cultures have been maintained and deposited in BIOTEC and MFLU culture collections. These isolates will be correctly named and resolved the taxonomic confusion of the genus with the help of pylogenatically informative morphological characters and molecular data. At the later stages the culture will be used to screen novel secondary metabolites and write papers on *Pestalotiopsis* morphology and molecular phylogeny.

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## 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.* So the present project is mainly focus on the isolation of *Pestalotiopsis* found in different substrata around Northern Thailand specially important plant pathogen and chemically important endopyhtes. These cultures were deposited in BIOTEC and MFLU culture collections. These isolates were correctly named and resolved the taxonomic confusion of the genus with the help of pylogenatically informative morphological characters and molecular data. At the later stages culture will be used to screen novel secondary metabolites and write papers on *Pestalotiopsis* morphology and molecular phylogeny.

**วงศ์วานวิวัฒนาการและความหลากหลายทางชีวโมเลกุลของราในสกุล *Pestalotiopsis* ในประเทศไทย**

## บทคัดย่อ

เชื้อราในกลุ่ม *Pestalotiopsis* มีความสำคัญเนื่องจากเชื้อราในกลุ่มนี้หลายชนิดเป็นเชื้อก่อโรคที่สำคัญกับพืชเศรษฐกิจ และนอกจากนี้ หลายสายพันธุ์ดำรงชีวิตแบบ endophyte ที่มีความสามารถในการผลิตสารเมทาบอไลต์ได้หลากหลายชนิด อย่างไรก็ตาม การจัดจำแนกสายพันธุ์เชื้อราในกลุ่มนี้ยังค่อนข้างสับสน เนื่องจาก 1) เชื้อรา *Pestalotiopsis* ไม่มีความจำเพาะเจาะจงต่อสิ่งมีชีวิตเจ้าบ้านที่ชัดเจน 2) ลักษณะของ conidia มีความหลากหลาย และเหมือนกันในบางชนิด และ 3) การจัดจำแนกโดยวิธีของ Steyaert และ Guba พบว่าไม่ถูกต้องนัก เนื่องจากใช้เกณฑ์การจัดจำแนกโดยอิงกับสิ่งมีชีวิตเจ้าบ้านเป็นหลัก ดังนั้น จำนวนสายพันธุ์ของเชื้อรา *Pestalotiopsis* น่าจะมีน้อยกว่าที่ได้มีการบันทึกไว้

ด้วยเหตุผลดังกล่าว การตรวจสอบชนิด และการจัดจำแนกเชื้อรา โดยเฉพาะการพิสูจน์สายพันธุ์มาตรฐานเพื่อนำมาใช้อ้างอิง (epitypification) จึงมีความสำคัญ และในปัจจุบัน นอกเหนือจากการใช้ข้อมูลทางด้านสัณฐานวิทยาแล้ว การใช้ข้อมูลทางด้านพันธุกรรมก็เป็นสิ่งที่สำคัญและหลีกเลี่ยงไม่ได้ในการระบุชนิดของเชื้อรา

งานวิจัยนี้มีจุดมุ่งหมายที่จะแยกและคัดเลือกเชื้อรา *Pestalotiopsis* จากแหล่งตัวอย่างต่างๆ ทางภาคเหนือของประเทศไทย โดยมุ่งเน้นไปที่สายพันธุ์สำคัญที่สามารถก่อโรคพืช และสายพันธุ์ที่เป็น endophyte สายพันธุ์ของเชื้อรา *Pestalotiopsis* ที่แยกได้จากงานวิจัยนี้จะถูกเก็บไว้ในคลังจุลินทรีย์ของมหาวิทยาลัยแม่ฟ้าหลวง และของศูนย์พันธุวิศวกรรมและเทคโนโลยี เพื่อใช้ในการศึกษาด้านอนุกรมวิธานตลอดจนการศึกษาสารเมทาบอไลต์ที่มีความสำคัญในด้านต่างๆ อาทิ การเกษตร และอุตสาหกรรม

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# CHAPTER 1
# INTRODUCTION

## 1.1 Background

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. It is hypothesized 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.

## 1.2 Objectives of this study

- To document the diversity of *Pestalotiopsis* species mainly in northern Thailand and to isolate strains for conservation in the MFU and BIOTEC Culture Collections
- To construct phylogenetic trees to examine the inter-relationships of *Pestalotiopsis* species and to determine their relationships
- To use a polyphasic approach and epitypification to stabilize the nomenclature of *Pestalotiopsis* species

## 1.3 Scope of work

The project was carried out as follows:

Part A: Acquisition of *Pestalotiopsis* strains and documentation of diversity in mainly northern Thailand

For this part, samples of living and decaying plants were collected in forests and other habitats in Thailand and thus used to identify *Pestalotiopsis* species. The fungal isolates obtained were placed in the BIOTEC Culture Collection for future studies. The primary objective of this part is to acquire strains for the polyphasic study and to further our knowledge of Thai *Pestalotiopsis* species.

Part B: Polyphasic approach to study of *Pestalotiopsis* species

This section aimed to focus on the morphology (shape, size, colour, septation, appendages) of collections, cultural characteristics and growth rates in different media, plant pathogenicity testing, and metabolite profiling as well as multigene analysis.

Part C: Epitypification of important species

Some certain isolates of *Pestalotiopsis* were selected and used to epitypify species. The strains selected included those important species based on their mode of life and taxonomic purpose.

Section D: Chemical profiles of important species

We expect to establish chemical profiles for strains of some species in the genus and work further on chemicals that look unique or chemically interesting.

# CHAPTER 2
# LITERATURE REVIEWS

*Pestalotoiopsis* species are anamorphic fungi (coelomycetes) producing an appendage-bearing conidia currently classified in the family Amphisphaeriaceae (Barr 1975, 1990; Kang et al. 1998, 1999). Based on molecular data, it has been shown that *Pestalotiopsis* fungal group is monophyletic (Jeewon et al. 2002, 2003, 2004). Several species of *Pestalotiopsis* are common in tropical and temperate ecosystems and may cause plant disease (Das et al. 2010). They are also often isolated as endophytes (Liu et al. 2006; Wei et al. 2007; Watanabe et al. 2010), or occur as saprobes (Yanna et al. 2002; Hu et al. 2007; Liu et al. 2008a). This genus has received much attention from the scientific community. However, this not because of its pathogenic nature (Hyde and Frohlich 1995; 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. 1996a, 2002; Ding et al. 2008a, b; Aly et al. 2010; Xu et al. 2010). This Chapter aims to present an overview of this fungal genus including i) historical aspects; ii) morphological and molecular studies; iii) life mode of taxa; iv) species numbers; and v) biochemical production by selected species.

## 2.1 Historical background

In 1839, De Notaris introduced the genus *Pestalotia* De Not. based on the generic type *Pestalotia pezizoides* De Not., which occurred on the plant leaves of *Vitis vinifera* in Italy. This mould species is characterized by 6-celled conidia with four deeply olivaceous central cells, distosepta, hyaline terminal cells and simple or branched appendages arising from the apex (Figure 2-1).

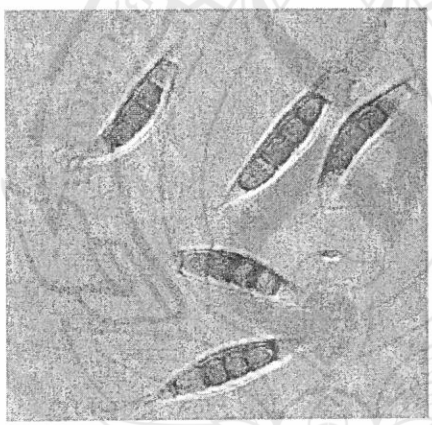

Figure 2-1 Conidia of *Pestalotia pezizoides* De Not. BPI0406483

In 1949, Steyaert revised *Pestalotia* and reclassified this genus into three groups based on the conidial types. Two new genera were also introduced: *Truncatella* Steyaert for 4-celled conidial forms and *Pestalotiopsis* Steyaert for the 5-celled forms, whereas the 6-celled forms remained in *Pestalotia*. *Pestalotia* was considered to be a monophyletic genus and it was suggested that the type species could be distinguished from *Pestalotiopsis* by its cupulate conidiomata and distoseptate median cells. Besides, Steyaert (1949) further classified *Pestalotiopsis* into additional sections based on the number of apical appendages including the Monosetulatae, Bistulatae, Trisetulatae and Multisetulatae. Conidia with a single setulae (apical appendage) were included in the Monosetulatae, which was further

divided into forms with simple and branched setulae. Conidia with two setulae or on average two setulae were included in the Bistulatae. Conidia with three setulae or on average three setulae were included in the Trisetulatae. Conidia with more than three setulae were included in the Multisetulatae. At that time, 46 species of *Pestalotiopsis* were provided with descriptions and *Pestalotiopsis guepinii* (Desm.) Steyaert was considered to be the type species of this newly introduced genus. *Pestalotiopsis guepinii* is morphologically characterized by 4-euseptate and fusiform conidia with a hyaline basal cell. Steyaert's introduction of the genus *Pestalotiopsis* was not supported by Moreau (1949), Servazzi (1953) and Guba (1956, 1961). Steyaert (1953a, b, 1961, 1963), however, published further evidence in support of his new genus with answers to the criticisms made by others.

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). However, morphological characters used to differentiate species of *Pestalotiopsis* and similar genera are limited (Hu et al. 2007). It has been shown that the morphological characters used 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). Satya and Saksena (1984) observed *Pestalotiopsis glandicola* (Castagne) Steyaert and *P. versicolor* var. *polygoni* and found that the intensity of the median cells varied with culture and host and concluded that colour of median cells cannot be used to judge their taxonomic position.

Jeewon et al. (2003) and Tejesvi et al. (2009) compared morphology with sequence data and showed that species of *Pestalotiopsis* display considerable diversity in morphology and that isolates grouped together based on similarities in conidial morphology. Hu et al. (2007) found that conidial characters such as conidial length, median cell length, conidial width and colour of median cells were stable characters within Pestalotiopsis; however, the length of the apical and basal appendages varied. Jeewon et al. (2003) evaluated the morphological characters that could be used to differentiate species of *Pestalotiopsis*. They suggested that melanin granule deposition within the cell matrix providing pigmentation to the median cells has taxonomic value; this agreed with the findings of Griffiths and Swart (1974a, b) which suggested that the colour of median cells was useful for distinguishing species of *Pestalotiopsis*. Tejesvi et al. (2009) also agreed that species of *Pestalotiopsis* can be distinguished on the basis of morphological characters rather than host-specificity or geographical location. 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*. However the pigmentation can be effected by environmental conditions, different stages of spore maturity and the observer's expertise (Liu et al. 2010a), hosts, medium, and even different generations through subculturing (Purohit and Bilgrami 1969; Satya and Saksena 1984; Hu et al. 2007). The pigmentation of the median cell however, can be stable even within a successive subculture; when using standard conditions culture on autoclaved carnation leaf segments (Liu et al. 2010a).

Presently, according to the Index Fungorum database (available at the following website, http://www.indexfungorum.org/names/names.asp), there are 235 *Pestalotiopsis* names, while in MycoBank (www.mycobank.org/mycotaxo.aspx) 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).

**2.2 Morphological characteristics**

Conidial morphology is the most widely used taxonomic character for the genus *Pestalotiopsis* (Figure 2-2). Most species are divided into different groups based on the size of the conidia. The length and width are good taxonomic markers for the genus and stable within the different media and the generations in most cases (Hu et al. 2007). Colour of the median cells is still a widely used character, and all species separate into three groups based on this such as concolorous, versicolorous umber olivaceous and versicolorous fuliginous olivaceous. Molecular evidence indicates that it is more precise to group species according to concolorous and versicolorous rather than the above three groups (Jeewon et al. 2003). 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.

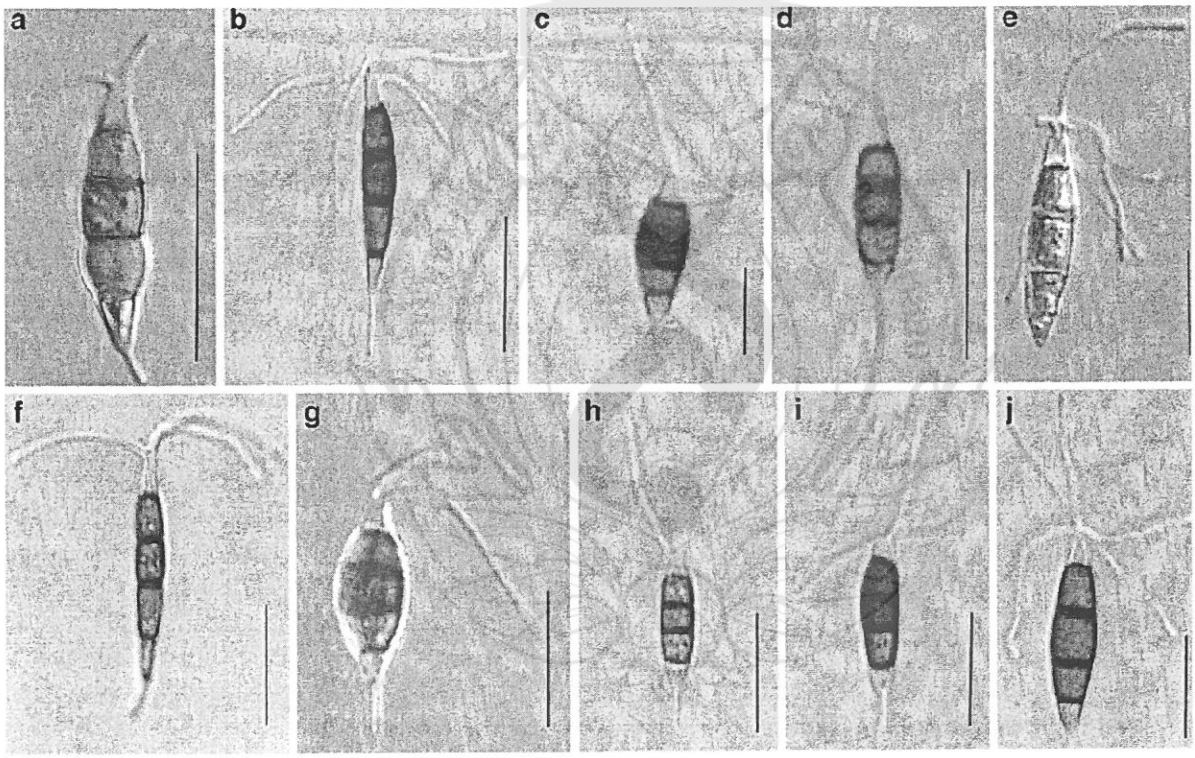


Figure 2-2 Examples of conidial characters commonly used for *Pestalotiopsis* species identification. Colour of the median cells: light concolorous (a), dark concolorous (b), and versicolorous (c). Size of the conidia: small conidia (d), large conidia (e), relatively long conidia (f), and relatively broad conidia (g). Number of apical appendages: two apical appendages (h), three apical appendages (i), and five apical appendages (j). Scale bars = 20 μm.

## 2.3 Molecular data

Hu et al. (2007) showed that the ITS gene is less informative than the β-tubulin gene in differentiating endophytic species of *Pestalotiopsis* in *Pinus armandii* and *Ribes* spp. Thus, Hu et al. (2007) pointed out that the β-tubulin genes resolves *Pestalotiopsis* phylogeny better than the ITS gene. Besides, a combination of both the β-tubulin and ITS genes provided better phylogenetic resolution, and they suggested that at least two genes should be used to resolve the phylogeny of species of *Pestalotiopsis*. However, Liu et al. (2010a) disagreed with Hu et al. (2007) concerning the ITS region as being less informative when compared to the β-tubulin region. They indicated that proper analysis and alignment of the ITS region can be a useful character in grouping *Pestalotiopsis* to different types of pigmentation, which can be used as a key character for the phylogeny of the species. Random amplification of polymorphic DNA (RAPD) can also be used to detect genetic diversity in species of *Pestalotiopsis* (Tejesvi et al. 2007a). Tejesvi et al. (2009) showed that the ITS region is more informative than internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP). They used five restriction enzymes (Alu I, Hae III, Ava II, Hpa II and Taq I) in their ITS-RFLP analysis and showed that ITS-RFLP profiles were distinctly different in *P. virgatula* (Kleb.) Steyaert and P. theae (Sawada) Steyaert and intraspecific polymorphism highly variable in *P. microspora* (Speg.) G.C. Zhao & N. Li. Based on the ITS sequence, pathogenic and endophytic strains clustered into distinct groups and these clusters were irrespective of the host, parts of the host or location.

## 2.4 Mode of life of *Pestalotiopsis*

Several *Pestalotiopsis* species are pathogens but they are not highly host-specific and taxa may have the ability to infect a range of hosts (Hopkins and McQuilken 2000; Keith et al 2006). 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 (Pirone 1978; Kwee and Chong 1990; Xu et al. 1999; Tagne and Mathur 2001; Sousa et al. 2004; Espinoza et al. 2008). Pirone (1978) considered that species of *Pestalotiopsis* are weak or opportunistic pathogens and may cause little damage to ornamental plants; however, Hopkins and McQuilken (2000) pointed out that some species of *Pestalotiopsis* may cause serious damage to pot grown plants and the number of known infected plant species is generally increasing. Pathogenic species of *Pestalotiopsis* initially make contact with the host where the infection occurs (inoculum), probably by means of the conidia or fragmented spores (Espinoza et al. 2008). These inocula may survive during harsh weather conditions and may cause primary infections. Secondary inoculum produced on diseased tissue may cause secondary infections and increase the severity of the disease. The source of the inoculum can be wild plantations (Keith et al. 2006), flowers (Pandey 1990), crop debris, disease stock plants, used growing media, soil and contaminated nursery tools (McQuilken and Hopkins 2004), splashed water droplets (Hopkins and McQuilken 1997; Elliott et al. 2004) and also spores in the air (Xu et al. 1999).

*Pestalotiopsis* species have also constantly been isolated as endophytes from plant tissues (Wei and Xu 2004; Liu et al. 2006; Wei et al. 2005, 2007; Tejesvi et al. 2009; Watanabe et al. 2010).

Species have also been recorded as saprobes (Guba 1961; Wu et al. 1982; Agarwal and Chauhan 1988; Yanna et al. 2002; Liu et al. 2008a) where they are recyclers of dead plant materials (Okane et al. 1998; Osono and Takeda 1999; Tokumasu and Aoiki 2002). This fungal group has rarely known to cause disease in humans (Sutton 1999).

Some species of *Pestalotiopsis* have also been isolated from extreme environments and these isolates have been shown to produce bioactive metabolites (Tejesvi et al. 2007b). *Pestalotiopsis microspora* isolated from *Taxus* sp. from the foothills of Himalayas produced taxol (Strobel et al. 1996a), *P. microspora* isolated from Sepik River drainage system in Papua New Guinea produced isopestacin (Strobel et al. 2002) and *Pestalotiopsis* sp. obtained from the gut of a grass hopper (Chondracris rosee) produced two new phytotoxic g-lactones, pestalotines A and B (Zhang et al. 2008).

**2.5 Useful metabolites from *Pestalotiopsis***

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 (Aly et al. 2010; Xu et al. 2010). Several researches have reported that *Pestalotiopsis* species can produce an enormous number of secondary metabolites that may have medicinal, agricultural and industrial applications. The bioactive compounds produced include 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
# METHODOLOGY

## 3.1 Collection of the samples

*Pestalotiopsis* isolates were collected from the leaf spots and diseased fruits of various hosts in Chiang Mai and Chiang Rai. To induce sporulation, diseased leaves were placed in sterilized Petri dishes with moistened sterile filter paper. Acervuli were rehydrated in water for examination and sectioning. Specimens were then examined under a Leica MZ16A stereo microscope and fine forceps were used to move one or two acervuli from the specimen, which were mounted in water and lactic acid. Observations and microphotographs were made using the light microscope (Nikon Ei800 and Leica DM3000). Hand sections were taken with a razor blade and thin (4–10 μm); the sections were transferred to a drop of water, a drop of lactic acid or a drop of cotton blue for examination and photography. The type cultures obtained from the CBS and other collaborating institutes were also loaned and used for comparative study.

## 3.2 Morphological examination

Single spore isolation was carried out as described by Chomnunti et al. (2011). A conidiomata was immersed in 300 μl of sterile distilled water on a slide and left for a few minutes so that the conidia were discharged. A conidial suspension was made, small drops were placed on water agar (WA) in Petri dishes and kept at room temperature for 8–12 h for conidia to germinate; single germinating conidia were transferred to potato dextrose agar (PDA) plates. The plates were incubated at 25°C for 7 to 10 days. Colonies grown on PDA were transferred to PDA slants, and stored at 4°C for further study. Sporulation was induced by placing sterilized carnation leaves on the surface of PDA with growing mycelia. All microscopic measurements were observed and recorded and 30 conidial measurements were taken for the isolate.

## 3.3 DNA extraction and PCR

Total genomic DNAwas extracted from fresh cultures using a modified protocol of Guo et al. (2000). Fresh fungal mycelia (500 mg) was scraped from the margin of a PDA plate incubated at 25°C for 7 to 10 days and transferred into a 1.5 ml centrifuge tube with 100 μl 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 were ground using a glass pestle for 5 min and an extra 500 μl 2X CTAB preheated (60 °C) was added and incubated in a 65 °C water bath for 30 min with occasional shaking. 500 μl of phenol:chloroform (1:1) was added to each tube and shaken thoroughly to form an emulsion. The mixture was spun at 11,900g for 15 min at 25 °C in a microcentrifuge and the supernatant phase decanted into a fresh 1.5 ml tube. Supernatant containing DNAwas re-extracted with phenol: chloroform (1:1) at 4 °C until no interface was visible. 50 μl of 5 M potassium acetate was added into the supernatant followed by 400 μl of isopropanol and inverted gently to mix. The genomic DNAwas precipitated at 9,200g for 2 min at 4 °C in a microcentrifuge. The DNA pellet was washed with 70 % ethanol twice and dried using SpeedVac® (AES 1010; Savant, Holbrook, NY, USA) until dry. The DNA pellet was then resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The ITS sequences including 5.8S region were amplified using primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). Partial β-tubulin gene region was amplified with primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5'

ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997) and tef1 was amplified using the primer pairs EF1-526F (5'-GTCGTYGTYATY GGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRATACCACCRATCTT-3') (Rehner 2001). In addition to above three gene regions, selected LSU, SSU, actin, GS, GPDH, RPB1and CAL regions were amplified using primer pairs listed in Table 3-1.

Table 3-1 Primers used in this study

| DNA sequence | Primers |
|---|---|
| **LSU** | LR OR /5(Rehner and Samuels 1994; Moriya et al. 2005) |
| **SSU** | NS 1/4 (White at al. 1999) |
| **ACT** | ACT 512F/783R (Carbone and Kohn 1999) |
| **GS** | GS F1/R1 (Stephenson et al. 1997; Guerber et al. 2003) |
| **GPDH** | GDF1/GPD2LM (Myllys et al. 2002; Guerber et al. 2003) |
| **RPB1** | RPB1Af/Ac/Cr (http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.pdf) |
| **CAL** | CL 1/2; CAL 228F/737R (Carbone and Kohn 1999; O'Donnell et al. 2000) |

PCR was then performed with the 25 μl reaction system containing 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), 1.0 μl of DNA template. The thermal cycling program was as follows: For ITS an initial denaturing step of 95°C for 3 min, followed by 35 amplification cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 90 s and a final extension step of 72°C for 10 min. For β-tubulin PCR conditions were 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, an 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 LSU, SSU, Actin, GS, GPDH, RPB 1and CAL regions were tested under different optimal conditions (not shown). The PCR products were verified by staining with Goldview (Guangzhou Geneshun Biotech, China) on 1% agarose electrophoresis gels.

### 3.4 Phylogenetic analysis

DNAStar and SeqMan were used to obtain consensus sequences from sequences generated fromforward and reverse primers. Single locus dataset and combination of multi-locus dataset of three gene regions were aligned using CLUSTALX (v. 1.83) (Thompson et al. 1997). The sequences were further aligned using default settings of MAFFTv6 (Katoh et al. 2008; mafft.cbrc.jp/alignment/server/) and manually adjusted using BioEdit (Hall 1999) to allow maximum alignment and minimum gaps.

A maximum parsimony analysis (MP) was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees were set up to 5,000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) were

calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees was 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 & Hasegawa 1989) were performed to determine whether the trees inferred under different optimality criteria were significantly different. Trees were viewed in Treeview (Page 1996).

**3.5 Analysis of fungal metabolites**

Four *Pestalotiopsis* isolates, namely MFLUCC 10-0140, 10-0141, 11-0565, and 12-0233, were cultured on potato dextrose agar (PDA) at 30°C for 14 days. To extract the metabolites, the fungal cultures were blended with 30ml ethyl acetate using a blender. The suspension was incubated at room temperature for 24h to separate the solid and solvent phase. The solvent phase was collected and air-dried yielding the crude extracts stored at 4°C until use.

The fungal metabolites were then subject to further analysis using GC/MS (Agilent 6890 / HP 5973). The conditions used were as follows: a HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30m × 0.25 mm i.d., and 0.25 μm film thickness), injector and detector temperature at 70°C and 180 °C with a rate of 2°C/min, and ionization energy of 70 eV. Purified helium gas was used as carrier gas at a constant flow rate of 1 ml/min.

In addition, the crude extracts were also tested for their antimicrobial activity using the disc diffusion assay. The testing microbes used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Bacillus subtilis*, *Candida albicans*, and *Candida utilis*.

# CHAPTER 4
# RESULTS AND DISCUSSION

## 4.1 *Pestalotiopsis* in GenBank

At present, there are 235 and 232 *Pestalotiopsis* names deposited in the Index Fungorum and in MycoBank, respectively. The reason for the large number of names is historical and may not reflect the actual number of species (Jeewon et al. 2004). Similarly to other pathogenic fungal genera, *Pestalotiopsis* species were historically named according to the host from which they were first observed. If a new host occurrence was found, a new species was described. It is therefore strongly thought that the actual number of *Pestalotiopsis* may be fewer than 50. There is also an additional drawback in taxonomic study due to the limited availability of type cultures. It is also hypothesized that the use of gene sequences (particularly the ITS, rRNA gene, etc.) might cause a difficulty to clarify species names reliably. An attempt was therefore carried out to determine if there is a confusion in phylogenetic relationship of *Pestalotiopsis* when using the ITS sequence data available in GenBank database.

Initially, the ITS sequences of 48 species of *Pestalotiopsis* were downloaded from GenBank (Table 4-1) and aligned using Clustal X. The alignment was optimized manually to allow maximum alignment and maximum sequence similarity. Gaps were treated as missing data. Phylogenetic analysis was then carried out based on the aligned dataset using PAUP* 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved and figured in Treeview (Figure 4-1).

Table 4-1 Lists of the ITS sequences of *Pestalotiopsis* used in this study. The GenBank accession numbers were provided and type species were marked in bold.

| Species | Accession numbers | Species | Accession numbers |
|---|---|---|---|
| *P. clavispora* | AY682928 | *P. neglecta* | EU342212 |
| *P. clavispora* | AY924263 | *P. neglecta* | FJ037759 |
| *P. clavispora* | DQ812921 | *P. neglecta* | GU595050 |
| *P. clavispora* | GU362540 | ***P. pallidotheae*** | **AB482220** |
| *P. disseminate* | AY687870 | *P. photiniae* | AY682937 |
| *P. disseminata* | DQ001000 | *P. photiniae* | AY682943 |
| *P. disseminata* | DQ195782 | *P. photiniae* | AY682946 |
| *P. disseminata* | EF055196 | *P. photiniae* | DQ812939 |
| *P. disseminata* | HM535728 | *P. photiniae* | EU030345 |
| *P. disseminata* | HM535738 | *P. virgatula* | AY924281 |
| *P. disseminata* | HM535752 | *P. virgatula* | DQ812936 |
| *P. disseminata* | HM535759 | *P. virgatula* | DQ813436 |
| ***P. hainanensis*** | **GQ869902** | *P. virgatula* | HM535725 |
| ***P. jesteri*** | **AF377282** | *P. vismiae* | EF055220 |
| ***P. kunmingensis*** | **AY373376** | *P. vismiae* | EF055221 |
| *P. microspora* | AY924278 | *P. vismiae* | EF055222 |
| *P. microspora* | AY924285 | *P. vismiae* | EU273510 |
| *P. microspora* | DQ000996 | *P. vismiae* | EU326213 |
| *P. microspora* | FJ459945 | *P. vismiae* | HM535710 |
| *P. microspora* | FJ478120 | *P. vismiae* | HM535751 |
| *P. microspora* | FJ487936 | *P. theae* | AY924265 |
| *P. neglecta* | AY682930 | *P. theae* | DQ812917 |
| *P. neglecta* | DQ812935 | *P. theae* | EF423551 |
| *P. neglecta* | EF055209 | *Truncatella angustata* | DQ093715 |

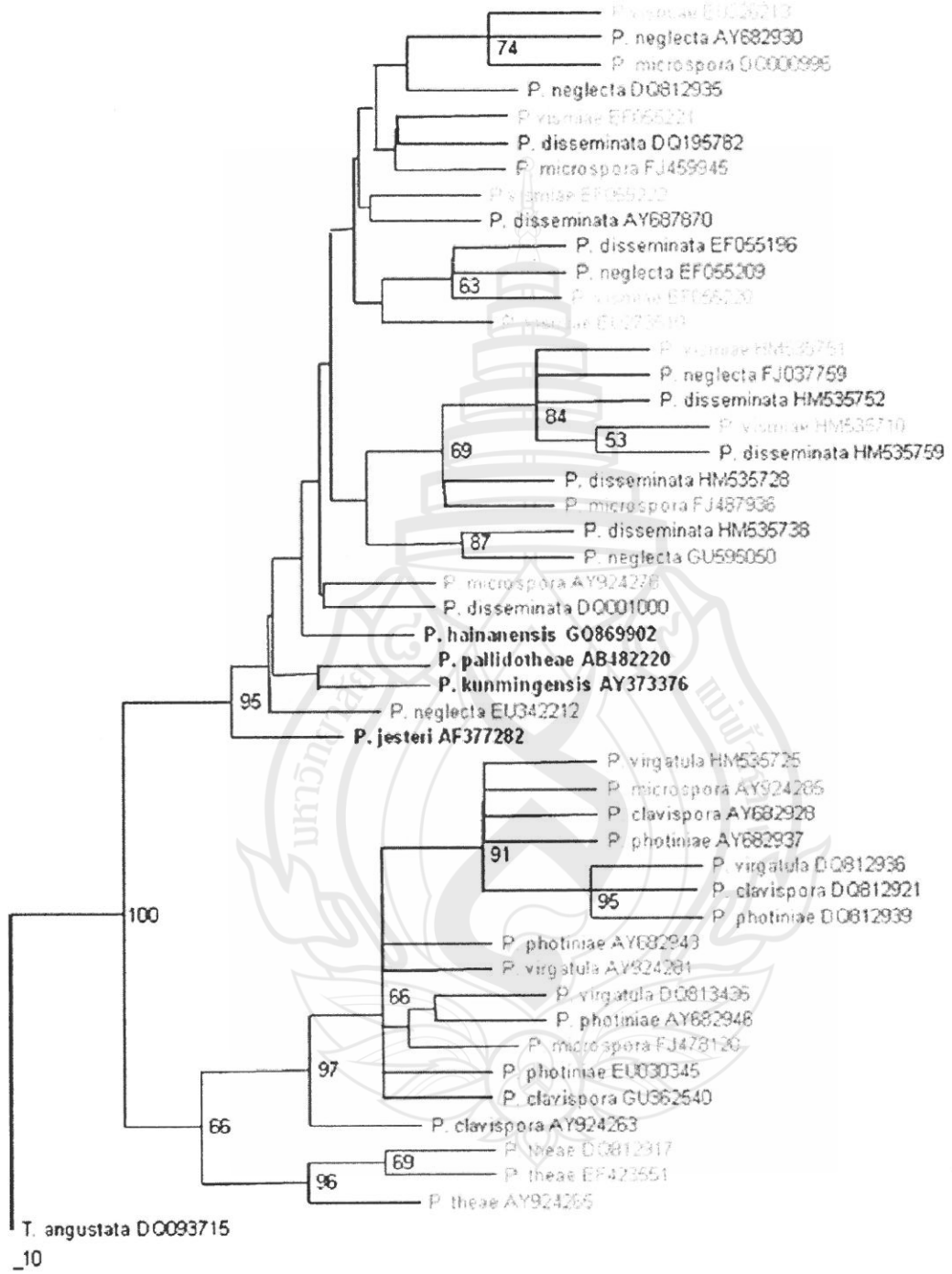


Figure 4-1 Maximum parsimony phylogram generated from ITS sequence analysis of *Pestalotiopsis* species. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Type sequences of *Pestalotiopsis* were in black and bold.

Based on key morphological characteristics of *Pestalotiopsis* species used in taxonomy, pigmentation is a highly weighted character in the lineage of species of *Pestalotiopsis* and which can be differentiated into two main groups based on the colour of the median cells (Jeewon et al. 2003; Liu et al. 2010a). This criterion is in agreement with the species concept proposed by Guba (1961) and Steyaert (1949), based on versicolorous median cells as well as those species characterized by concolorous median cells. Jeewon et al. (2003) showed that species such as *P. theae* with dark colored concolorous median cells with knobbed apical appendages should be included in the versicolorous group. Jeewon et al. (2003) argued that the arrangement of Guba (1961) that groups the versicolorous assemblages of species into umber olivaceous and fuliginous olivaceous depends on the color intensity of the median cells. This statements was followed by Liu et al. (2010a) and they proposed the use of "brown to olivaceous" and "umber to fuliginous" colour median cells as valid for the taxonomy of the genus instead of the use of the "concolorous" and "versicolor" median cells grouping system proposed by Steyaert (1949) and Guba (1961).

As a result, *Pestalotiopsis clavispora*, *P. disseminata*, *P. microspora*, *P. neglecta*, *P. photiniae*, *P. theae*, *P. virgatula* and *P. vismiae* can be divided into two groups depending mainly on the colour of the median cells. One group is the versicolorous group, consisting of *P. clavispora*, *P. photiniae* and *P. virgatula*, and dark concolorous median cells with knobbed apical appendages containing the *P. theae* group. The other group consists of species with concolorous median cells (i.e., *P. disseminata*, *P. microspora*, *P. neglecta* and *P. vismiae*.

According to Figure 4.1, an example of the confusion resulting from molecular data is present. Almost all strains that separate into two main clades depend on the concolorous and versicolor system, and only *P. microspora* strains AY924295 and FJ478120 cluster in the wrong clade. However, within the two main groups, the respective species distributions are scattered and most species overlap with each other. Because of the limitation of characters used to differentiate species (Hu et al. 2007) and many overlapping characters (Sutton 1980), identification to species in *Pestalotiopsis* is presently difficult. For an example according to Guba (1961), *P. disseminata*, *P. microspora*, *P. neglecta* and *P. vismiae* within the concolorous group have the same conidia size (18–26×5–8 µm). *Pestalotiopsis vismiae* can be differentiated as it has two apical appendages, while *Pestalotiopsis microspora* is differentiated from *P. neglecta* and *P. dissementa* by the length of the apical appendages. *Pestalotiopsis neglecta* and *P. dissementa* can be distinguished from each other only by the shape of the conidia. Most of above characters vary when in culture and following successive subculturing (Hu et al. 2007). Within the versicolorous group, *P. clavispora* and *P. photiniae* are morphologically very similar (conidia size 19–26×6–8.5 µm), while *P. virgatula* can be differentiated from *P. clavispora* and *P. photiniae* by its relatively small conidia (17–23×6–8 µm). However, these characters overlap and thus identification to these species is rather difficult. For this reason, naming of species is difficult and highly subjective and many sequences for *Pestalotiopsis* deposited in GenBank are likely to be wrongly named.

The species of *Pestalotiopsis* sequenced with ITS having a high PCR and sequence success rate (S. Maharachchikambura, personal communication). However, the ITS could not fulfill the role as candidate gene for species discrimination, as the data did not have high variation between species. Thus, possible cryptic taxa could not be discriminated from one another. *Pestalotiopsis* species identification depends mostly on the conidial characteristics. It has been shown that most of the key conidial characters used in species level separation are not stable and vary with host range, generation, culture and other environmental conditions. Thus same species can be identified as two different species depend on the above conditions. Thus species wrongly named and misidentified sequence may be deposited in GenBank (same gene could be deposited into the database many times with different names) and

different version of the same gene could be submitted many times with different accession number. Furthermore there are only a few type cultures and, therefore, it is impossible to use gene sequences in GenBank to clarify species names reliably. Therefore epitypification with molecular work is needed to understand the species and what distinguishes them. Re-examination of type materials, establishment of epitypes with living cultures and investigation of new protein coding gene region with higher resolution is needed to develop a strong species-based taxonomic system for the genus *Pestalotiopsis*.

**4.2 Diversity of *Pestalotiopsis* in Thailand**

In this project, 54 *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 are 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 diameter.

The species identification was performed in accordance with the Monograph of *Pestalotia* and *Monochaetia* (Guba 1961) and Steyaert (1961). Initially, the fungal species were grouped based on the colour of the median cells (i.e., concolorous and versicolorous). Then the isolates were divided using the size of the conidia and subsequently further divided based on the length of apical appendages. Furthermore some isolates were identified to species level using the presence of knobbed apical appendages, the position of apical appendages arise from apical cell, number of apical appendages, branching nature of apical cell and presence or absence of basal appendages (Figure 4-2). However, it should be noted that there are still many isolates whose identities remained unsolved.

Table 4-2 shows all isolates of *Pestalotiopsis* obtained from this study. At present, the isolates were determined morphologically and it was found that these fungal strains were separated into different morphological group. Of 53 isolates, only 12 isolates were able to identify at species level. Interestingly, it should be noted that two species were described as new species, namely *Pestalotiopsis furcata* and *P. samarangensis* and one species was also epitypified and designated as *P. theae*.

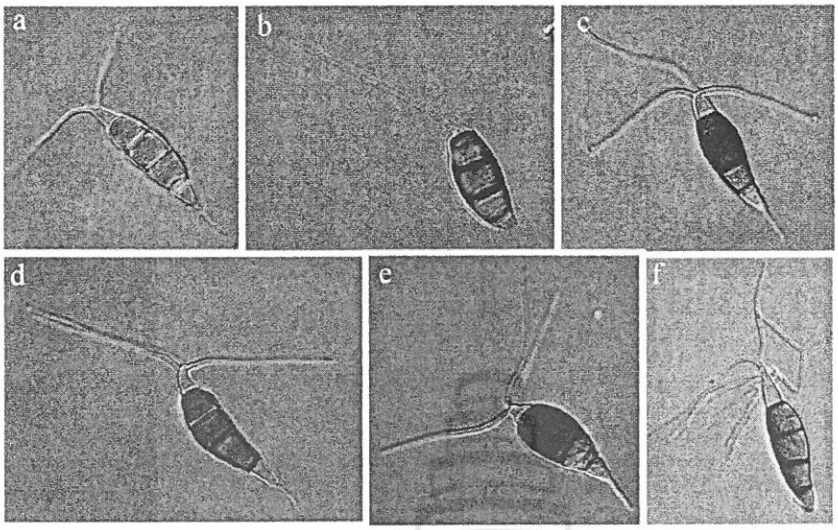


Figure 4-2 Morphological characteristics used to identify *Pestalotiopsis* isolates. Key criteria include: (a) isolates having light concolorous median cells; (b) isolates with single apical appendages; (c) isolates with knobed apical appendages; (d) isolates having dark concolorous median cells; (e) isolates having versicolorous median cells; and (f) isolates with branched apical appendages.

Table 4-2 List of *Pestalotiopsis* isolates collected during this study

| Isolates | Species identified | Collection site |
|---|---|---|
| 10-0146 | *Pestalotiopsis adusta* | Chiang Rai |
| 10-0147 | *Pestalotiopsis palmarum* | MFU, Chiang Rai |
| 10-0148 | *Pestalotiopsis samarangensis* | MFU, Chiang Rai |
| 10-0149 | *Pestalotiopsis heterocornis* | Huay Mae Sak Waterfall, Chiang Rai |
| 10-0150 | *Pestalotiopsis disseminata* | MFU, Chiang Rai |
| 10-0151 | *Pestalotiopsis palmarum* | Huay Mae Sak Waterfall, Chiang Rai |
| 10-0152 | *Pestalotiopsis* sp. | Orchids nursery, Chiang Mai |
| 10-0153 | *Pestalotiopsis palmarum* | Mushroom Research Centre (MRC), Chiang Mai |
| 10-0154 | *Pestalotiopsis* sp. | MFU, Chiang Rai |
| 10-0155 | *Pestalotiopsis furcata* | MRC, Chiang Mai |
| 10-0156 | *Pestalotiopsis* sp. | MRC, Chiang Mai |
| 10-0157 | *Pestalotiopsis palmarum* | MFU, Chiang Rai |
| 10-0158 | *Pestalotiopsis palmarum* | MRC, Chiang Mai |
| 10-0159 | *Pestalotiopsis* sp. | Chiang Rai |
| 10-0160 | *Pestalotiopsis* sp. | MRC, Chiang Mai |
| 10-0161 | *Pestalotiopsis theae* | Khun Korn Waterfall, Chiang Rai |
| 12-0055 | *Pestalotiopsis theae* | MRC, Chiang Mai |
| 10-0784 | *Pestalotiopsis* sp. | Chiang Rai |
| 10-0899 | *Pestalotiopsis* sp. | Chiang Rai |
| 10-0900 | *Pestalotiopsis* sp. | Chiang Mai |
| 10-0901 | *Pestalotiopsis* sp. | Chiang Mai |
| 10-0902 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0029 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0030 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0101 | *Pestalotiopsis* sp. | Chiang Rai |

| | | |
|---|---|---|
| 11-0111 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0224 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0235 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0248 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0251 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0259 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0260 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0285 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0286 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0287 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0453 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0497 | *Pestalotiopsis* sp. | Chiang Mai |
| 11-0500 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0559 | *Pestalotiopsis* sp. | Chiang Rai |
| 11-0565 | *Pestalotiopsis foedans* | Chiang Rai |
| 11-0567 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0082 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0083 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0121 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0122 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0125 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0130 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0133 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0136 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0152 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0153 | *Pestalotiopsis* sp. | Chiang Rai |
| 12-0156 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0201 | *Pestalotiopsis* sp. | Chiang Mai |
| 12-0233 | *Pestalotiopsis samarangensis* | Chiang Mai |

This present study is one of the pioneering projects focusing on the diversity of *Pestalotiopsis* species in Thailand. Fifty three isolates were obtained from this study. Based on the species identification guide of Guba (1961) and Steyaert (1961), it can be clearly seen that the data obtained only from morphological characteristics are not sufficient to identify this fungal group at species level. It is expected that further analysis using molecular approach would reveal an insight in clarifying their species identity.

### 4.3 New Species and Epitypes of *Pestalotiopsis*

4.3.1 New species from *Camellia sinensis*

***Pestalotiopsis furcata*** sp. nov.

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 (Mean = 35.5 × 9.7 µm), basal cell obconic, hyaline or slightly olivaceous, thin- and smooth-walled, 4.9–6.4 µm long (Mean = 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 (Mean = 23.4 µm) (second cell from base 7–9 µm (Mean = 7.9 µm); third cell 7.5–9.1 µm (Mean = 8.2 µm); fourth cell 7.2–9.2 µm (Mean = 8.0 µm); apical cell hyaline, conic to cylindrical 6.3-8.44 µm long (Mean = 7.48 µm); 5–9 tubular apical appendages, some appendages branched, arising from the upper portion of the apical cell, 20–35 µm long (Mean = 27.7 µm), unequal; basal appendages absent.

Colonies on PDA reaching 7 cm after 7 days at 25oC, edge entire, whitish, with dense, aerial mycelium on surface, fruiting bodies black, gregarious; reverse of culture white (Figure 4-3).

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).

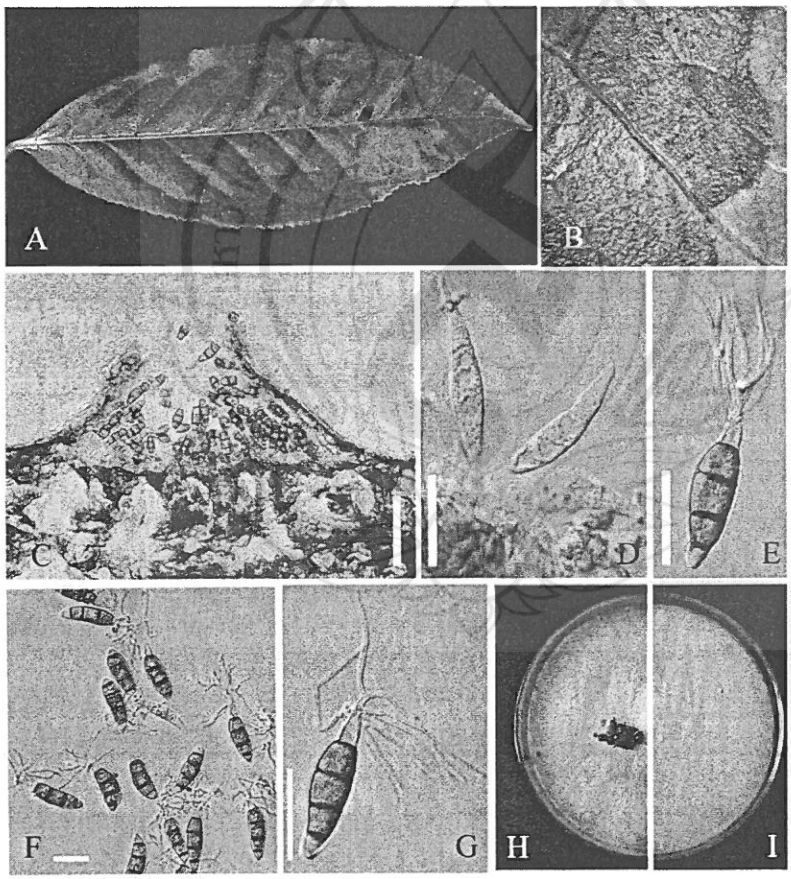


Figure 4-3 *Pestalotiopsis furcata* (holotype). Species appearance: (A) Blight on leaf of *Camellia sinensis*; (B) Conidiomata, split irregularly; (C) Section of conidiomata; (D)

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.

4.3.2 New species in *Syzygium samarangense*

***Pestalotiopsis samarangensis*** sp. nov.

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 (Mean = 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 (Mean = 4 μm); apical cell 2.5–4.6 μm long (Mean = 3.4 μm), conical, hyaline, thin and smooth-walled; three median cells 12.8–13.8 μm long (Mean = 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 (Mean = 4.8 μm); third cell darker brown, 3.7–5 μm long (Mean = 4.1 μm; the fourth cell darkest, 4.5–5.3 μm (Mean = 4.9 μm); three apical appendages 12–18 μm long (Mean = 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 25oC, edge entire, whitish aerial mycelium, fruiting-bodies black, gregarious; reverse of culture white (Figure 4-4).

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

MATERIAL EXAMINED: THAILAND, Chiang Mai Province, 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).

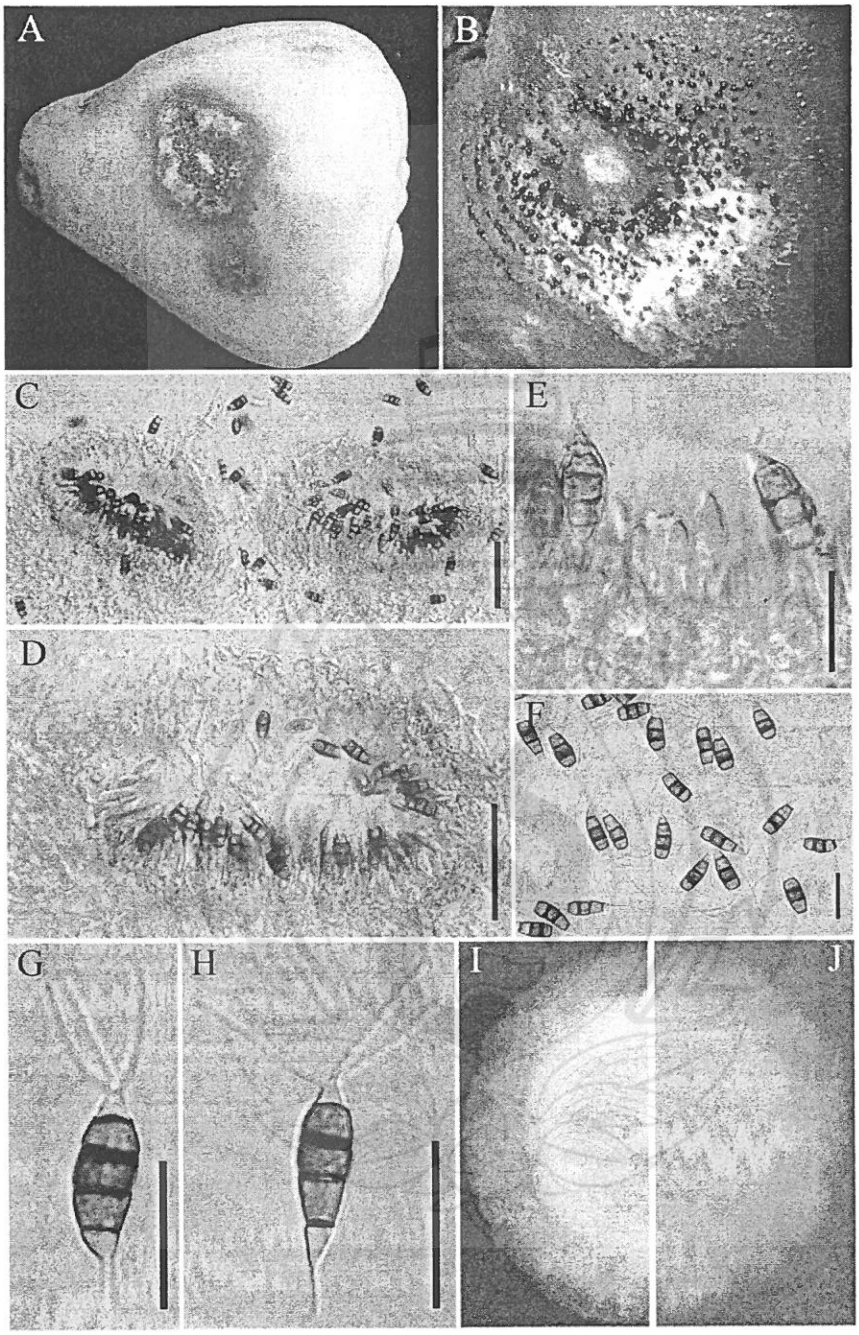


Figure 4-4 *Pestalotiopsis samarangensis* (holotype). Characteristics: (A), (B) Fruit rot of wax apple; (C), (D) Acervular conidiomata, epidermal to superficial in origin; (E) Conidiogenous cells; (F) - (H) Versicoloured conidia; (I), (J) Colony on PDA top. Scale bars: (C), (D) = 50 μm, (E) - (H) = 20 μm.

4.3.3 Epitype designated from *Camellia sinensis*

***Pestalotiopsis theae***

Conidiophores growing in clusters, simple, short, filiform, fugacious, smooth, thin-walled, hyaline, 4–8 × 1–2 µm (Mean = 6 × 1.5 µm). Conidia fusiform to ellipsoid, straight to slightly curved, 4-septate 22.5–28 × 6.7–8.2 µm (Mean = 25.5 × 7.6 µm), basal cell conic or obconic, hyaline, thin and smooth walled, 3.9–5.3 µm long (Mean = 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 (Mean = 16.7 µm) (second cell from base 5–7.2 µm (Mean = 6.3 µm); third cell 4.8–6 µm (Mean = 5.4 µm); fourth cell 5–6.8 µm (Mean = 5.7 µm)); apical cell hyaline, cylindrical 4.2–5.9 µm long (Mean = 5.2 µm); 3–4 apical appendages, tubular, arising from the upper portion of the apical cell, 22.5–31 µm long (Mean = 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 (Figure 4-5).

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.

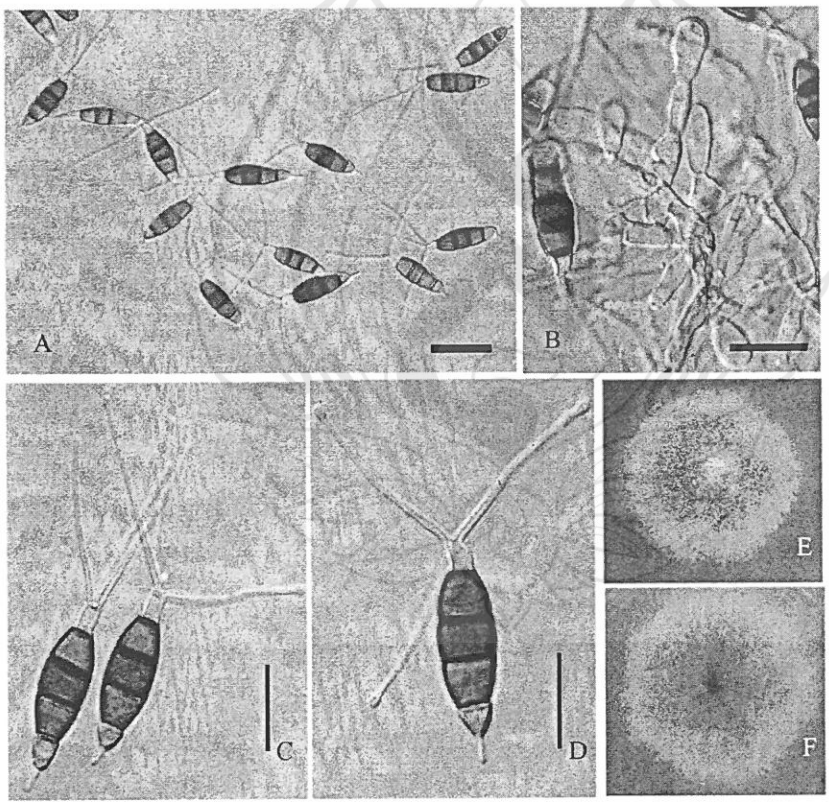


Figure 4-5 *Pestalotiopsis theae* (epitype). Appearance: (A) Conidia in culture; (B) Conidiogenous cells; (C), (D) Conidia; (E), (F) Colony in culture; (E) from above, (F) from below. Scale Bars: (A), (B) = 20 µm; (C), (D) = 15 µm.

### 4.4 Analysis of *Pestalotiopsis* metabolites

Four *Pestalotiopsis* strains namely MFLUCC 10-0140, 10-0141, 11-0565, and 12-0233, were selected for this experiment. The strains used belonged to the species known to produce active compounds and/or possible new species. Detailed information of these strains are presented in Table 4-3.

Table 4-3 Characteristics of *Pestalotiopsis* strains used in metabolite analysis

| ***Pestalotiopsis* spp.** | **Hosts / Places of collections** |
|---|---|
| *P. samarangensis* 12-0233 | *Syzygium samarangense* / Thailand |
| *P. foedans* 11-0565 | Leaf litter / Thailand |
| *P. microspora* 10-0140 | *Parrotia persica* / Iran |
| *P. palmarum* 10-0141 | *Parrotia persica* / Iran |

*Pestalotiopsis* strain 12-0233 is a new species as described in Section 4.3.2. The other three strains (11-0565, 10-0140, and 10-0141) are the species known to produce important metabolites (Xu et al. 2010). It should be noted that these three strains were identified by morphological characteristics. Two strains from Iran were used for comparative study.

The crude extracts of these fungi were identified using the GC/MS as shown in Table 4-4. Classification of these chemicals based on functional group is also shown in Figure 4-6.

Table 4-4 GC-MS analysis of four species of *Pestalotiopsis* extracts.

| ***Pestalotiopsis* strains** | **Volatile compound** | **RT (min)** | **Amount (%)** | **Quality (%)** |
|---|---|---|---|---|
| **12-0233** | **Acid and Ester** | | | |
| | 2-Ethylhexyl acrylate | 11.95 | 1.55 | 83 |
| | Palmitic acid | 31.94 | 18 | 91 |
| | Linolic acid | 40.27 | 17.23 | 89 |
| | Elaidic acid | 40.53 | 26.76 | 98 |
| | Glyceryl monooleate | 50.35 | 6.34 | 53 |
| | cinnamyl cinnamate | 56.64 | 3.98 | 42 |
| | 5-(dimethylamino)naphthalene-1-sulphonic acid | 66 | 3.65 | 9 |
| | **Alcohol** | | | |
| | (1S,2R)-2-methylcyclohexanethiol | 57.16 | 1.54 | 7 |
| | **Alkene** | | | |
| | 1-Cyclohexylnonene | 60.28 | 1.2 | 38 |
| | 10-Demethylsqualene | 61.55 | 6.37 | 59 |
| | **Aromatic** | | | |
| | 2,4-dimethyl-1H-imidazole | 46.14 | 1.8 | 5 |
| | Bis(2-ethylhexyl) phthalate | 53.75 | 4.32 | 64 |
| | **Ketone** | | | |
| | Cyclodecaneone oxime | 41.42 | 2.11 | 9 |
| | 1-oxacyclotetradecan-2-one | 51.68 | 2.52 | 27 |
| | 3-(4-chlorophenyl)-1-[4-(2- methylphenyl) piperazin-1-yl]-3-phenylpropan-1-one | 56.52 | 2.65 | 47 |
| **11-0565** | **Alcohol** | | | |
| | Phenethyl alcohol | 8.98 | 88.63 | 91 |
| | **Aromatic** | | | |
| | 2-Methylpyrrolidine | 11.96 | 2.6 | 45 |
| | **Ester** | | | |
| | Bis(2-ethylhexyl) phthalate | 53.82 | 11.37 | 83 |

Table 4-4 GC-MS analysis of four species of *Pestalotiopsis* extracts.

| *Pestalotiopsis* strains | Volatile compound | RT (min) | Amount (%) | Quality (%) |
|---|---|---|---|---|
| **10-0140** | **Alcohol** | | | |
| | Isocurcumenol | 22.09 | 9.69 | 93 |
| | S(+)-1-indanol | 23.44 | 0.92 | 32 |
| | **Alkene** | | | |
| | 10-Dimethyl-7-(1'-methylethenyl)-10-mercaptobicyclo[4.4.0]dec-4-ene | 22.39 | 1.5 | 45 |
| | **Alkyne** | | | |
| | 3-Decyn-1-ol | 27.37 | 7.28 | 35 |
| | **Aromatic** | | | |
| | Ethylbenzene | 3.54 | 0.41 | 90 |
| | 6-Methylxanthotoxin | 21.53 | 31.17 | 83 |
| | 2,2,3,7- Tetrametlytricyclo (5.2.O.O.(1,6)) undec-3-ene | 22.7 | 1.75 | 43 |
| | 4-(oxiranylmethoxy)-1H-indole | 24.4 | 11.39 | 27 |
| | Imidazo[1,2-a]pyrimidin-5(3H)-one, 2,8-dihydro-6,8-dimethyl- | 24.89 | 6.32 | 30 |
| | 1,3-Cyclopentadiene,1,3-bis(1-methylethyl)- | 26.76 | 4.86 | 38 |
| | **Ester** | | | |
| | Ethyl Bicyclic Phosphate | 25.69 | 4.82 | 46 |
| | **Ketone** | | | |
| | Germacrone | 23.61 | 14.97 | 96 |
| | Spiro[5.5]undec-8-en-1-one | 24.8 | 4.46 | 14 |
| **10-0141** | **Acid and Ester** | | | |
| | Palmitic acid | 31.88 | 15.97 | 43 |
| | Aceticaciddecenylester | 50.36 | 4.8 | 32 |
| | 5-(dimethylamino)naphthalene-1-sulphonic acid | 65.97 | 4.46 | 5 |
| | **Alcohol** | | | |
| | Isopulegol | 40.45 | 19.94 | 49 |
| | **Alkene** | | | |
| | 7,7',8,8',11,11',12,12',15,15'-decahydro-psi,psi-carotene | 61.56 | 13.74 | 72 |
| | **Alkyne** | | | |
| | 1-Chloro-6-heptadecyne | 40.23 | 21.32 | 58 |
| | **Aromatic** | | | |
| | Benzyl 2-Deoxy-2-phthalimido-4,6-O benzylidene-D-glucopyranoside | 53.75 | 7.04 | 64 |
| | **Pyrazine** | | | |
| | 3-Trifluoromethyl-5-(phenyl)pyrazole | 23.04 | 8.53 | 4 |
| | 6,7-Dihydro-5-methyl-5(H)-cyclopentapyrazine | 23.17 | 4.19 | 58 |

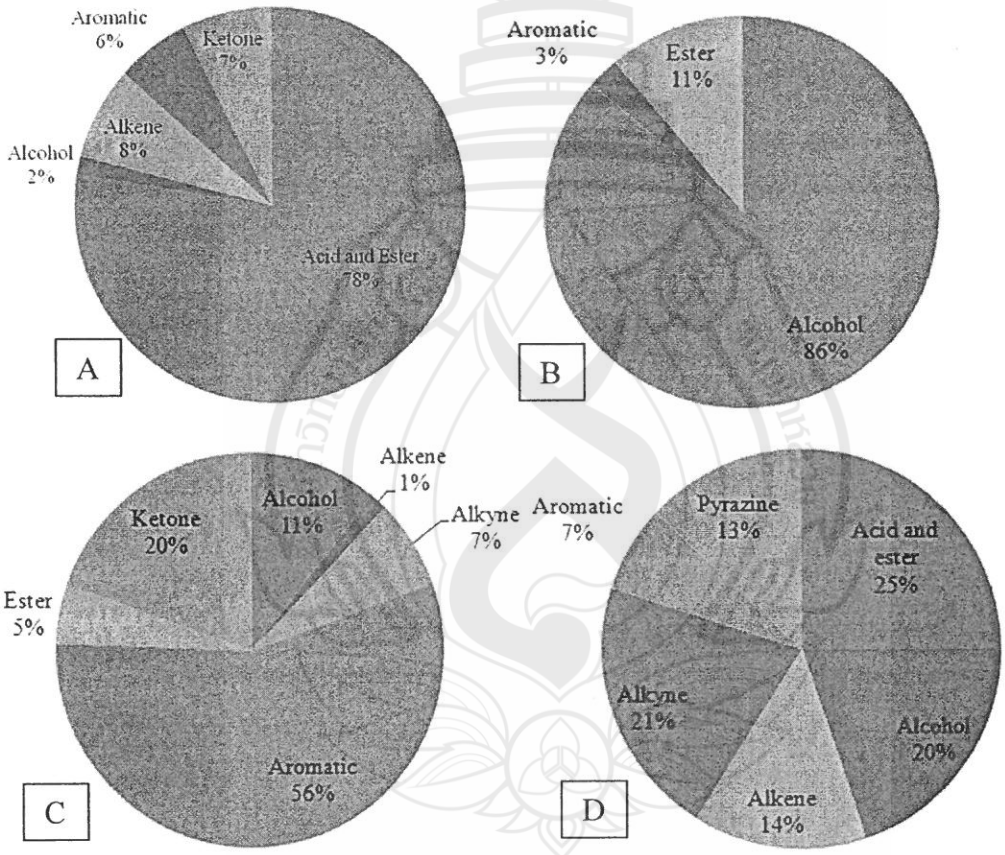


Figure 4-6 Distribution of chemical compounds based on functional group of *Pestalotiopsis* strains 12-0233 (A), 11-0565 (B), 10-0140 (C), and 10-0141 (D).

For antimicrobial activity, the crude extracts of these *Pestalotiopsis* strains were also effective against the microbes used; the activity was different depending on the testing microbes (see Table 4-5 and Figure 4-7).

Table 4-5 Antimicrobial activity of *Pestalotiopsis* strains against some microbes.

| | **Crude extracts of *Pestalotiopsis* strains*** | | | |
|---|---|---|---|---|
| **Microbial strains** | **12-0233** | **11-0565** | **10-0140** | **10-0141** |
| *Candida albicans* | 2.80±0.53 | - | - | - |
| *Candida utilis* | 1.05±0.10 | - | 1.45±0.10 | 1.77±0.16 |
| *Pseudomonas aeruginosa* | 1.08±0.17 | 1.17±0.19 | - | 1.95±0.51 |
| *Bacillus cereus* | 1.32±0.29 | - | 2.12±0.10 | 2.25±0.66 |
| *Escherichia coli* | 1.22±0.22 | 1.38±0.12 | 2.18±0.12 | 2.52±0.04 |
| *Staphylococcus aureus* | 1.13±0.37 | - | 1.38±0.08 | 1.48±0.75 |
| *Micrococcus luteus* | 1.93±0.20 | 0.83±0.14 | 1.23±0.16 | 1.45±0.10 |

*The activity was expressed as a diameter of an inhibition zone (mm.); – means no activity; values represent mean ± SD.

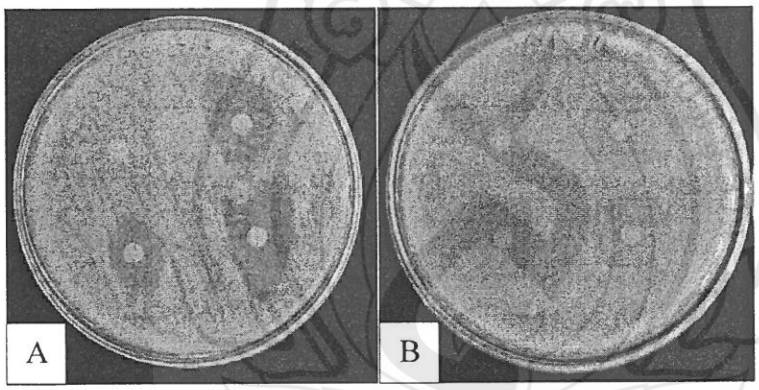


Figure 4-7 Representatives of antimicrobial activity of *Pestalotiopsis* extracts. (A) *Pestalotiopsis* strain 12-0233 on *Candida albicans*; (B) strain 11-0565 on *Escherichia coli*.

# CHAPTER 5
# CONCLUSION

In this study, the *Pestalotiopsis* fungal group was determined in terms of its taxonomy and phylogenetic relationship. Initially, the fungal isolates were screened and isolated from various kinds of plant materials collected from the Northern part of Thailand. For this project, 54 *Pestalotiopsis* strains were obtained and pre-characterized using morphological characteristics. Of these, two new species (*P. furcata* and *P. samarangensis*) were discovered in which their detailed descriptions were provided and illustrated. In addition, an epitype of *P. theae* was proposed. The discovery of new species and proposed epitype has been established in this study and is expected to be useful for the taxonomic study of this fungal group in future study. The *Pestalotiopsis* isolates obtained in this study have presently been deposited in BIOTEC and MFU culture collection.

In addition, the chemical profiles of four *Pestalotiopsis* were determined using GC/MS technique. Different fungal strains showed different profiles. The results obtained are expected and should be further developed as an alternative means to differentiate the fungal species, the so-called 'chemotaxonomy'. These fungal metabolites were also active when testing against some microbial pathogens.

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# *Pestalotiopsis*—morphology, phylogeny, biochemistry and diversity

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**Abstract** 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. In this review, *Pestalotia*, *Pestalotiopsis* and some related genera are evaluated; 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. It has not even been established whether *Pestalotia* and *Pestalotiopsis* are distinct genera, as no isolates of the type species of *Pestalotia* have been sequenced, and they are morphologically somewhat similar. When selected GenBank ITS accessions of *Pestalotiopsis clavispora, P. disseminata, P. microspora*, *P. neglecta*, *P. photiniae, P. theae, P. virgatula* and *P. vismiae* are aligned, most species cluster throughout any phylogram generated. Since there appears to be no living type strain for any of these species, it is unwise to use GenBank sequences to represent any of these names. Type cultures and sequences are available for the recently described species *P. hainanensis, P. jesteri*, *P. kunmingensis* and *P. pallidotheae*. It is clear that the important species in *Pestalotia* and *Pestalotiopsis* need to be epitypified so that we can begin to understand the genus/genera. 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.

**Keywords** Epitypify · Host occurrence · *Pestalotia* · *Pestalosphaeria* · Pigmentation · Secondary metabolites · Taxol

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## Introduction

*Pestalotoiopsis* Steyaert is an appendage-bearing conidial anamorphic form (coelomycetes) in the family Amphisphaeriaceae (Barr 1975, 1990; Kang et al. 1998, 1999), and molecular studies have shown that *Pestalotiopsis* is monophyletic (Jeewon et al. 2002, 2003, 2004). Species of *Pestalotiopsis* are common in tropical and temperate

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# A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species

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**Abstract** *Pestalotiopsis* is a taxonomically confused, pathogenic and chemically creative genus requiring a critical re-examination using a multi-gene phylogeny based on ex-type and ex-epitype cultures. In this study 40 isolates of *Pestalotiopsis*, comprised of 28 strains collected from living and dead plant material of various host plants from China were studied by means of morphology and analysis of ITS, β–tubulin and *tef1* gene sequence data. Based on molecular and morphological data we describe 14 new species (*Pestalotiopsis asiatica*, *P. chinensis*, *P. chrysea*, *P. clavata*, *P. diversiseta*, *P. ellipsospora*, *P. inflexa*, *P. intermedia*, *P. linearis*, *P. rosea*, *P. saprophyta*, *P. umberspora*, *P. unicolor* and *P. verruculosa*) and three species are epitypified (*P. adusta*, *P. clavispora* and *P. foedans*). 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. This work provides a backbone tree for 22 ex-type/epitypified species of *Pestalotiopsis* and can be used in future studies of the genus.

**Keywords** β-tubulin · Epitype · ITS · Phylogeny · Saprobe · *tef1*

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RESEARCH ARTICLE / *ARTIGO*

# A destructive new disease of *Syzygium samarangense* in Thailand caused by the new species *Pestalotiopsis samarangensis*

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**ABSTRACT**

A new fungal species, *Pestalotiopsis samarangensis,* was isolated from fruit rot in *Syzygium samarangense* from markets in Chiang Mai and Chiang Rai provinces, Thailand. Initially small, circular, black, slightly sunken spots developed on fruits. Later, the spots enlarged rapidly, became sunken, and resulted in a soft decay of the fruit flesh. Molecular analysis of the ITS, β-tubulin, and *tef1* combined gene sequences distinguish *P. samarangensis* from other species in the genus. Pathogenicity testing proved that wounding triggers the disease symptoms and thus careful handling of fruits during transport and storage helps to prevent the disease.
**Key words:** β-tubulin, fruit rot, ITS, neighbor-joining, phylogenetic, *tef1*.

## INTRODUCTION

*Syzygium samarangense* Merr. (Myrtaceae) is widely grown for its fruits throughout Cambodia, Laos, India, Philippines, Samoa, Sri Lanka, Taiwan, Thailand, and Vietnam (Srisaard, 2003; Vara-Ubol et al., 2006). In Thailand, the wax apple fruit is commonly known as chomphu and harvested the year round with a peak during January to March from the cultivars 'Dang Indo', 'Phet Ban Plew', 'Phet Jin Da', 'Phet Nam Pueng', 'Phet Sai Rung', 'Phet Sam Phran', 'Thub Thim Chan', and 'Thun Klao'. In 2004, Thailand earned US$ 26.5 million from 69,608 tons of wax apple planted over 10,240 ha (Shü et al., 2008). The fruits have a thin, delicate skin and are thus easily susceptible to pest and disease attack. Diseases of wax apple include *Pestalotiopsis* fruit rot (*Pestalotiopsis eugeniae* (Thüm.) S. Kaneko), shoot dieback (*Fusarium* sp.), anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.), *Phytophthora* fruit rot (*Phytophthora palmivora* (E.J. Butler) E.J. Butler), *Dothiorella* fruit rot (*Dothiorella* sp.), and *Pseudocercospora* fruit rot (*Pseudocercospora* sp.) (Janick & Paull, 2008; Lan, 2001), although these species need to be confirmed by molecular data (Phoulivong et al., 2010; Ko Ko et al., 2011).

*Pestalotiopsis* spp. are important plant-pathogenic species known mostly from the tropics, where they cause leaf blights (Guba, 1961) in many plant species (Hyde & Fröhlich, 1995; Xu et al., 1999; Das et al., 2010; Maharachchikumbura et al., 2011). Species may also cause rots of fruit and other post-harvest diseases (Ullasa & Rawal, 1989; Korsten et al., 1995; Xu et al., 1999). Several post-harvest diseases are caused by species of *Pestalotiopsis*, e.g., postharvest decay of mangos by *P. glandicola* (Castagne) Steyaert (Ullasa & Rawal, 1989), fruit rot of grapevine by *P. menezesiana* (Bres. & Torrend) Bissett as well as *P. uvicola* (Speg.) Bissett (Xu et al., 1999), and fruit rot of rambutan by *Pestalotiopsis* sp. (Sangchote et al., 1998). Scabby fruit canker of guava is caused by *P. psidii* (Pat.) Mordue (Kaushik et al., 1972).

We surveyed market fruit disease of wax apple in Chiang Mai and Chiang Rai provinces in Thailand in 2010 and 2011, and constantly observed a distinctive fruit rot disease. In this study, we introduce this new *Pestalotiopsis* fruit rot disease of wax apple, with a description of morphological and molecular characteristics of the fruit rot agent.

## MATERIALS AND METHODS

### Symptoms and sample collection

Surveys of post-harvest disease of wax apples were conducted in markets of Chiang Mai and Chiang Rai provinces from January to August in 2010 and from March to December in 2011. Wax apple fruits with disease symptoms were carried to the laboratory and photographed.

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