TAXONOMY AND PHYLOGENY OF *Phyllosticta*

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Phyllosticta is one of the major plant pathogenic genera, whose species have a worldwide distribution. *Phyllosticta* species are responsible for numerous diseases including leaf spots and black spots on fruits. Several species have also been reported as saprobes and/or as endophytes of many host plants. Species recognition in *Phyllosticta* has historically been based on morphology, culture characters and host association. Although there have been several taxonomic revisions of this genus, there is still considerable confusion in the classification and identification of *Phyllosticta* and *Guignardia* species. This thesis provides additional knowledge on the taxonomy and relationships of *Phyllosticta* species based on morphological and phylogenetic data.

The diversity of the genus *Phyllosticta* in Thailand was investigated in this study. Plant tissues (i.e., leaves and fruits) were collected and used to isolate the *Phyllosticta* species. Their host and fungal mode of life were also recorded. Of 43 *Phyllosticta* isolates, 39 isolates were identified as *P. capitalensis*; other species were also present including 2 isolates of *P. cordylinophila*, 1 isolate of *P. citrimaxima* sp. nov. and 1 isolate of *P. mangiferaceae* sp. nov.

I then focused on *P. capitalensis* taxonomy due to its common occurrence (based on this study). The morphology of the species was observed and recorded; *P. capitalensis* CPC20252 was fully described as a representative for this species in this study. The phylogeny was further explored using multigene analysis (ITS, ACT and TEF). Apart from the data of Thai *P. capitalensis*, sequences of other *P. capitalensis* strains from the CBS were included. Based on the phylogenetic tree, the data revealed that *P. capitalensis* was monophyletic exhibiting a broad host range and worldwide distribution.

In addition, my study revealed 15 new species (i.e., *Phyllosticta citrimaxima* sp. nov. and *P. mangiferaceae* sp. nov. were isolated from Thailand) based on analysis of multigene; ITS, LSU, ACT, TEF and GPDH sequence data for 160 *Guignardia*/*Phyllosticta* isolates. This finding also confirmed that species identification in *Phyllosticta* cannot be based only on morphological characters. Twelve species of *Phyllosticta* were also investigated and thus designated as epitypes (based on phylogenetic tree analysis).

Finally, several type species of *Guignardia* and *Phyllosticta* were studied solely by morphological characters. Their detailed descriptions and illustrations are given with an expectation to use as a key reference for future study.

Keywords: Endophytes/Guignardia/Morphology/Molecular Phylogeny/Multi-Gene Analysis/Plant Pathogenic Fungi/*Phyllosticta*/Secondary Metabolites

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CHAPTER 1

INTRODUCTION

The genus *Phyllosticta* Pers. ex Desm. is a taxonomically confused group of microfungi comprising mostly important phytopathogens with a wide host range (van der Aa, 1973; van der Aa, Vanev, Aptroot, Summerbell, & Verkley, 2002). Although the generic concept of *Phyllosticta* has been refined and species names were enumerated in a monographic treatment by van der Aa et al. (2002), species recognition still remains problematic (Hyde, Abd-Elsalam & Cai, 2010; Glienke et al., 2011). Several species of *Phyllosticta* have also been reported as endophytes and saprobes (van der Aa et al., 2002; Baayen et al., 2002; Okane, Lumyong, Nakagiri & Ito, 2003; Wulandari et al., 2009; Wulandari, To−Anun, Lei, Abd−Elsalam & Hyde, 2010; Glienke et al., 2011). Species of *Phyllosticta* (sexual state *Guignardia* Viala & Ravaz) cause leaf spot symptoms and fruit diseases on a range of hosts including some economically important crops and ornamentals such as citrus, banana, apple, grapes, cranberry, orchids, *Ficus* sp., *Buxus* sp. and maple (Uchida & Aragaki, 1980; Paul & Blackburn, 1986; Baayen et al., 2002; McManus, 1998; Olatinwo, Hanson & Schilder, 2003; Paul, Van Jaarsveld, Korsten & Hattingh, 2005; Liu, Ding, Deng & Chen, 2009). *Phyllosticta* species are also potential biocontrol agents (Yan, Sikora & Zheng, 2011), and have been reported to produce novel bioactive metabolites such as phyllostine and phyllostoxin (Evidente, Cimmino, Andolfi, Vurro, Zonno & Motta, 2008). Molecular data has vastly improved the knowledge of species relationships and taxonomic classifications in the past decade with reference to different complex groups of plant pathogenic fungi (Crous & Groenewald, 2005; Shenoy, Jeewon & Hyde, 2007; Rossman & Palm-Hernández, 2008; Udayanga et al., 2011). Similarly, Phyllosticta (and its sexual Guignardia state) is an important genus requiring modern revisionary treatment employing morphological characters and a molecular phylogenetic approach, as the understanding of species is less advanced; molecular data are

expected to reveal cryptic novel species (Crous & Groenewald, 2005; Hyde, Abd-Elsalam et al., 2010; Hyde, Chomnunti et al., 2010).

1.1 History

The genus *Phyllosticta* Pers. was established by Persoon (1818) when he introduced the generic name *Phyllosticta* for *Sphaeria lichenoides* DC. Over the past 200 years numerous species have been added to the genus, often based on host association so that about 3,200 names have been associated with *Phyllosticta* at various times (http://www.indexfungorum.org/names/Names.asp). Desmazieres (1847) validated *Phyllosticta* Pers., and Donk (1968) designated *Phyllosticta convallariae* Pers. as the type species. Many of the 3,100 names do not refer to what is now considered to be *Phyllosticta sensu stricto*. Initially, many fungi with unicellular conidia, similar to those of *Phoma* were named as either *Phoma* or *Phyllosticta*, depending on the location of conidiomata on the host. Those fruiting on leaves were described as *Phyllosticta* while those occurring on other parts of the plant were placed in *Phoma*. van der Aa (1973) provided a key for 46 species of *Phyllosticta* he accepted, and this has been widely followed. Furthermore, in their monographic study, van der Aa et al. (2002) accepted 190 species in this genus, while Kirk, Cannon, Minter and Stalpers (2008) estimated that there are only 92 *Phyllosticta* species. Since the revision by van der Aa et al. (2002), a further 17 new species have been described (Table 1.1).

Table 1.1 History of the Study of *Phyllosticta*

Year	Event	References
1818	*Phyllosticta* introduced as generic name for *Sphaeria lichenoides* DC.	Persoon (1818)
1847	*Phyllosticta* Pers. was validated	Desmazières (1847)
1849	*Phyllosticta* Pers. ex Desm. typified with *Phyllosticta* cruenta (Kunze ex Fr.) Kickx	Kickx (1849)
1968	*Phyllosticta convallariae* Pers. designated as the type species	Donk (1968)
1927	A compilation of *Phyllosticta* names published	Petrak and Sydow (1927)
1973	*Phyllosticta* outlined with 46 accepted species	van der Aa (1973)
2002	A further revision of the species described in *Phyllosticta* with notes on each of the 191 accepted species	van der Aa et al., (2002)

Table 1.1 (Continued)

Year	Event	References
2003-2013	*P. ardisiicola* Motohashi, I. Araki & C. Nakash.	Motohashi, Araki and Nakashima (2008)
	P. aspidistricola Motohashi, I. Araki & C. Nakash.	
	P. kerriae Motohashi, I. Araki & C. Nakash.	
	P. fallopiae Motohashi, I. Araki & C. Nakash.	
	P. citriasiana Wulandari, Crous & Gruyter	
	Phyllosticta (*Guignardia*) *musicola* N.F. Wulandari, L. Cai & K.D. Hyde	Wulandari et al. (2009)
	P. bifrenariae O.L. Pereira, C. Glienke & Crous	Wulandari, To-Anun, Lei et al. (2010)
	P. brazilianiae D. Stringari, C. Glienke & Crous	
	P. citribraziliensis C. Glienke & Crous	Glienke et al. (2011)
	P. cavevdishii M.H. Wong & Crous	
	P. maculate M.H. Wong & Crous	
	P. citrichinaensis X.H. Wang, K.D. Hyde & H. Y. Li	Wong, Crous, Henderson, Groenewald and Drenth (2012)
	P. hostae Y.Y. Su & L. Cai	
	P. schimae Y.Y. Su & L. Cai	Wang et al. (2012)
	P. ilicis-aquifolii Y.Y. Su & L. Cai	Su and Cai (2012)
	P. styracicola K. Zhang & L. Cai	Zhang, Crous, Schoch and Hyde (2012)
	P. hubeiensis K. Zhang & L. Cai	

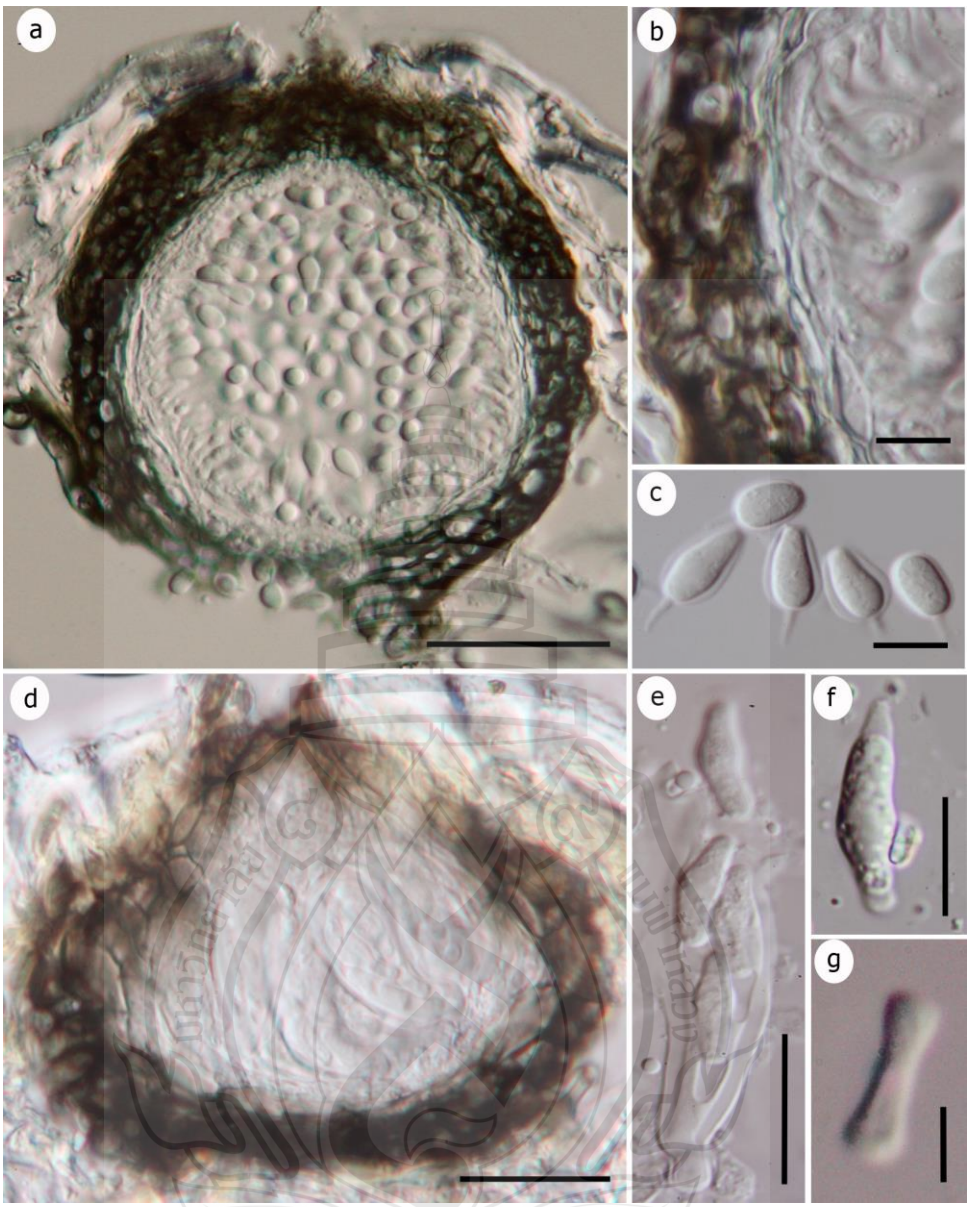
1.2 Using *Phyllosticta* Versus *Guignardia*

The name *Phyllosticta* (asexual state) and *Guignardia* (sexual state) have been used separately following the dual classification system used by mycologists over several decades (Hawksworth, 2004; McNeil et al., 2005; Shenoy, Jeewon & Hyde, 2007). For instance, *Phyllosticta musarum* (Cooke) Aa and *Guignardia musae* Racib. are the same biological species but have different names, *P. musarum* being the asexual state and *G. musae* being the sexual state (van der Aa, 1973; Wulandari, To-Anun & Hyde, 2010). However, with the use of molecular data it is now possible to link asexual and sexual states (Berbee & Taylor, 2001) and the use of the dual nomenclature system of classification in fungi has become redundant (Hawksworth, 2011). Therefore, a single name should be adopted and there are various views to which names should be followed, i.e. the oldest, the sexual state name, the most conserved name, and there is a view maintaining both names (Berbee & Taylor, 2001; Seifert & Rossman, 2010; Hyde, McKenzie & KoKo, 2011). Our view is that we should generally adopt the oldest name for each genus, which will soon be enforced in the International Code of Nomenclature for algae, fungi and plants, but also taking into account which name is the most important and commonly used. *Phyllosticta* Pers. (1818) is a much older name than *Guignardia* Viala and Ravaz (1892) and generally *Phyllosticta* species are known to cause important diseases (e.g. leaf spot, citrus black spot, black rot of horse chestnut). There are also many more species of *Phyllosticta* than *Guignardia*. There are exceptions, for example *Guignardia candeloflamma* K.D. Hyde is only known in it teleomorph state (Wulandari To-Anun & Hyde, 2010), while banana freckle is caused by both states (Wulandari, To-Anun & Hyde, 2010). Because *Phyllosticta* is the oldest name and generally more important as the causal agent of disease we chose to adopt this name and treat all *Guignardia* species as synonyms of *Phyllosticta*, in the sense of Glienke et al. (2011). Because of this decision we use the name *Phyllosticta* throughout this review unless we specifically refer to a *Guignardia* species. *Leptodothiorella,* which previously represented the spermatial state of some *Phyllosticta* species (e.g. *Leptodothiorella aesculicola* (Sacc.) Sivan.), are also treated as synonyms of *Phyllosticta* (van der Aa, 1973).

1.3 Morphological Characteristics to Differentiate Species

Phyllosticta pycnidia are usually globose, subglobose or tympaniform, flattened above, and closely connected with the subepidermal pseudostroma (Figures 1.1a, b). They are mostly unilocular but occasionally may be multilocular (van der Aa, 1973). The conidia are commonly hyaline, one-celled, ovoid, obovate or ellipsoid, or short cylindrical, seldom pyriform, globose or subglobose, 10–25 µm long , and usually covered by a slime layer and bearing a single apical appendage (Figure 1c) (van der Aa, 1973). Cultural characteristics when grown on specific media may also be used as differentiating characters. In the case of *P. citricarpa* colonies can be characterised after 14 days at 25 °C in the dark on OA as flat, spreading, olivaceous-grey, becoming pale olivaceous-grey towards the margin, with sparse to moderate aerial mycelium; surrounded by a diffuse yellow pigment in the agar medium (Wulandari et al., 2009).

The sexual state *Guignardia* can be characterized by erumpent ascomata, which are globose to pyriform in section, often irregularly shaped, unilocular, and with a central ostiole forming by dehiscence when mature. The peridium is thin, comprising a few layers of angular cells. Asci are 8-spored, bitunicate, clavate to broadly ellipsoid, with a wide, slightly square apex, tapering gradually to a small pedicel, and with a well-developed ocular chamber. Ascospores are ellipsoid to limoniform, sometimes slightly elongated, aseptate, hyaline, often guttulate or with a large central guttule, and some have mucilaginous polar appendages (van der Aa, 1973; Wulandari, To-Anun, McKenzie & Hyde, 2011; Figure 1.1d-f).



Notes. a. Vertical section through pycnidium. b. Pycnidial wall with conidiogenous cells. c. Conidia. d. Section of ascoma. e. Bitunicate and fissitunicate ascus. f. Ascospore. g. *Leptodothiorella* spermatial state. Bars: a, b, d, e = 50 µm, c, f, g =10 µm.

Figure 1.1 Comparison of *Phyllosticta* and *Guignardia* States

A spermatial state is often present in the life cycle of *Guignardia* species, and readily forms in culture. Spermatia are cylindrical to dumbbell-shaped with guttules at each end (Figure 1.1g). In the past several spermatial states were officially named. For instance, the spermatial state of *Melanops concinna* Syd. (= *Guignardia concinna* (Syd.) Aa, van der Aa 1973) was described as *Leptodothiorella concinna* Sydow (1926).

1.4 Molecular Studies Advance the Understanding of *Phyllosticta*

The rapid development of molecular phylogenetic tools have improved our understanding of several other coelomycetous genera such as *Colletotrichum* (Cai et al., 2009; Crouch, Clarke & Hillman, 2009; Hyde et al., 2009), *Phomopsis* (Santos & Phillips 2009, Udayanga et al., 2011), *Phoma* (Aveskamp, De Gruyter, Woudenberg, Verkley & Crous, 2010; De Gruyter et al., 2010), *Fusicoccum* (Crous et al., 2006), *Diplodia* (Phillips et al., 2008) and *Pestalotiopsis* (Liu, Ding, Deng & Chen, 2009), to name but a few. There have also been several molecular phylogenetic studies concerning *Phyllosticta* species that have helped to facilitate the identification of species and resolution of species complexes (Baayen et al., 2002; Okane et al., 2003; Motohashi, Inaba, Anzai, Takamatsu & Nakashima, 2009; Wulandari et al., 2009; Glienke et al., 2011).

1.4.1 Internal Transcribed Spacer rDNA Sequence Studies in *Phyllosticta*

ITS rDNA sequences are often used to infer phylogenetic relationships in many groups of fungi including *Phyllosticta* (Okane et al., 2003; Motohashi et al., 2009; Wulandari et al., 2009). Motohashi et al. (2009) evaluated the phylogenetic relationships among Japanese species of *Phyllosticta sensu stricto* and its teleomorph *Guignardia* using 18S rDNA sequence data. They observed that *Phyllosticta sensu stricto* is a monophyletic clade. In the same study, ITS and 28S rDNA sequences were used in a phylogenetic analysis of *Phyllosticta* strains from various host plants. Results from this study revealed isolates to cluster in two subgroups based on molecular data as, (1) cultures from a wide range of host plants mainly derived as

endophytes from symptomless plants (*P. capitalensis* complex, see below) and (2) relatively host-specific strains (often isolated as foliar pathogens from diverse plants).

ITS- Restriction Fragment Length Polymorphism (RFLP), and ITS sequence analysis were used to examine genetic variation of foliar endophytic *Phyllosticta* strains from different tropical trees (Pandey, Reddy & Suryanarayanan, 2003). Although ITS-RFLP failed to infer genetic diversity among isolates used, the ITS phylogram supported the identity of *P. capitalensis* as a common foliar endophyte and pathogen with wide range of hosts. In a similar study, the diversity of strains of *Guignardia* (or *Phyllosticta*) was evaluated using rDNA ITS sequence data (Okane et al., 2003). *Guignardia endophyllicola* (anamorph *Phyllosticta capitalensis*) was shown to have an extensive host range. The taxon was identified in 53 isolates from the same number of different plants belonging to 43 genera. Phylogeny based on rDNA ITS sequence analyses derived from 18 tropical endophytic strains from different plants confirmed conspecificity of Brazilian isolates with *Phyllosticta captalensis* (as *G. mangiferae*) (Rodrigues & Sieber, 2004). In the ITS sequence comparison, some *Guignardia* and *Phyllosticta* strains from unrelated hosts were more closely related than other isolates derived from closely related plants. However, the diversity across the wide range of hosts should be evaluated by incorporating more genes in analyses and isolating strains from a wide range of hosts in future studies.

The population structure and phylogenetic relationships of *Guignardia citricarpa* (citrus black spot) were investigated by Baayen et al. (2002) using ITS, Amplified Fragment Length Polymorphism and morphological comparison. The observations supported the historic distinction between slow growing pathogenic isolates and fast growing non-pathogenic isolates, which proved to belong to *P. capitalensis* (as *G. mangiferae*), the ubiquitous endophyte reported in various studies (Okane et al. 2003, Rodriguez, White, Arnold & Redman, 2009; Glienke et al., 2011). Numerous synonyms for *P. capitalensis* have been used in earlier studies. We have used the name as it appeared in the original publication, although the need for careful refinement using the currently accepted name is recommended.

1.4.2 Studies Employing Multi-locus Analyses in *Phyllosticta*

Identification of *Phyllosticta* species is problematic as few morphological characters are available to differentiate species. Although ITS sequence data have been widely used for species discrimination, multi-locus phylogenies might resolve cryptic species (Wulandari et al., 2009). A combined phylogenetic analysis based on the ITS rDNA, translation elongation factor 1 (TEF1), and actin (ACT) genes resolved three species, namely *P. mangiferae*, *P. citricarpa* and a new species, *P. citriasiana* (Wulandari et al., 2009), the latter causing tan spot of *Citrus maxima* in Asia. Glienke et al. (2011) investigated the genetic diversity of endophytic and pathogenic *Phyllosticta* species, with particular emphasis on *Phyllosticta citricarpa* and *Guignardia mangiferae* occurring on *Citrus*. Combined DNA sequence analysis based on ITS rDNA, translation elongation factor 1 (TEF1), actin (ACT) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes resolved nine well-supported clades related to seven known species and two apparently undescribed species. Epitypes were designated for *P. citricarpa* collected from Australia and *Phyllosticta capitalensis* collected from Brazil (Glienke et al., 2011). Furthermore, *P. brazilinae*, *P. bifinariae* and *P. citribraziliensis* were described as novel species based on morphology and a multilocus phylogeny. The combined gene analysis further revealed that the allocation of various synonyms for the endophytic, non-pathogenic isolates occurring on wide range of hosts would be more correctly referred to as *P. capitalensis*. Further work is needed, however, to resolve whether this taxon is a complex of cryptic species.

1.5 Multi-locus Phylogeny Inferred from Available Sequences in GenBank

A selected set of ITS rDNA, ACT, and TEF1 sequences, including the available ex-type, and ex-epitype materials were downloaded from GenBank (Table 1.2). The sequences were aligned using Bioedit, alignment was optimized manually and the genes were combined to perform phylogenetic analysis. Parsimony analysis was carried out by PAUP v. 4.0b10 (Swofford, 2002). Ambiguously aligned regions

were excluded from all analyses and the gaps were treated as missing data. Trees were figured in Treeview (Page, 1996). One of the most parsimonious trees generated from combined gene analysis for 35 strains is provided (Figure 1.2).

The phylogenetic tree based on GenBank sequences including sequences originating from seven ex-type cultures, and other sequences are identified as appeared in recent publications. We have used 35 isolates in the multilocus phylogenetic tree, as there is a lack of sequence data of all three genes for all known ex-type cultures, some of which therefore had to be excluded from the analyzed dataset (Table 1.2). However, we recommend the improvement of the multi-locus phylogenetic analysis by using more phylogenetically informative genes, and more ex-type isolates in future work. Incorporation of more ex-type sequences will accelerate the accurate identification of other species from various geographical locations and a wide range of hosts.

Table 1.2 Details of *Guignardia* and *Phyllosticta* Isolates and GenBank Accession Number of Their Sequence Data

Species	Strain[1]	Substrate	Country	ITS	ACT	TEF1
G. bidwellii	CBS 111645	*Parthenocissus quinquefolia*	USA	EU683672	-	EU683653
G. citricarpa	CBS 102345	*Citrus aurantium,*	Brazil	FJ538311	FJ538427	FJ538369
G. citricarpa	CBS 122482	*Citrus sinensis*	Zimbabwe	FJ538317	FJ538433	FJ538375
G. citricarpa	CBS 122384	*Citrus limon*	South Africa	FJ538316	FJ538432	FJ538374
G. citricarpa (ex-epitype)	CBS 127454	*Citrus limon*	Australia	JF343583	JF343667	JF343604
G. mangiferae	CBS 115046	*Myradcrodruon urundeuva*	Brazil	FJ538322	FJ538438	FJ538380
G. mangiferae	CBS 115047	*Aspidosperma polyneuron*	Brazil	FJ538323	FJ538439	FJ538381
G. mangiferae	CBS 114751	*Spondias mombin*	Brazil	FJ538349	FJ538465	FJ538407
G. mangiferae	CBS 115049	*Bowdichia nitida*	Brazil	FJ538324	FJ538440	FJ538382
G. mangiferae (ex-type)	IMI 260576	*Mangifera indica*	India	JF261459	JF343641	JF261501
G. psidii (ex-type)	CBS 100250	*Psidium guajava*	Brazil	FJ538351	FJ538467	FJ538409
G. vaccinii	CBS 126.22	*Oxycocus macrocarpus*	U.S.A	FJ538353	FJ538469	FJ538411
P. bifrenariae (ex-type)	VIC30556; CBS 128855	*Bifrenaria harrisoniae*	Brazil	JF343565	JF343649	JF343586
P. brazilianiae	LGMF 333	*Mangifera indica*	Brazil	JF343574	JF343658	JF343595
P. brazilianiae	LGMF 334	*Mangifera indica*	Brazil	JF343566	JF343650	JF343587

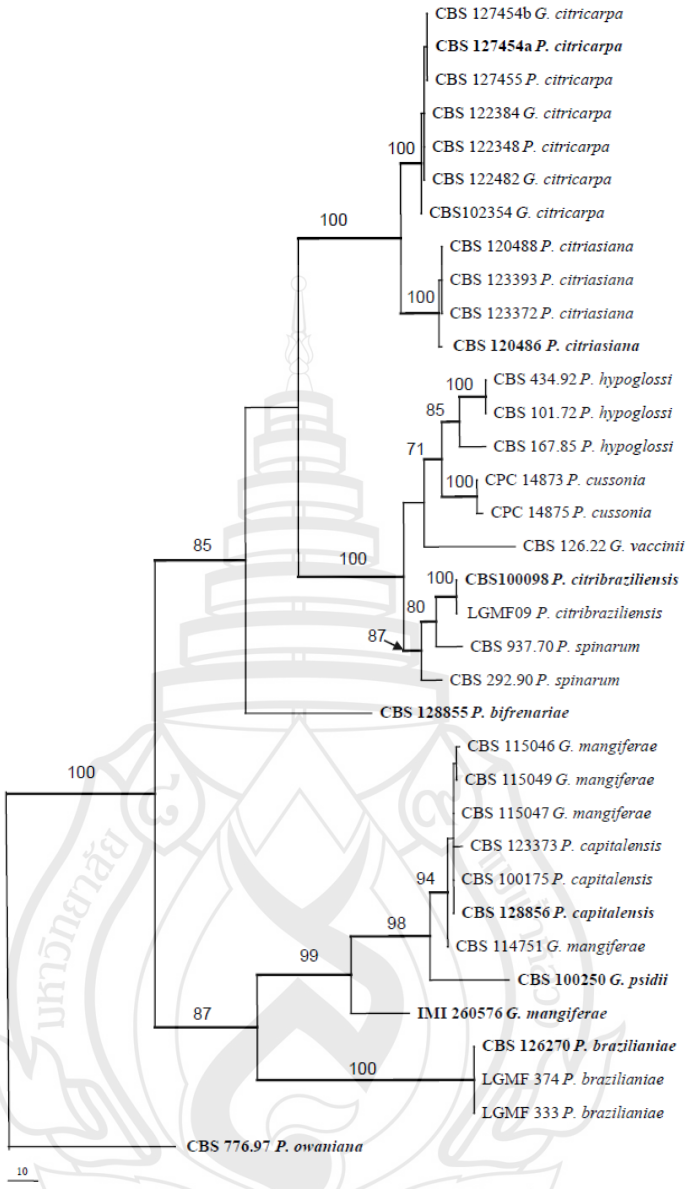
Table 1.2 (continued)

Species	Strain[1]	Substrate	Country	ITS	ACT	TEF1
P. brazilianiae (ex-type)	CBS 126270	*Mangifera indica*	Brazil	JF343572	JF343656	JF343593
P. capitalensis	CBS 100175	*Citrus* sp.	Brazil	FJ538320	FJ538436	FJ538378
P. capitalensis	CBS 123373	*Musa paradisiaca*	Thailand	FJ538341	FJ538457	FJ538399
P. capitalensis (ex-epitype)	CBS 128856	*Stanhopea graveolens*	Brazil	JF261465	JF343647	JF261507
P. citriasiana	CBS 120488	*Citrus maxima*	Thailand	FJ538354	FJ538470	FJ538412
P. citriasiana	CBS 123393	*Citrus maxima*	Vietnam	FJ538358	FJ538474	FJ538416
P. citriasiana	CBS 123372	*Citrus maxima*	Vietnam	FJ538357	FJ538473	FJ538415
P. citriasiana (ex-type)	CBS 120486	*Citrus maxima*	Thailand	FJ538360	FJ538476	FJ538418
P. citribraziliensis (ex-type)	CBS 100098	*Citrus* sp.	Brazil	FJ538352	FJ538468	FJ538410
P. citribraziliensis	LGMF 09	*Citrus* sp.	Brazil	JF261436	JF343618	JF261478
P. citricarpa	CBS 122348	*Citrus sinensis*	Zimbabwe	FJ538315	FJ538431	FJ538373
P. citricarpa	CBS 127455	*Citrus sinensis*	Australia	JF343584	JF343668	JF343605
P. citricarpa (ex-epitype)	CBS 127454	*Citrus limon*	Australia	JF343583	JF343667	JF343604
P. cussonia	CPC 14873	*Cussonia* sp.	South Africa	JF343579	JF343663	JF343600
P. cussonia	CPC 14875	*Cussonia* sp.	South Africa	JF343578	JF343662	JF343599

Table 1.2 (continued)

Species	Strain[1]	Substrate	Country	ITS	ACT	TEF1
P. hypoglossi	CBS 101.72	*Ruscus aculeatus*	Italy	FJ538365	FJ538481	FJ538423
P. hypoglossi	CBS 434.92	*Ruscus aculeatus*	Italy	FJ538367	FJ538483	FJ538425
P. hypoglossi	CBS 167.85	*Ruscus hypoglossum*	Italy	FJ538366	FJ538482	FJ538424
P. owaniana	CBS 776.97	*Brabejum stellatifolium*	South Africa	FJ538368	FJ538484	FJ538426
P. spinarum	CBS 292.90	*Chamaecyparis pisifera*	France	JF343585	JF343669	JF343606
P. spinarum	CBS 937.70	*Hedera helix*	Italy	FJ538350	FJ538466	FJ538408

Note. [1]CBS Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; LGMF: Culture collection of Laboratory of Genetics of Mycroorganisms, Federal University of parana, Curitiba, Brazil; VIC: Culture Collection of Federal University of Vicosa, Vicosa, Brazil.



Note. Sequence Data of *Phyllosticta* spp downloaded from GenBank. Strictly consensus branches are thickened and bootstrap support values >70% are shown below or above the branch

Figure 1.2 Phylogram of *Phyllosticta* Generated from the Parsimony Analysis Based on Combined ITS, ACT, TEF1 Sequence Data for *Phyllosticta* spp.

1.6 Ecology of *Phyllosticta* Species

Phyllosticta species are important plant pathogens and, although taxa are also commonly identified as endophytes (Baayen et al., 2002; Rodrigues & Sieber, 2004), a few species have also been reported as saprobes. In some cases a species may occupy more than one life mode. For example, *P. capitalensis* was originally described on *Stanhopea* (*Orchidaceae*) from Brazil as a fungal pathogen. Recently, Silva, Pereira, Braga and Leli (2008) reported that *P. capitalensis* caused disease of leaves and pseudobulbs of *Bifrenaria harrisoniae* (*Orchidaceae*) in Brazil. *Phyllosticta capitalensis* has also been reported as an endophyte on ericaceous plants in Japan by Okane, Nakagiri and Ito (2001), and non-pathogenic strains have been isolated from *Citrus* sp. (Table 1.3) (Baayen et al., 2002; Glienke et al., 2011) .

Table 1.3 *Phyllosticta* Species Recorded as Endophytes

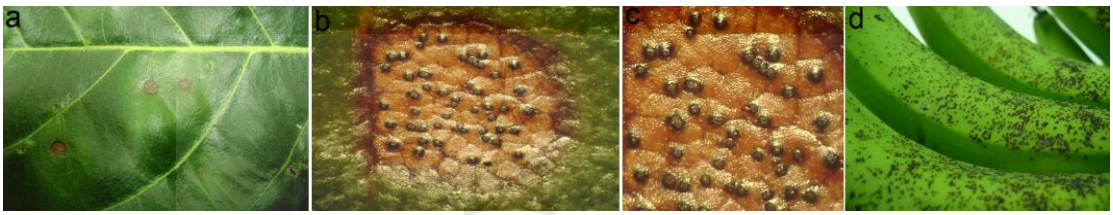
Species	Hosts	Country	Plant tissue	References
P. bifrenariae	*Orchidaceae*	Brazil	Leaf and bulb	Glienke et al. (2011)
P. brazilianiae	*Anacardiaceae*	Brazil	Leaf and fruit	Glienke et al. (2011)
P. capitalensis	Various hosts, woody plant	New Zealand	Leave and fruit	Baayen et al. (2002), Glienke et al. (2011)
P. ilicina	*Quercus ilex* (*Fagaceae*)	Switzerland	Leaves or needles	Collado, Platas and Peláez (1996)
P. spinarum	*Platycladus orientalis* (*Cupressaceae*)	USA	Leaves	Wijeratne et al. (2008)
Phyllosticta sp.	*Ginkgo biloba* (*Ginkgoaceae*)	Japan	Leaf, petiole, twigs	Thongsandee Matsuda and Ito (2011)
Phyllosticta sp.	*Abies grandis* (*Pinaceae*)	USA	Leaves or needles	Carroll and Carroll (1978)

1.7 Host Specificity of *Phyllosticta* and Disease Symptoms

Phyllosticta species may cause leaf spot on many plant species and it is not clear if they are generalists or host-specific; this may depend on the particular species or their life style. It is known that several species that cause diseases are host, genus or family specific, while endophytes may be generalists. For example *P. sphaeropsoidea* Ellis & Everh. causes leaf blotch disease specific to horse chestnut in Europe and North America (Hudson, 1987). *Phyllosticta citricarpa* causes leaf spot disease of *Citrus* species, while *P. citriasiana* can infect fruits of *Citrus maxima* (pomeloes), and causes tan spot and has only been isolated from pomeloes, but has never been found from lemons, mandarins and oranges, and *Phyllosticta musarum* is specific on *Musa* spp. (Wulandari et al., 2009; Wulandari, To−Anun, Lei et al., 2010; Wang et al., 2012). *P. capitalensis* is an endophyte of a wide range of hosts (Okane et al., 2003; Baayen et al., 2002).

Knowledge of disease symptoms on hosts are important for field identification by taxonomists as well as plant pathologist interested in disease occurrence, management and distribution. Generally, *Phyllosticta* species cause necrotic lesions on leaves, which are characteristically small, often 1-2 mm in diameter, circular, brown in the middle and dark brown or sometimes reddish at the margin (Figure 1.3a). One to more than 10 pycnidia are often found in one lesion (Figure 1.3b, c). Pycnidia on leaves are usually black, globose or subglobose, and semi-immersed (Figure 1.3c). After infection by *Phyllosticta* the leaf may become dry in the centre of the lesion, causing the infected tissue to drop out, forming a hole, and hence this is known as target spot or shot hole spot. Leaf spot often occur in living leaves in the late dry and wet seasons or in winter in temperate countries. There are four types of leaf spot symptoms - hard spot, false melanose, freckle spot and virulent spot (Kotzé, 2000). In July 1984, *Phyllosticta* species were the cause of problems on *Muehlenbeckia adpressa* in Victoria, Australia. Virtually all mature leaves of plants contained distinctive necrotic spots for an area of 10 sq. m. Spots were roughly circular to elliptical in shape and were tan with a maroon margin (Paul & Blackburn, 1986). Freckle disease occurs on several species and varieties of banana (Figure 1.3d).

Characteristic spots (pycnidia and ascomata) form on fruit, giving the lesion a sandpaper texture. Leaves of banana will turn yellow when infected with this fungus (Wulandari, To−Anun, Lei et al., 2010).



Notes. a. Leaf spot. b–c. Lesion on adaxial surface. d. Banana freckle disease

Figure 1.3 *Phyllosticta* sp. on Living Leaf of Jackfruit

1.8 *Phyllosticta* Species as Endophytes

Endophytes are fungi that asymptomatically colonize plant tissues during some phase of their life cycle (Petrini, 1991; Hyde & Soytong, 2008; Saikkonen, 2007), but may turn pathogenic during host senescence (Rodriguez & Redman, 2008; Rodriguez et al., 2009). The relationship may be symbiotic, antagonistic, neutral or mutualistic (Hyde & Soytong, 2008; Aly, Debbab & Proksch, 2011). Endophytes are horizontally transmitted, and transfer to their host plants via airborne spores. However, some endophytes may also be vertically transmitted to the next host plant generations via seeds (Hartley & Gange, 2009). Although the first discovery of endophytes dates back to 1904, they did not receive much attention until the recent recognition of their pharmaceutical and ecological significance (Gunatilaka, 2006). Recent development of screening technologies revealed the great potential of endophytes as a major source of biologically active compounds (Strobel & Daisy, 2003; Huang, Cai, Hyde, Corke & Sun, 2008; Xu, Ebada & Proksch, 2010; Tan & Zou, 2001). Investigations related to endophytic microorganisms in plants and

especially tropical hosts have increased, due to the significance of using endophytes in biological control and the discovery of biologically active compounds (Le Calvé et al., 2011).

Although *Phyllosticta* species have been reported as endophytes there are relatively few reports of *Phyllosticta* species being recorded as endophytes in recent studies. In two volumes of the journal Fungal Diversity (Volume 41, 2010; Volume 47, 2011) there were 13 manuscripts devoted to biodiversity of fungal endophytes and only one (Lin et al., 2010) reported an endophytic *Guignardia* species. *Phyllosticta capitalensis* however has commonly been recorded as an endophyte in several studies (Baayen et al., 2002; Glienke et al., 2011; Okane et al., 2003) and was reported as an endophyte in more than 20 hosts in eight countries (Wulandari, To−Anun, Lei et al., 2010; Glienke et al., 2011). Therefore, the species is thought to be one of the most common endophytic species of *Phyllosticta* (Glienke et al., 2011). There are few records of other *Phyllosticta* species recorded as endophytes and they are usually listed as unidentified *Phyllosticta* sp. (Pandey et al., 2003). Some *Phyllosticta* species reported as endophytes are listed in Table 1.3.

1.9 *Phyllosticta* Species as Saprobes

Most fungi have the ability to grow as saprobes, and degrade organic material from dead plant material as a food source. Plant pathogenic fungi can often survive as saprobes between growing seasons (Trigiano, Windham & Windham, 2004). For example, *Phyllosticta carpogena* and *P. ericae* occurred as saprobes on *Rubus* sp. (*Rosaceae*) and *Erica carnea* (*Ericaceae*), respectively (van der Aa et al., 2002) (Table 1.4).

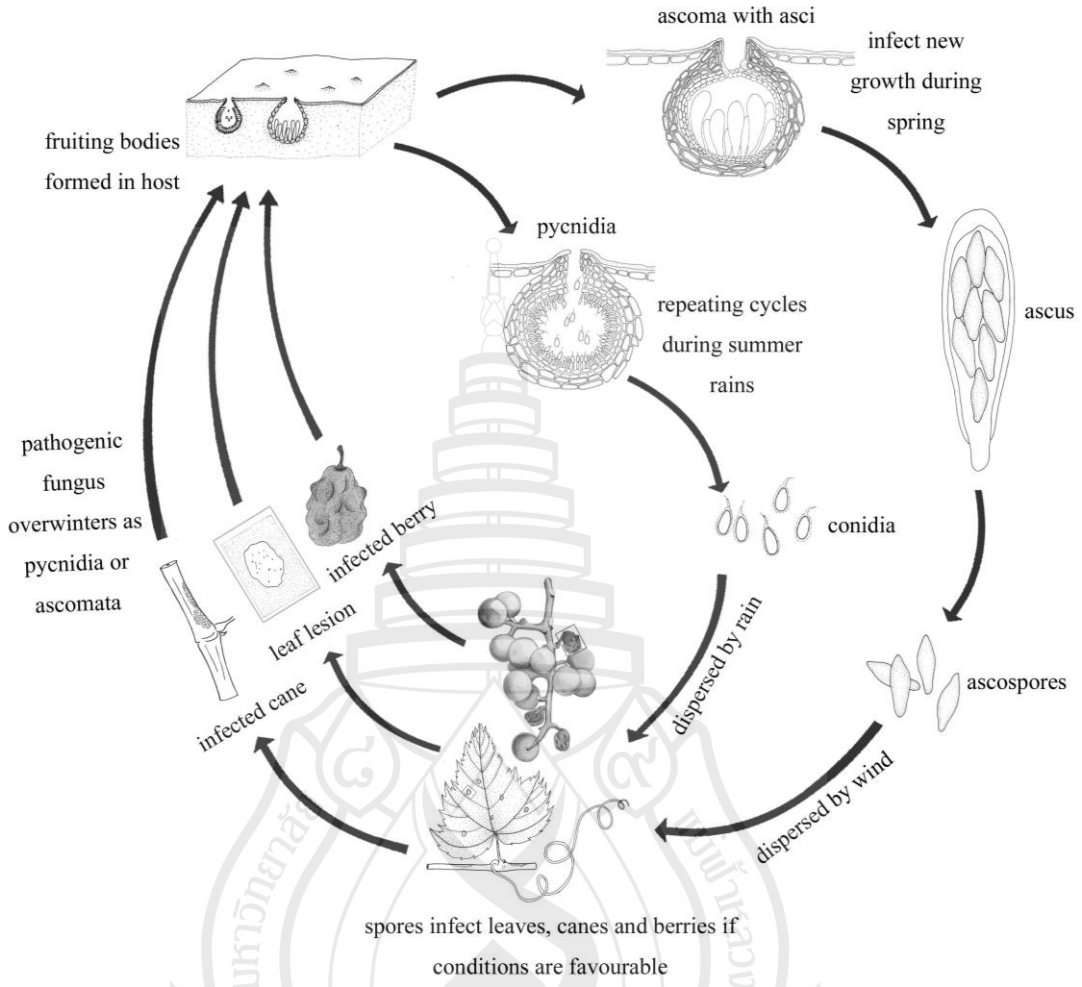
Table 1.4 *Phyllosticta* Species Recorded as Saprobes in Selected Studies.

Species	Host	Country	Plant organ	References
P. acetosellae	*Rumicis acetosellae*	England	Leaves	Smith and Ramsbottom (1913)
P. capitalensis	*Magnolia liliifera*	Thailand	Senescent leaves	Okane et al. (2003)
P. cocoicola	Palm	-	Leaves	Punithalingam (1974), Taylor and Hyde (2003)
P. pyrolae	*Pyrola rotundiforia*	America	Leaves	Ellis and Everhart (1889)

1.10 Life Cycle of *Phyllosticta* and Its Teleomorph

Concepts concerning life cycles of plant pathogens may have significant practical consequences for plant pathologists and taxonomists. Herein, we provide a schematic diagram of the life cycle of a typical species of *Phyllosticta* (*Phyllosticta ampelicida*) (Figure 1.4).

After infection by the *Phyllosticta* or *Guignardia* propagules, pycnidia and/or ascomata develop under the leaf tissue, and produce Leaf spot on the host. During the wet season conidia and ascospores, and sometimes the spermatial stage are present. Subsequently spores are released and ejected from the pycnidia and ascomata. The spores are carried by rain and wind to other leaves and young fruits. Germ tubes develop from spores and grow into leaves and develop within the plant tissue. The disease spreads by transmission by warm wind and rain during the wet season.



Note. Schematic representation of the life cycle of *Phyllosticta* and its sexual state (Wilcox, 2003)

Source http://www.nysipm.cornell.edu/factsheets/grapes/ diseases/ grape_br.pdf

Figure 1.4 Life Cycle of *Phyllosticta* and Its Sexual State

1.11 Secondary Metabolites from Species of *Phyllosticta*

Fungi are well-known as a good source of important metabolites, some of which are useful in the pharmocological industry and agriculture (Pearce, 1997; Smith & Casey, 2008; Aly, Debbab, Kjer & Proksch, 2010; Xu et al., 2011; Udayanga et al., 2011). Both novel and previously known metabolites have been isolated from species of *Phyllosticta* (Table 5, 6). Metabolites produced by *Phyllosticta* species include phyllostin and phyllostoxin. Phyllostictines A-D that were isolated from *P. cirsii* (Evidente, Cimmino, Andolfi, Vurro, Zonno, Cantrell et al., 2008). Phytotoxins, including phyllosinol, brefeldin, and PM-toxin (Sakamura, Niki, Obata, Sakai & Matsumoto, 1969; Sakai, Sato, Niki & Sakamura, 1970) were extracted from *Phyllosticta maydis* (Comstock, Martinson & Gengenbach, 1973) and *Phyllosticta medicaginis* (Entwistle, Howard & Johnstone, 1974). Phyllostictines A-D have been tested on five cancer cell lines which displayed growth-inhibitory activity (Le Calvé et al., 2011). Five new metabolites were isolated from *P. spinarum*, reported by Wijeratne et al. (2008) namely (+)-(5S,10S)-4′hydroxymethylcyclozonarone, 3-etotauranin, 3-hydroxytauranin, 12-hydroxytauranin, phyllospinarone.

Taxol was initially known as a phytochemical derived from the bark of *Taxus brevifolia* (Western Yew) and is an expensive and important diterpenoid anti-cancer intensive treatment drug used against breast, ovarian and lung cancers (Wani, Taylor, Wall, Coggon & McPhail, 1971). It has been reported that the molecule has anti-tumour activity in several experimental trials. Taxol is produced by various fungal strains of *Pestalotiopsis* (Strobel et al., 1996; 1997), *Phomopsis* (Kumaran & Hur, 2009) and *Phyllosticta* (Kumaran, Muthumary & Hur 2008a; 2008b; Kumaran & Hur, 2009) in culture media under various conditions. Taxol has been reported from *P. citricarpa* from *Citrus medica* and *P. dioscoreae* from *Hibiscus rosa-sinensis* (Kumaran et al., 2008a; Kumaran, Muthumary & Hur, 2009). Species of *Phyllosticta* are therefore potential sources for discovery of pharmaceutical, medical and agricultural novel compounds.

Table 1.5 Metabolites Produced by *Phyllosticta* Species

Compounds	Properties	*Phyllosticta* spp.	References
Befeldin	Bioactive metabolite	*P. medicaginis*	Entwistle et al. (1974)
Phyllosinal	Bioactive metabolite	*Phyllosticta* sp., *P. maydis*	Sakamura et al.(1969), Sakai et al.(1970)
Phyllostictine	Mycoherbicide	*P. cirsii*	Evidente, Cimmino, Andolfi, Vurro, Zonno, Cantrell et al. (2008)
Phyllostin	Anti-microbial, anti-cancer	*P. cirsii*	Evidente, Cimmino, Andolfi, Vurro, Zonno and Motta (2008), Le Calvé et al. (2011)
Phyllostoxin	Mycoherbicide	*P. cirsii*	Evidente, Cimmino, Andolfi, Vurro, Zonno and Motta (2008)
PM−toxin	Mycoherbicide	*P. maydis*	Comstock et al. (1973)
Tauranine	Anti-cancer activity	*P. spinarum*	Wijeratne et al. (2008)
Taxol	Anti-cancer activity	*P. tabernaemontanae*	Kumaran and Hur (2009)
(+)−(5S,10S)−4′ hydroxymethylcy clozonarone	Inhibition of cell proliferation	*P. spinarum*	Wijeratne et al. (2008)
3−ketotauranin	Inhibition of cell proliferation	*P. spinarum*	Wijeratne et al. (2008)
3 –hydroxytauranin	Inhibition of cell proliferation	*P. spinarum*	Wijeratne et al. (2008)
12−hydroxytauranin	Inhibition of cell proliferation	*P. spinarum*	Wijeratne et al. (2008)
Phyllospinarone	Inhibition of cell proliferation	*P. spinarum*	Wijeratne et al. (2008)

1.12 *Phyllosticta* in Biocontrol

Biocontrol is “the control of unwanted organisms such as weeds by the use of other organisms, as through the use of organisms that are natural predators, parasites, or pathogens. Fungi are commonly used as biological control agents (Charudattan & Dinoor, 2000; Mortensen, 1998; Trujillo, 2005; Rosskopf, Charudattan, DeValerio & Stall, 2000). *Phyllosticta* species may have potential for use as biocontrol agents although there are presently few examples (Table 1.6). A strain of *Phyllosticta* (Ph511) was shown to produce compounds that had high effect on motility of the second stage juveniles of *Meloidogyne incognita* and has potential in parasitic nematode control (Yan et al., 2011). *P. cirsii*, a pathogen isolated from diseased leaves of *Cirsium arvense* and evaluated as a potential biocontrol agent of this noxious perennial weed, also produces different phytotoxic metabolites with potential herbicidal activity when grown in liquid cultures (Evidente, Cimmino, Andolfi, Vurro, Zonno, Cantrell et al., 2008). The metabolites reported are Phyllostictines A-D which are potential mycoherbicides (Berestetskiy et al. 2008, Evidente, Cimmino, Andolfi, Vurro, Zonno & Motta 2008, Evidente, Cimmino, Andolfi, Vurro, Zonno, Cantrell et al., 2008).

Table 1.6 *Phyllosticta* Species Used in Biocontrol

Species	Host	Compound	Used against	References
Phyllosticta cirsii	*Cirsium arvense*	Phyllostictine A−D	*Cirsium arvense* (weed)	Evidente, Cimmino, Andolfi, Vurro, Zonno and Motta (2008)
Phyllosticta sp.	*Cirsium arvense*	Phyllostin (8−hydroxy−3−methyl −2−oxo−2,3,4a,5,8,8a− hexahydro−benzo[1,4]d ioxine−6 carboxylic acid methyl ester)	*Cirsium arvense*	Tuzi Andolfi, Cimmino and Evidente (2010)
Phyllosticta sp.	*Cucumis sativus*	*Phyllosticta* Ph511	*Meloidogyne incognita* root knot nematode	Yan et al. (2011)

1.13 Need for Epitypification of *Phyllosticta* Species

An epitype is a fresh specimen (usually with accompanying culture) selected to serve as a representative type when such authentic material has been recollected, and confirmed to represent the same species as the original type material (Phillips, Oudemans, Correia & Alves 2006; Hyde, Abd-Elsalam et al., 2010, Hyde, Chomnunti et al.,2010; Abd-Elsalam et al., 2010). This practice enables mycologists to link older names to DNA data derived from fresh collections. The sole purpose of epitypification, is to move mycology into the culture and DNA era. When an epitype is designated the original material that the epitype supports must be explicitly cited. Several authors have discussed the urgent need for epitypication in plant pathogenic genera (Verkley, Crous, Groenewald, Burun & Aptroot, 2004; Crous, 2005; Crous, Braun & Groenewald, 2007; Shenoy et al., 2007; Hyde, Abd-Elsalam et al., 2010; Hyde, Chomnunti et al., 2010).

1.14 Notes on Selected Species of *Phyllosticta*

Molecular data has to date proven to be inadequate in resolving species in the genus *Phyllosticta* (Wulandari et al., 2009). Either there are very few species of *Phyllosticta* with some having a very wide host range or the genes that we are presently using do not resolve species complexes. Differentiation of the 192 species accepted by van der Aa (1973) and van der Aa et al. (2002) was based on morphological data with often minor differences, and molecular evidence is not available to support this differentiation. Most species also lack living cultures and their uniqueness cannot be confirmed. For this reason it is not possible to list which species can be stated as currently in use in *Phyllosticta*, as has been done for *Cochliobolus* (Manamgoda, Cai, Bahkali, Chukeatirote & Hyde, 2011), *Colletotrichum* (Hyde et al., 2009), *Fusarium* (Summerell et al., 2011) and *Phomopsis* (Udayanga et al., 2011).

Below we discuss alphabetically selected *Phyllosticta* names which includes the generic type, an earlier name for the generic type, plant pathogens and endophytes resolved using molecular data, all taxa introduced since van der Aa et al. (2002) and some other taxa that we believe warrant discussion and is partly based on the most recent literature. This cannot be considered as a thorough account of the generic species, but provides a starting point towards establishing the number of acceptable species in future revisionary treatments of *Phyllosticta* as phylogenetic data accumulates and helps to resolve the species.

The account of selected species names provides authorities and publication details in Index Fungorum (http://www.indexfungorum.org/names/Names.asp). Synonyms are not given as these can be searched for in *Index Fungorum*. The teleomorph is given where known. This is not a rigorous list as it is impossible to verify at this stage whether collections of each taxon on a host are correctly identified. We have annotated the notes with host range, symptoms and known distribution, and additional notes on pathological, taxonomic and phylogenetic research. Also the additional notes emphasize the need for molecular data in future studies. We recommend that other resolved species are added to this selected list based on future

studies of *Phyllosticta* employing molecular and morphological data as has been done in *Colletotrichum* (Phoulivong, 2011).

When referring to *Phyllosticta* species one also has to consider the teleomorph *Guignardia*. However, although there is molecular data for this teleomorphic genus, it is mostly for the endophyte incorrectly identified as *G. mangiferae* (e.g. *P. capitalensis*). We therefore do not discuss *Guignardia* species below. However, *Guignardia psidii* Ullasa & Rawal, was shown to be distinct in the molecular analysis of Wang et al. (2012). This strain (CBS 100250) was isolated from a fruit of *Psidium guajava* collected from Sao Paulo, Brazil. The *Phyllosticta* state is unknown. Several new species of *Guignardia* have also been described in recent years (e.g. *G. musicola* N.F. Wulandari, L. Cai & K.D Hyde, *G. bispora* N.F. Wulandari & K.D. Hyde, Wulandari, To-Anun & Hyde, 2010; Wulandari et al., 2011) and these also need recollecting and sequencing to establish their relationships with species of *Phyllosticta* and whether they can be considered as distinct species.

Phyllosticta ampelicida (Engelm.) Aa, Stud. Mycol. 5: 28 (1973)

Sexual state: *Guignardia bidwellii* (Ellis) Viala & Ravaz, Bull. Soc. mycol. Fr. 8:63 (1892)

Hosts: *Vitis* spp., *Ampelopsis* spp., *Cissus* spp., *Parthenocissus* spp. (*Vitaceae*).

Disease symptoms: Black rot

Distribution: Asia, Canada, South America, UK and USA

Notes: This *Phyllosticta* species is linked to the generic type of *Guignardia* and has a *Leptodothiorella* spermatial state. The black rot fungus can infect all parts of the vine, although the most significant losses are caused by berry infection (Reddik, 1911; Ellis et al., 2004). In warm humid climates, susceptible varieties can experience complete loss if the pathogen is left uncontrolled. Sequences of reference isolates of this species are available in GenBank but it has not yet been epitypified.

Phyllosticta ardisiicola Motohashi, I. Araki & C. Nakash., Mycoscience 49(2): 139 (2008)

Hosts: *Ardisia crenata* (*Myrsinaceae*)

Disease symptoms: Leaf spot

Distribution: Japan

Note: This species was introduced as new species based on its morphological differences with taxa from related hosts (Motohashi et al., 2008). Molecular data should be included in future work to confirm the status with closely related species.

Phyllosticta aspidistricola Motohashi, I. Araki & C. Nakash., *Mycoscience* 49(2): 140 (2008)

Hosts: *Aspidistra elatior* (*Liliaceae*)

Disease symptoms: Leaf spot

Distribution: Japan

Note: This species were introduced as new species base on its morphological differences with taxa from related hosts (Motohashi et al., 2008). Molecular data should be included in future work to confirm the status with closely related species.

Phyllosticta beaumarisii A.P. Paul & M.D. Blackburn, Australas. Pl. Path. 15: 41 (1986)

Spermatial state: *Leptodothiorella* sp.

Hosts: *Muehlenbeckia adpressa* (*Nyctaginaceae*)

Disease symptoms: Leaf spot with distinctive necrotic lesions

Distribution: Australia

Note: The disease is prevalent during autumn and winter. Mature diseased leaves age and abscise when a new flush of growth occurs in spring. Pathogenicity testing has shown that *P. beaumarisii* is the causal agent of the disease, and not other species of *Phyllosticta* associated with the host (Paul & Blackburn, 1986). Although Yip (1987) provided a full description and illustration of the species, molecular data is needed in future studies to confirm its status as a distinct species.

Phyllosticta bifinariae O.L. Pereira, C. Glienke & Crous, Persoonia 26: 52 (2011)

Hosts: *Bifrenaria harrisoniae* (*Orchidaceae*)

Disease symptoms: Leaf spot

Distribution: Brazil

Notes: This isolate was originally thought to be representative of *P. capitalensis* but was found to be ecologically and phylogenetically distinct and a pathogen of *Bifrenaria harrisoniae* (Glienke et al., 2011).

Phyllosticta brazilianiae D. Stringari, C. Glingke & Crous, Persoonia 26: 53 (2011)

Hosts: *Mangifera indica* (*Anacardiaceae*)

Disease symptoms: Symptomless endophyte

Distribution: Brazil

Note: The species is ecologically distinct from *P. anacardiacearum* being an endophyte, and failed to induce Leaf spot despite repeated inoculation on mango (Glienke et al., 2011). Molecular data has also shown that it is distinct from other closely related species.

Phyllosticta capitalensis Henn., Hedwigia 48: 13 (1908)

Hosts: *Phyllosticta capitalensis* was originally described on *Stanhopea* (*Orchidaceae*) from Brazil by Hennings (1908) although now thought to occur in wide range of hosts.

Disease symptoms: Leaf spot (when cause disease)

Distribution: Worldwide

Note: *Phyllosticta capitalensis* is the most recently proposed name for the entities that were formerly incorrectly referred to as *Guignardia mangiferae* (Baayen et al., 2002; Glienke et al., 2011). The taxon is frequently isolated as an endophyte and has a wide host range and geographic distribution. Okane, Nakagiri and Ito (2001) identified an endophytic *Phyllosticta* strain in ericaceous plants from Japan, *as Phyllosticta capitalensis*, describing the teleomorph as a new species, *G. endophyllicola*. Baayen et al. (2002) recognized the common endophytic species associated with a wide host range of plants based on ITS sequence similarities, which was similar to *G. endophyllicola* in morphology. Although several names were available for this species, they opted to call the species *G. mangiferae* (a pathogen on *Mangifera indica* (*Anacardiaceae*) in India), while the anamorph was referred to as *P. capitalensis*. Although no clear argument was presented for choosing the name *G. mangiferae* for this fungus, the choice of the anamorph name was based on the fact that two isolates from *Orchidaceae* (CBS 398.80, CBS 226.77) clustered in the same clade in their study. A comprehensive study of endophytic and pathogenic *Phyllosticta* species on *Citrus* was carried out by Glienke et al. (2011). Their combined phylogenetic tree revealed the *P. capitalensis sensu lato* clade to be genetically distinct from a reference

isolate of *G. mangiferae* isolated from India. Several names were available for this clade, the oldest being *P. capitalensis*. Glienke et al. (2011) therefore, suggested that endophytic, non-pathogenic isolates occurring on a wide host range would be more correctly referred to as *P. capitalensis*. However, more genes need to be analyzed to fully resolve the morphological variation still observed within this clade.

Phyllosticta citriasiana Wulandari, Crous & Gruyter, Fungal Diversity 34: 31 (2009).

Hosts: *Citrus maxima* (*Rutaceae*)

Disease symptoms: tan spots (produces shallow lesions with a small central grey to tan crater usually with dark brown margin on fruits)

Distribution: Asia (China, Thailand, Vietnam)

Notes: The tan spot symptom usually appears after the fruit has started to ripen and sometimes it can occur after harvest. Combined gene analysis, morphological and culture based characters were employed to distinguish the species from *P. citricarpa* and other species considered (Wulandari et al., 2009). Recent studies on endophytic and pathogenic species of *Phyllosticta* from citrus in different regions of the world shows that the morphological, cultural and biochemical characters for species were consistent with the results of phylogenetic analysis of related taxa (Glienke et al., 2011; Wang et al., 2012). A specific primer pair Pca8/ITS4 was also designed and selected, and a PCR protocol was used to detect *P. citriasiana* in a recent study (Wang et al., 2012).

Phyllosticta citribraziliensis C. Glienke & Crous, Persoonia 26: 54 (2011)

Hosts: *Citrus limon* (*Rutaceae*)

Disease symptoms: Symptomless endophyte

Distribution: Brazil

Notes: This species is closely related to *P. spinarum* but phylogenetically distinct. Also *P citribraziliensis* is morphologically distinguished in having larger conidia, a thick mucilaginous sheath surrounding its conidia and branched conidiophores.

Phyllosticta citricarpa (McAlpine) van der Aa, Stud. Mycol. 5: 40 (1973)

Sexual state: *Guignardia citricarpa* Kiely

Hosts: *Citrus aurantius, C. limon, C. delicoisa, C. reticulata, C. sinensis* (*Rutaceae*)

Disease symptoms: Black spot of citrus, foliar and fruit diseases, premature fruit drop

Distribution: Asia, Africa, Australia, USA (Florida)

Notes: *P. citricarpa* causes foliar and fruit disease of *Citrus* spp. *G. citricarpa* (anamorph *P. citricarpa*) which causes citrus black spot is regulated as a quarantine pest in the European Union and the USA (Wang et al., 2012). This pathogen can infect the rind of Citrus fruit causing disease lesions (Kiely, 1948a). Serious infection near the pedicel of the developing fruit possibly will lead to premature fruit drop (Baayen et al., 2002). The first report of Black spot on Citrus orchards was near Sydney, Australia and it was described as *Phoma citricarpa* McAlpine (McAlpine, 1899). The teleomorph was described as *Guignardia citricarpa* Kiely (Kiely, 1948b). van der Aa (1973) classified the anamorph as *Phyllosticta citricarpa* (McAlpine) Van der Aa. The species was recollected from Australia and an epitype was designated and the distinctiveness from *P. citriasiana* was confirmed (Glienke et al., 2011).

Phyllosticta citrichinaensis H.X. Wang, K.D. Hyde & H.Y. Li, Fungal Diversity (2012)

Hosts: *Citrus* spp. (*Rutaceae*)

Distribution: China

Disease symptoms: small grey, red-brown or brown spots and freckles on leaves, melanose like black spots on fruits

Notes: This taxon has been isolated as an endophyte and is also weak pathogen (Wang et al., 2012). *P. citrichinaensis* differs from the other four *Phyllosticta* species associated with citrus in its morphological, cultural and biochemical characteristics.

Phyllosticta convallariae Pers., Traité sur les Champignons Comestibles (Paris): 148 (1818)

Hosts: *Polygonatum* spp., *Convallaria* (*Convallariaceae*), *Maianthemum* (*Liliaceae*).

Disease symptoms: Red leaf spot

Distribution: Asia, Europe

Note: This is the generic type of *Phyllosticta* and has been clearly designated in Donk (1968). This species causes disease called reddish-brown Leaf spot on its host and has a *Leptodothiorella* spermatial state (http://www.uni-graz.at/~oberma/fungi-of-austria/*Phyllosticta*-convallariae.html) and needs recollecting and epitypifying. An earlier name was found in *Phyllosticta cruenta* (van der Aa, 1973) but this needs confirmation based on sequencing collections from the original host.

Phyllosticta cruenta (Fr.) J.J. Kickx, Fl. Crypt. Flandres 1: 412 (1867)

Sexual state: *Guignardia reticulata* (DC.: Fr.) Aa

Hosts: *Polygonatum* spp., *Convallaria* (*Convallariaceae*), *Maianthemum* (*Liliaceae*).

Disease symptoms: Leaf spot

Distribution: Asia, Europe

Notes: *P. cruenta* is the earlier name for type of *Phyllosticta convallariae* and in older literature, it was considered to be an intermediate form between *Phyllostictina* and *Dothiorella* (van der Aa et al., 2002). This taxon should be recollected and epitypified as its distinctiveness from *P. convallariae* needs confirmation.

Phyllosticta cussoniae Cejp, Bothalia 10(2): 341 (1971)

Sexual state: *Guignardia cussonia* Crous

Host: *Cussonia* spp.

Disease symptoms: On leaves causing a prominent leaf spot.

Distribution: South Africa

Notes: In the phylogenic tree presented in Glienke et al. (2011) the isolates of this species clusters in a distinct clade and appears to represent a distinct taxa. Representative isolates were obtained from South Africa by P.W. Crous

Phyllosticta dioscoreae Cooke, Grevillea 6(no. 40): 136 (1878)

Sexual state: *Guignardia dioscoreae* A.K. Pande, Sydowia 22(5-6): 367 (1969) [1968]

Host: *Dioscorea* spp. (*Dioscoreaceae*)

Disease symptoms: Leaf spot

Distribution: Africa (South Africa), Asia, Australia, South America (Brazil), South Pasific (Figi, Tonga, Solomon Islands),USA.

Notes: An isolate identified as *Phyllosticta dioscoreae* from *Hibiscus rosa-sinensis* has been reported to produce the anti-cancer compound taxol (Kumaran , Muthumary & Hur, 2009), but the species was identified on basis of morphological characters and therefore needs confirmation with molecular data. This species commonly causes Leaf spot on *Dioscorea* spp. with its *Guignardia* state usually being produced (N. Wulandari, pers. comm.) and should be recollected and epitypified to establish if it is a distinct species.

Phyllosticta fallopiae Motohashi, I. Araki & C. Nakash., Mycoscience 49(2):141 (2008)

Hosts: *Fallopia japonica*

Disease symptoms: Leaf spot

Distribution: Japan

Note: This species were introduced as a new species based on its morphological differences with taxa from related hosts. Molecular data are needed in future studies to establish its uniqueness (Motohashi et al., 2008).

Phyllosticta hypoglossi (Mont.) Allesch., Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1(6): 163 (1898)

Hosts: Living leaves and dead cladodes *Ruscus spp.* (*Liliaceae*)

Disease symptoms: Occurring on living and dead cladodes and stems, though distinct spots not reported.

Distribution: Asia, Europe (France, Italy, Portugal, Turkey, Ukraine)

Notes: The spermatial state is a *Leptodothiorella* sp. The taxon was originally described from France, and the sequenced isolates used by Glienke et al. (2011) collected from Italy could potentially be used for epitypification.

Phyllosticta kerriae Motohashi, I. Araki & C. Nakash., Mycoscience 49(2): 141 (2008)

Sexual state: Unknown

Hosts: *Kerria japonica*

Disease symptoms: Leaf spot

Distribution: Japan

Note: This species were introduced as new species base on its morphological differences with taxa from related hosts therefore the molecular based re evaluation is needed to establish its uniqueness (Motohashi et al., 2008).

Phyllosticta minima (Berk. & M.A. Curtis) Underw. & Earle, Bulletin of the Alabama Agricultural Experiment Station 80: 168 (1897)

Hosts: *Acer* spp. (Maples) (*Sapindaceae*)

Disease symptoms: Leaf spot (black or purple eye spot).

Distribution: Asia (China), North America

Notes: This pathogen has been reported as a common causative agent of leaf spots on *Acer* spp. (Nelson & Johnson (2005); see more information at website http://www.unce.unr.edu/publications/files/ho/2005/fs0547.pdf). *Phyllosticta gallarum* has been recorded causing similar Leaf spot on *Caragana* spp. Recollection and taxonomic re evaluation is needed to confirm if this species can be differentiated from closely related species.

Phyllosticta musarum (Cooke) Aa, Stud. Mycol. 5: 72 (1973)

Basionym: Sphaeropsis musarum Cook, Grevillea 8 (no.47): 93 (1880)

Sexual state: *Guignardia musae* Racib.

Hosts: *Musa* spp. (*Musaceae*).

Disease symptoms: Leaf spot, fruit spot, banana freckle, banana black spot

Distribution: Widespread

Notes: The pathogen **(***Phyllosticta musarum* or the sexual state *Guignardia*) infects leaves and also affects the external appearance of the fruit, decreasing its quality and marketability. Severe infections of the disease may cause premature death of the older leaves on some banana cultivars. Preinfection studies shows that the pathogen seems to penetrate directly through the epidermal cuticle layer of the host by forming appressoria and infection pegs (Pu et al., 2008). Wulandari, To−Anun, Lei et al. (2010) investigated the problem of the occurrence of the species epithet (“musae”) on separate occasions related to sexual state based on herbarium specimens and fresh collectios and distingushed three different species including taxonomic novelties. However the *Phyllosticta/Guidnardia* species from banana needs to re-evaluated based on morphological and molecular approach in future studies.

Phyllosticta owaniana G. Winter, Hedwigia 24: 31 (1885)

Host: *Brabejum stellatifolium* (*Proteaceae*)

Disease symptoms: Leaf spot

Distribution: South Africa

Notes: *Phyllosticta telopeae* Yip has been reported from *Telopea speciosissima* (*Proteaceae*) and is distingushed from *P. owaniana* by its larger conidia and much longer appendages. Both of the species are accepted in van der Aa et al. (2002). The sequences of the type of *P. owaniana* has been used in phylogenetic analysis but as the outgroup which shows the species to be significantly different from other *Phyllosticta* species. An epitype will be designated based on fresh collections (Glienke et al., 2011).

Phyllosticta solitaria Ellis & Everh., Proc. Acad. nat. Sci. Philad. 47: 430 (1895)

Hosts: *Malus* spp., *Crataegus* spp., *Pyrus* spp., *Rosaceae*

Disease symptoms: Leaf spot, fruit blotch, twig canker.

Distribution: Asia (India, China), Africa (Zimbabwe, South Africa), Europe (Greece), North America (USA), South America (Brazil)

Notes: Although the teleomorph is unknown, Guba (1925) have noticed the fructifications on fallen leaves in spring (van der Aa et al., 2002). *Phyllosticta solitaria* causes a serious blotching of apples which reduces fruit quality. The ability of the fungus to withstand long periods of cold storage should be noted. Re-evaluation of the pathogen and epitypification is needed in future studies.

Phyllosticta sphaeropsoidea Ellis & Everh., Bull. Torrey bot. Club 10(7): 97 (1883)

Sexual state: *Guignardia aesculi* (Peck) V.B. Stewart

Hosts: *Aesculus* spp. (*Hippocastanaceae*).

Disease symptoms: leaf blotch (disease known as buckeye blotch or horse chestnut blotch), black rot , brown leaf margin and necrotic tissue

Distribution: Asia, Europe, North America

Notes: *Guignardia aesculi* (sexual stage), initiates leaf infections in early spring, while *P. sphaeropsoidea* (asexual stage) perpetuates infections during the summer. Infections from both stages combine to cause horse chestnut leaf blotch

(Gillman, 2005; Pastricakova, 2004). Recollection from various host species of *Aesculus* and various geographical locations are needed to establish the uniqueness of the taxon with molecular data.

Phyllosticta spinarum **(**Died**.)** Nag Raj & M. Morelet, Bull. Soc. Sci. nat. Arch. Toulon et du Var 34(219): 12 (1978)

Basionym: *Phoma spinarum* Died., Krypt.-Fl. Brandenburg (Leipzig) 9: 148 (1912)

Hosts: *Juniperus* sp, *Chamaecyparis pisifera*, *Platycladus orientalis* (*Cupressaceae*), *Hedera helix* (*Araliaceae*)

Disease symptoms: None reported, presumed endophyte

Distribution: Europe (Germany, France, Italy), USA

Notes: This was originally described from *Juniperus* sp. in Germany while the isolates sequenced in Glienke et al. (2011) were from *Chamaecyparis pisifera* and *Hedera helix* (from France and Italy). The endophytic isolate putatively identified as *P. spinarum* from *Platycladus orientalis* is known to produce novel secondary metabolites (Wijeratne et al., 2008).

Phyllosticta vaccinii Earle, Bull. Torrey bot. Club 24: 31 (1897)

Sexual state: *Guignardia vaccinii* Shear

Hosts: *Vaccinium* spp. (*Ericaceae*).

Disease symptoms: Blast or blight of flowers and young fruits; early rot of fruits in storage

Distribution: Asia (China), North America

Notes: Weidemann, Boone and Burdsall. (1982) listed the anamorph of *Guignardia vaccinii* as *Phyllosticta elongata*, but van der Aa et al. (2002) lists both *P. elongata* and *P. vaccinii* as anamorphs. A strain of this species (CBS165.86) has been sequenced by Duong (2008) and appears to be a distinct species in phylogenetic analysis. However, future work is needed to establish the uniqueness of taxa.

1.15 Research Objectives

To clarify the species of *Phyllosticta* associated with disease in a range of hosts in Thailand and internationally.

To understand the relationship between morphology and phylogeny characters of *Phyllosticta* species and their relationships with hosts.

To find a group of genes or preferably a single gene that will resolve species in the genus *Phyllosticta* and *Guignardia.*

CHAPTER 2

MORPHOLOGY OF SELECTED GENERA OF *BOTRYOSPHEARIALES*

2.1 Introduction

Botryosphaeria was introduced by Cesati and Notaris (1863). Saccardo (1877) emended the initial generic description and transferred the hypocreaceous species amongst them to *Gibberella* and *Lisea*. Because Cesati and Notaris (1863) did not designate a type species, von Höhnel (1909) suggested *Botryosphaeria berengeriana* De Not., while Theissen and Sydow (1915) suggested *B. quercuum* (Schwein.) Sacc., which could be regarded as generic lectotype. Neither proposal was accepted because these species were not included in the original description of the genus (Cesati & Notaris, 1863). Therefore, Barr (1972) proposed *B. dothidea* (Moug.) Ces. & De Not, one of the species originally included by Cesati and Notaris (1863), as the lectotype of this genus. This proposal has generally been accepted and Slippers et al. (2004) proposed a neotype and epitype to stabilize the type species *B. dothidea* and provided a modern description of this genus based on these new types.

Species of *Botryosphaeria* are cosmopolitan in distribution and occur on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts; on woody branches, herbaceous leaves, stems and culms of grasses; and on twigs and in the thalli of lichens (Barr, 1987; Denman, Taylor, Kang, Pascoe & Michael, 2000; Mohali, Slippers & Wingfield, 2007; Lazzizera, Frisullo, Alves, Lopes & Phillips, 2008; Marincowitz Groenewald, Wingfield & Crous, 2008). Taxa range in habit from saprobic to parasitic orendophytic (Smith, Wingfield, Crous & Coutinho, 1996; Denman et al., 2000; Phillips et al., 2006; Slippers & Wingfield, 2007; Huang et al.,

2008, Pérez, Wingfield, Slippers, Altier & Blanchette, 2010; Ghimire, Charlton, Bell, Krishnamurthy & Craven, 2011; González & Tello, 2011) and cause die-back and canker diseases of numerous woody hosts (von Arx, 1987; Damm, Crous & Fourie, 2007; Phillips, Crous & Alves, 2007; Slippers & Wingfield, 2007; Alves, Crous, Correia & Phillips, 2008; Lazzizera, Frisullo, Alves & Phillips, 2008; Marincowitz et al., 2008; Zhou, Xie, Chen & Wingfield, 2008; Pérez et al., 2010; Adesemoye & Eskalen, 2011; Urbez-Torres et al., 2012). Species of *Botryosphaeria* have also been isolated from marine environments in sea grasses (Sakayaroj, Preedanon, Supaphon, Jones & Phongpaichit, 2010).

The *Botryosphaeriales* was introduced by Schoch et al. (2006) following molecular analysis, and comprises a single family *Botryosphaeriaceae*. This family however, has a rather varied past as can be seen from inclusion of genera by various uthors von Arx and Müller (1954) included 15 genera, but later reduced it to 14 genera by von Arx and Müller (1975). Barr (1987) was much more conservative and included only nine genera, mostly different from those of von Arx and Müller (1954), while Hawksworth, Kirk, Sutton and Pegler (1995) listed five genera and numerous synonyms of *Botryosphaeria*. With the use of molecular data it has been possible to add more new genera to the family *sensu* Hawksworth et al. (1995). Lumbsch and Huhndorf 2010) included 11 genera, while Hyde et al. (2011) and Wijayawardene, Mckenzie & Hyde (2012) listed 20 asexual genera. Phillips and Alves (2009) restudied the botryosphaeriaceous *Melanops*, epitypifying the generic type. In Liu et al. (2012), we accept 29 genera based on molecular data and examination of genetic types. In the chapter 1, illustrate 7 types species used as part of Liu et al. (2012) study.

2.2 Materials and Methods

2.2.1 Examination of Herbarium Material and Fresh Specimens

The type specimens of *Auerswaldiella*, *Leptoguignardia*, *Neodeightonia*, *Phyllachorella*, *Saccharata*, *Sivanesania*, *Spencermartinsia* and *Vestergrenia* were obtained from BPI, K, IMI, LISE, LPS, PREM and S. Fresh material was collected from Chiang Rai in Thailand. Observations and photomicrographs were made from material mounted in water using a Nikon ECLIPSE 80i microscope.Measurements were made with Tarosoft (R) Image Frame Work (Liu, Chomnunti et al., 2010).

2.3 Taxonomy

The results of detailed morphological characterization of type material of type genera of *Botryosphaeriaceae* is presented below. A list of possible synonyms are given for genera and species.

2.3.1 ***Auerswaldiella*** Theiss. & Syd., Ann. Mycol. 12: 278 (1914)

MycoBank: MB454

Possible synonyms:

= *Dimeriellina* Chardón, Bol. Soc. Venez. Cienc. Nat. 5(no. 40): 339 ('239') (1939)

= *Stichodothis* Petr., Ann. Mycol. 25: 198 (1927)

Saprobic on leaves. *Ascostromata* black, solitary, scattered, superficial on lower side, globose, rough, papillate, pulvinate, multiloculate, cells of ascostromata brown-walled *textura angularis*. *Peridium* of locules two-layered, outer layer composed of small heavily pigmented thick-walled cells of *textura angularis*, inner layer composed of hyaline thin-walled cells of *textura angularis. Pseudoparaphyses* hyphae-like, numerous, septate. *Asci* 8-spored, bitunicate, fissitunicate, cylindro-clavate, with a pedicel and an ocular chamber. *Ascospores* biseriate, hyaline to light

brown, obovoid to ellipsoidal with rounded ends, smooth–walled. *Asexual state* not established.

Notes: *Auerswaldiella* presently comprises nine epithets (Index Fungorum) with the latest species being introduced by Farr (1989). This unusual genus forms raised ascostromata on the surface of leaves comprising four to six locules with densely packed asci and unicellular hyaline to light brown ascospores. The asci are typical of *Botryosphaeriaceae*, however, the raised, pulvinate ascostromata on leaves and large numbers of pseudoparaphyses are atypical; the minute ascospores also differentiate this from other genera in *Botryosphaeriaceae*.

Generic type: *Auerswaldiella puccinioides* (Speg.) Theiss. & Syd., Ann. Mycol. 12: 278 (1914)

MycoBank: MB155192 (Figs. 2.1)

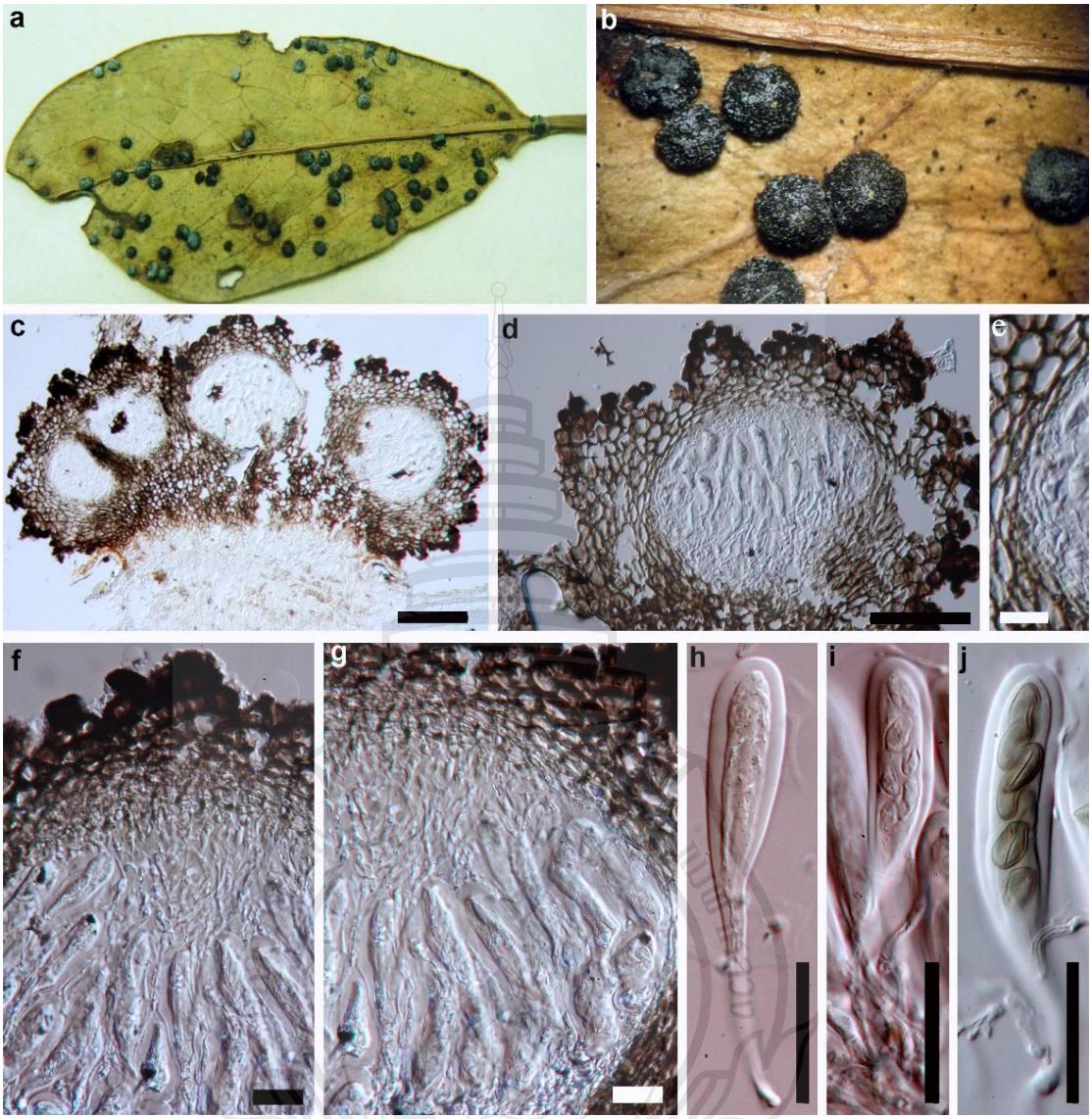
≡ *Auerswaldia puccinioides* Speg., Anales Soc. Ci. Argent. 19: 247 (1885)

= *Phyllachora viridispora* Cooke, Grevillea 13(no. 67): 65 (1885)

= *Dothidea viridispora* (Cooke) Berl. & Voglino, in Sacc., Syll. Fung. Addit. I-IV: 243 (1886)

= *Bagnisiella pruni* Henn., Hedwigia 48: 6 (1908)

Saprobic on lower surface of leaves. *Ascostromata* 0.8–0.9 mm diam, 0.4–0.5 mm high, black, raised on host tissue, solitary, scattered, superficial, pulvinate, globose, rough, multiloculate, containing 4–6 locules, with individual papillate ostioles, cells of ascostromata brown-walled *textura angularis*. *Locules* 320–370 × 450–500 µm. *Peridium* of locules two-layered, up to 30–40 µm wide, outer layer composed of small heavily pigmented thick-walled cells of *textura angularis*, inner layer composed of hyaline thin-walled cells of *textura angularis*. *Pseudoparaphyses* hyphae-like, septate, numerous. *Asci* 138–185 × 32–36 µm ($\bar{x}$ = 164 × 35 µm, n=15), 8-spored, bitunicate, fissitunicate, cylindro–clavate, with a long pedicel and wide shallow ocular chamber. *Ascospores* 9–12 × 3–6 µm ($\bar{x}$ = 11 × 5 µm, n=30), biseriate, hyaline to light brown, obovoid to ellipsoidal, flattened in one plane, with rounded ends, smooth–walled. *Asexual state* not established.



Notes. a–b. Ascostromata on the host. c–d, f–g. Sections of ascostromata. e. Peridium. h–j. Ascus with hyaline and light brown ascospores. Bars: c–d = 100 µm, e = 10 µm, f–g = 20 µm, h–j = 30 µm.

Figure 2.1 *Auerswaldiella puccinioides* on *Prunus sclerocarpa* Leaf

Material examined: PARAGUAY, Villa Rica; Mbocaiaté, on leaves of *Prunus sclerocarpa*, 15 January 1882, B. Balansa No 3443 (LPS 281, holotype).

Notes: The type specimen examined is relatively immature and it was very hard to find asci and ascospores. This is a very distinct fungus and should be recollected and epitypified. The smaller spores in Fig. 8 were not observed on the type specimen.

2.3.2 ***Leptoguignardia*** E. Müll., Sydowia 9: 216 (1955)

MycoBank: MB2777

Hemibiotrophic or *saprobic* on petioles. *Ascostromata* black, scattered, clustered or fusing in groups of 2–3, initially immersed, becoming erumpent but still under host tissue, ovoid to globose, coriaceous. *Papilla* central, ostiole with a pore. *Pseudoparaphyses* sparse, hyphae-like, not commonly observed in herbarium material. *Peridium* comprising small heavily pigmented thick-walled cells of *textura angularis*, *Asci* 8-spored, bitunicate, fissitunicate, with a short blunt pedicel, ocular chamber not clear. *Ascospores* hyaline, 2-septate, fusiform, asymmetrical, central cells widest, ends cells longer and tapering, smooth-walled. Asexual "Dothichiza"-like morph forming on same tissue. *Pycnidia* black, scattered, or fusing in groups or with locules, immersed, becoming erumpent, but still under host tissue, ovoid, coriaceous, scattered amongst locules. *Conidiogenous cells* hyaline, cylindrical, holoblastic. *Conidia* hyaline, 1-septate, septum nearer to apex, slightly constricted, ovoid with round ends.

Notes: *Leptoguignardia* was introduced by Müller (1955) and is monotypic represented by the generic type *Leptoguignardia onobrychidis* E. Müll. The taxon occurs on dead petioles of *Onobrychidis montanae* in France. There is no sequence data available for this species, but based on its ascomata and ascial characters, it fits well into *Botryosphaeriaceae*, although new collections are required to confirm this.

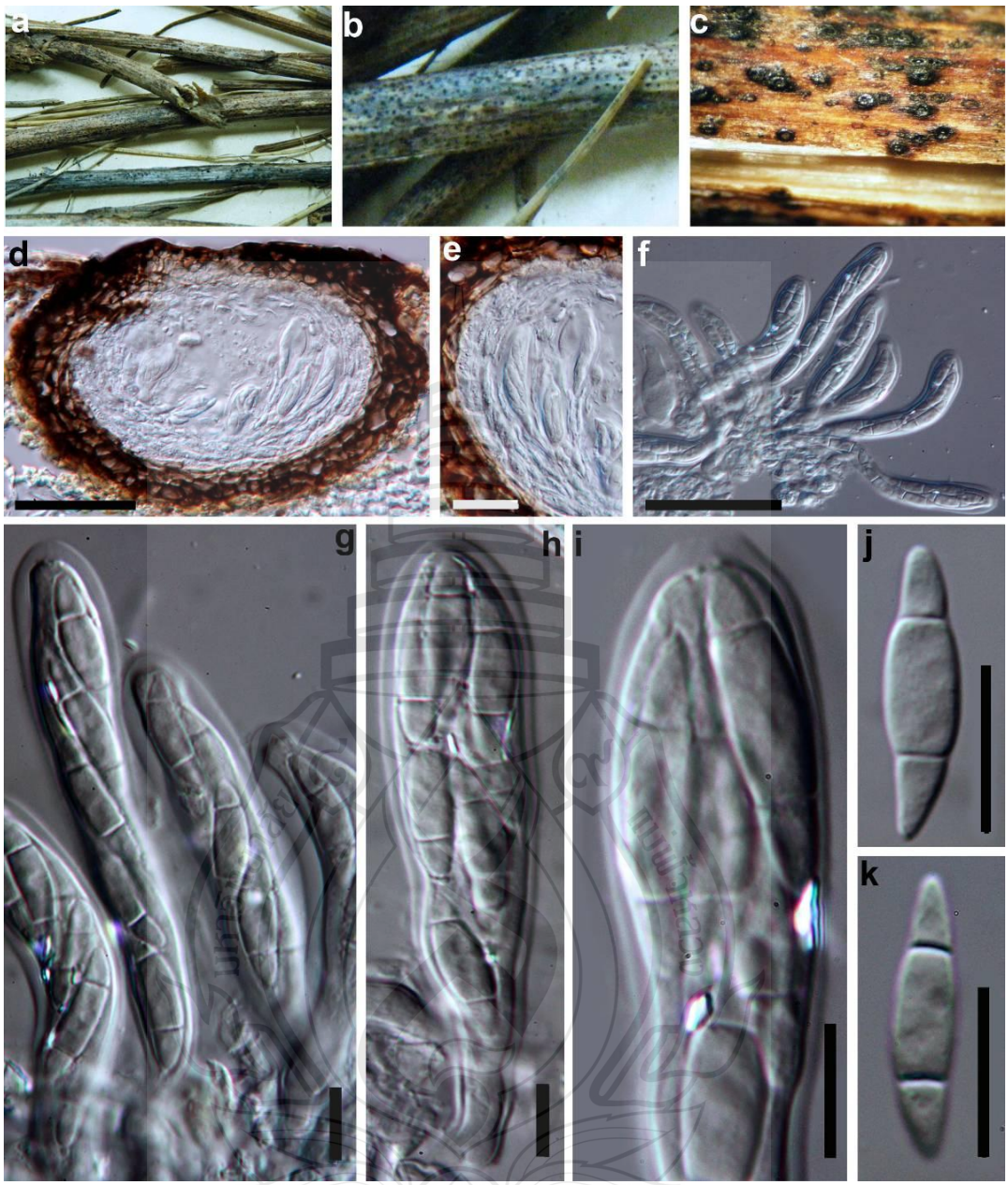
Generic type: *Leptoguignardia onobrychidis* E. Müll.

Leptoguignardia onobrychidis E. Müll., Sydowia 9: 217 (1955), (Figure 2.2, 2.3)

MycoBank: MB299536

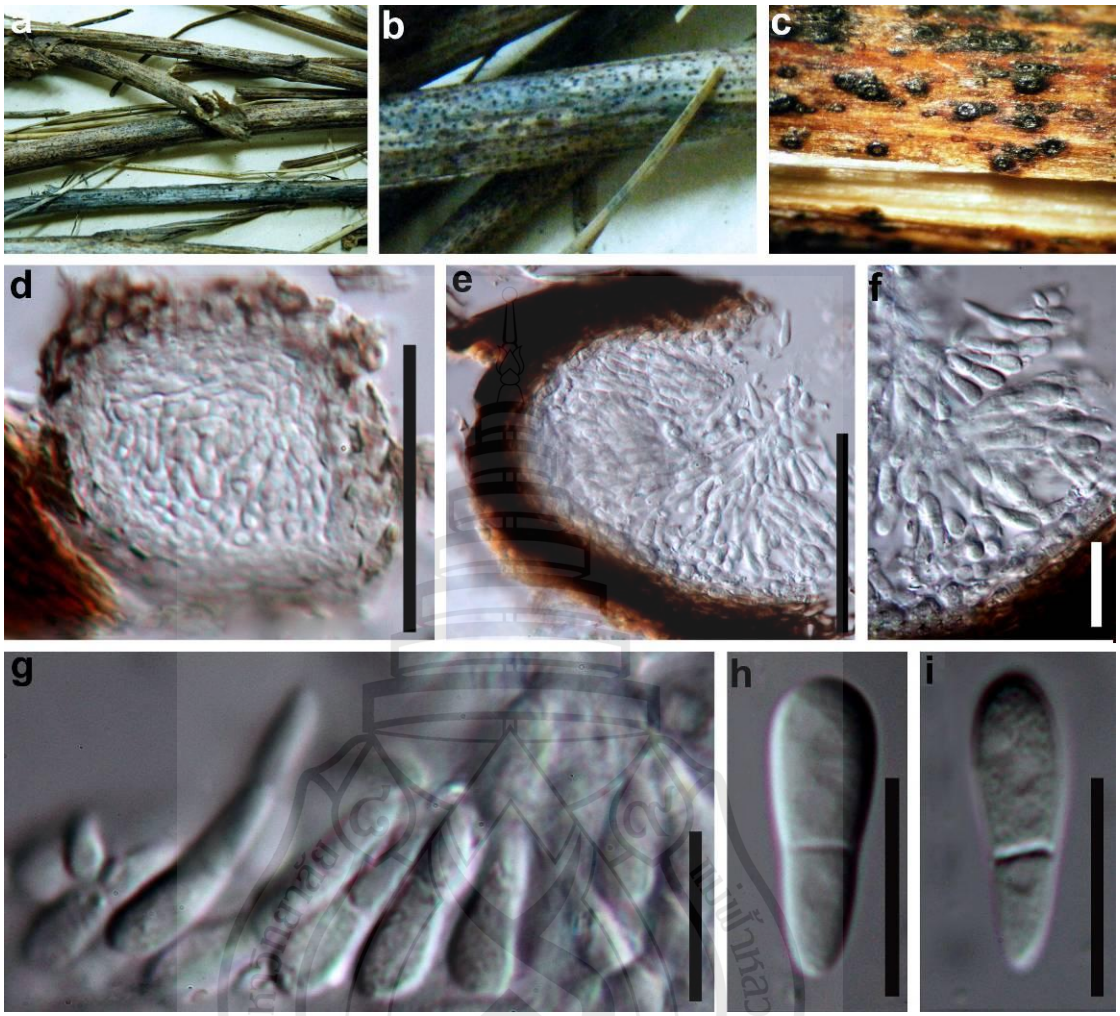
Hemibiotrophic or saprobic on petioles. *Ascostromata* 100–110 µm high 170–180 µm diam., black, scattered, clustered or fusing in groups of 2–3, initially immersed, becoming erumpent but still under host tissue, ovoid to globose,

coriaceous. *Papilla* central, ostiole with a pore opening, 38–40 µm long. *Peridium* up to 23 µm wide, comprising composed of small heavily pigmented thick-walled cells of *textura angularis. Pseudoparaphyses* sparse, hyphae-like, not commonly observed in herbarium material or visible in drawing in protologue. *Asci* 50–70 × 5–8 µm, 8-spored, bitunicate, fissitunicate, with a short blunt pedicel, ocular chamber not clear. *Ascospores* 30–33 × 7–8 µm, overlapping 1–2–seriate in base and 2–3 seriate at apex, hyaline, fusiform, asymmetrical, two-septate, central cells widest, ends cells longer and tapering, one end longer than other, but not related to position in ascus, constricted at the septum, smooth-walled and lacking a sheath. Asexual "Dothichiza"-like morph forming on same tissue. *Pycnidia* 116–150(–200) µm diam., 145–150 µm high, scattered, or fusing in groups or with ascomata, immersed, becoming erumpent, but still under host tissue, ovoid, black, coriaceous, scattered amongst ascomata. *Conidiogenous cells* hyaline, cylindrical, holoblastic. *Conidia* 11–16 × 2.7–4 µm ($\bar{x}$ = 13 × 3.5 µm), 1-sepate, septum nearer to apex, slightly constricted, hyaline, ovoid, and apical cells narrowing to the apex, basal cells widest, thin-walled.



Notes. a–c. Habit and appearance of ascostromata on host substrate. d–e. Section trough ascostromata showing developing of asci. f–i. Asci. j–k. Ascospores. Scale bars: d–f. = 50 µm, g–k**.** = 10 µm.

Figure 2.2 *Leptoguignardia onobrychidis* (Myc 2232, holotype)



Notes. a–c Habit and appearance of conidiomata on host substrate. d–f. Section through pycnidia. g. Conidiogenous cells. h–i. Conidia. Scale bars: d–f. = 50 µm, g-h. = 10 µm.

Figure 2.3 Asexual morph of *Leptoguignardia onobrychidis* (Myc 2232, holotype)

Material examined: FRANCE, Queyras, Abriés, on dead petioles of *Onobrychidis montanae* 12 June 1954, E. Müller & K.H. Richle (ZT, ZT Myc 2232, holotype, Myc 2231, Myc 2225).

Neodeightonia subglobosa C. Booth, in Punithalingam, Mycol. Pap. 119: 19 (1970) [1969], (Figs. 2.4).

MycoBank: MB318601

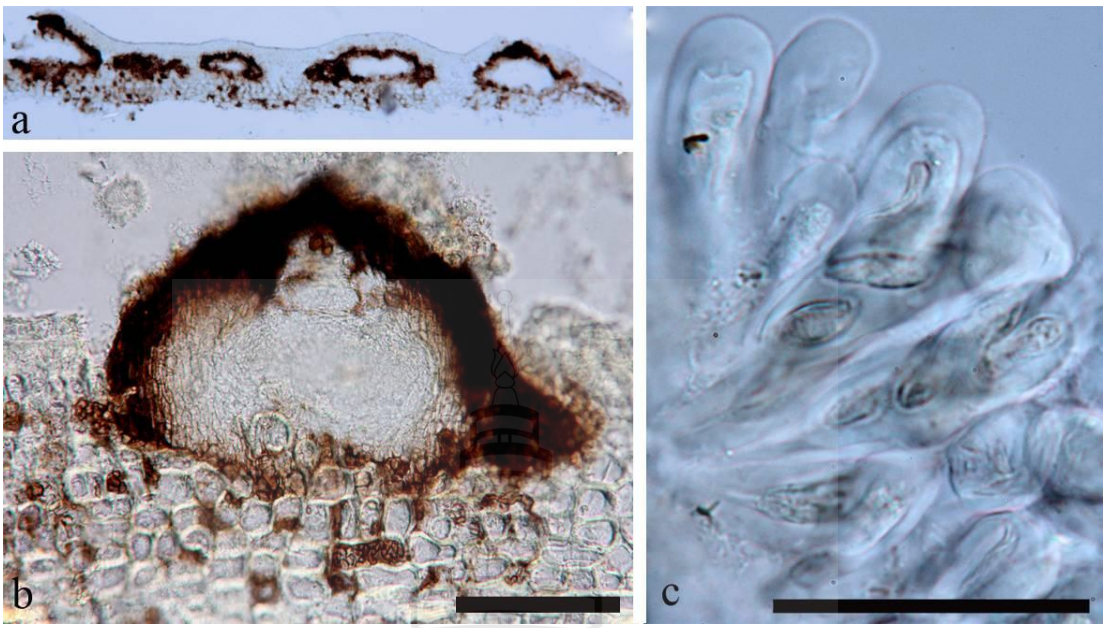
≡ *Botryosphaeria subglobosa* (C. Booth) Arx & E. Müll., Stud. Mycol. 9: 15 (1975)

≡ *Coniothyrium subglobosum* (Cooke) Tassi, Bulletin Labor. Orto Bot. de R. Univ. Siena 5: 25 (1902)

= *Macroplodia subglobosa* (Cooke) Kuntze, Revis. gen. pl. 3: 492 (1898)

≡ *Sphaeropsis subglobosa* Cooke, Grevillea 7(no. 43): 95 (1879)

Saprobic on dead bamboo. *Ascostromata* 140–200 μm high, 210–360 μm diam, dark brown, uniloculate, semi-immersed in host tissue, with protruding papilla or erumpent, developing under raised, dome-shaped regions. *Ostiole* 45–75 × 50–80 μm, central, papillate. *Peridium* 15–40 μm wide, comprising several layers of dark brown-walled cells of *textura angularis*. *Pseudoparaphyses* up to 3–5 μm wide, hyphae-like, cellular, numerous, embedded in a hyaline gelatinous matrix. *Asci* (70–) 81.5–100 (–117) × 18–22.5(–23) μm ($\bar{x}$ = 89.2 × 20.7 μm, n=20), 8-spored, bitunicate, fissitunicate, clavate to cylindro-clavate, with a short rounded pedicle, apically rounded with an ocular chamber (2.5–4.5 μm wide, n=5). *Ascospores* (19.5–) 21–26 (–28) × (6.5–) 7.5–9.5 (–10) μm ($\bar{x}$ = 23.4 × 8.5 μm, n=30), uniseriate at the base, biseriate at the apex, hyaline, aseptate, ellipsoidal to fusiform, usually widest in the middle, rough-walled, with bipolar germ pores, surrounded by distinctive structured mucilaginous sheath. *Pycnidia* 150–200 μm diam., brown to black, solitary or aggregated sometimes intermixed amongst ascostromata, unilocular or multilocular, spherical to globose, wall stromatic, composed of several layers of laterally compressed brown cells. *Conidia* (*phialospores*) 9–12 × 6–9 μm, mature ones light brown to dark brown, spherical to subglobose (asexual morph description follows Punithalingam, 1969).



Notes. a–b. section through ascostromata. c. developing asci. scale bars: b–c=50 µm.

Figure 2.4 *Neodeightonia subglobosa* **(**IMI 57769 c, holotype**)**

Phaeobotryon cercidis (Cooke) Theiss. & Syd., Ann. Mycol. 13: 664 (1915), (Fig. 2.5)

MycoBank: MB124247

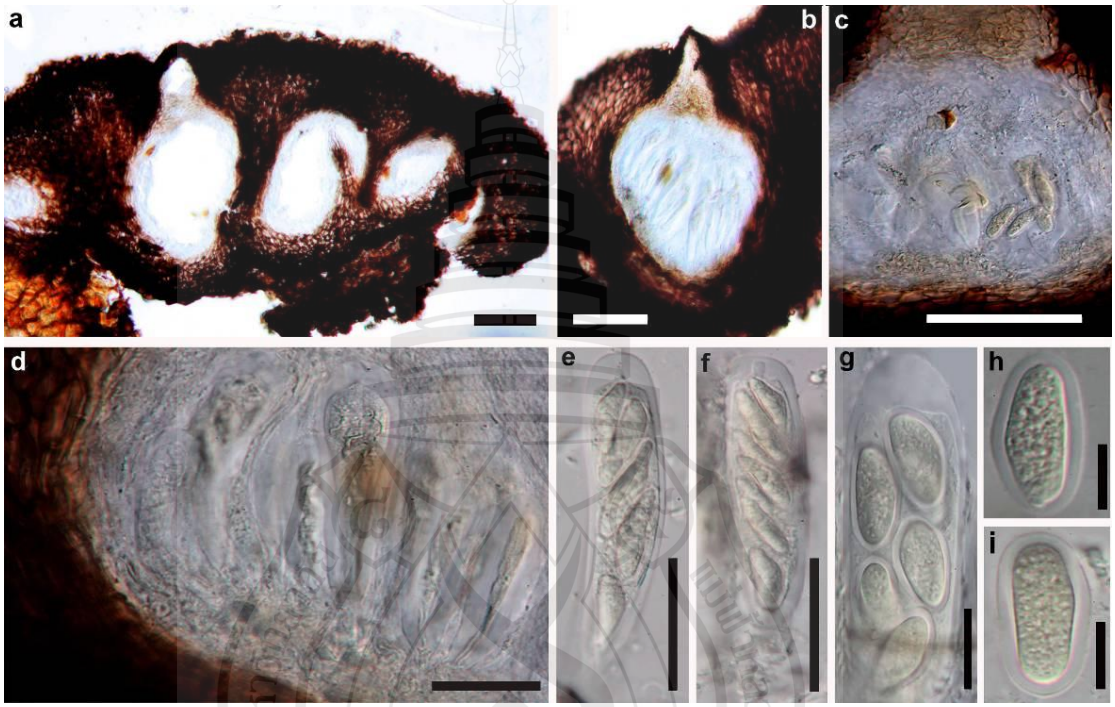
≡ *Dothidea cercidis* Cooke, Grevillea 13: 66. 1885, as 'Dothidea Bagnisiella'.

≡ *Bagnisiella cercidis* (Cooke) Berl. & Voglino, Add. Syll. Fung. 1–4: 223 (1886)

≡ *Auerswaldia cercidis* (Cooke) Theiss. & Syd., Ann. Mycol. 12: 270 (1914)

Saprobic on dead wood. *Ascostromata* 242−251 µm high × 218−253 µm diam, immersed, erumpent, but still under host tissue, subglobose to ovoid, rough, multilocular, with 3–4 *locules* in one ascostroma. *Ostiole* opening with a pore, 98−110 µm long. *Peridium* 200−250 µm wide, one-layered, composed of brown-walled cells of *textura angularis*. *Pseudoparaphyses* hyphae-like, septate, constricted

at septa. *Asci* 125−130 × 22−24 µm, 8-spored, bitunicate, fissitunicate, pedicellate, apically rounded with an ocular chamber. *Ascospores* 29−34 × 9−13 µm ($\bar{x}$ = 31 × 12 µm, n = 25), 1–2-seriate, ellipsoid to broad fusiform with broadly to narrowly rounded ends, hyaline, surrounded by a mucilaginous sheath. *Asexual state* not established.



Notes. a−b Section of ascostromata showing locules. c−d Locule. e−g Asci. h−i Ascospores with mucilaginous sheath. Scale bars: a−d = 100 µm, e−g = 50 µm, h−i = 10 µm.

Figure 2.5 *Phaeobotryon cercidis* (K134204, holotype)

Material examined: USA, Carolina, on bark of *Cercis canadensis*, ex Herb. MC Cooke No 795 (K134204, holotype).

Notes: The type material that we examined had hyaline, aseptate ascospores, surrounded by a mucilaginous sheath, which cncurs with the original description.

Theissen and Sydow (1915) reported that the ascospores became brown with age. It is possible that the material examined by us was not mature.

2.3.3 ***Phyllosticta*** Pers., Traité sur les Champignons Comestibles: 55, 147 (1818)

MycoBank: MB9384

Possible synonymys

= *CaudoPhoma* B.V. Patil & Thirum., Sydowia 20: 36 (1968) [1966]

= *Guignardia* Viala & Ravaz, Bull. Soc. Mycol. Fr. 8: 63 (1892)

= *Laestadiella* Höhn., Ann. Mycol. 16: 50 (1918)

= *Leptasteromella* Petr., Sydowia 20: 235 (1968) [1966]

= *Leptodothiorella* Höhn., Hedwigia 60: 173, 175 (1918)

= *Leptodothiorella* Aa, Stud. Mycol. 5: 13 (1973)

= *Leptophacidium* Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 127: 331 [3 repr.] (1918)

= *Macrophyllosticta* Sousa da Câmara, Anais Inst. sup. Agron. Univ. Téc. Lisboa 3: 36 (1929)

= *Montagnellina* Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 121: 387 [49 repr.] (1912)

= *Myriocarpa* Fuckel, Jb. Nassau. Ver. Naturk. 23-24: 116 (1870) [1869-70]

= *Pampolysporium* Magnus, Verh. Zool.-Bot. Ges. Wien 50: 444 (1900)

= *Phyllosphaera* Dumort., Comment. Bot.: 86 (1822)

= *Phyllostictina* Syd. & P. Syd., Ann. Mycol. 14: 185 (1916)

= *Polysporidium* Syd. & P. Syd., Ann. Mycol. 6: 528 (1908)

Endophytic or *pathogenic* on leaves of a wide range of hosts. *Ascomata* gregarious, circular, brown to black, coriaceous, with a central ostiole. *Asci* (6–)8-spored, bitunicate, fissitunicate, clavate, with a gelatinous pedicel and ocular chamber. *Ascospores* irregularly biseriate, hyaline, aseptate, ellipsoid to broadly fusoid, but much wider in the middle, smooth walled, usually with mucilaginous pads at one or both ends or surrounded by a mucilaginous sheath. *Pycnidia* circular, brown to black, coriaceous, with a central ostiole. *Peridium* comprising brown cells of

textura angularis. *Conidiogenous cells* lining wall of pycnidium, phialidic, cylindrical, hyaline. *Conidia* hyaline, ellipsoidal, aseptate, smooth-walled, surrounded by a mucilaginous sheath bearing a single apical appendage.

Notes: *Phyllosticta* has been reviewed by Wikee et al. (2011) and there have also been several other modern treatments of the genus (Wulandari et al., 2009; Glienke et al., 2011; Wong et al., 2012). The generic type (*Phyllosticta convallariae* Pers.) lacks any recent collections or sequence data and this is certainly required. The sexual state *Guignardia* is clearly linked to *Phyllosticta* and Wikee et al. (2011) proposed that *Phyllosticta* should be used for this genus with *Guignardia* listed as a synonym and this has already been used in *Index Fungorum* and by various authors (Glienke et al., 2011; Wikee, Wulandari, McKenzie & Hyde, 2011; Wong et al., 2012). As *Phyllosticta* is the older and more commonly used name there should be no difficulty in reaching a consensus on using *Phyllosticta* to represent all species in the biological genus with sexual and asexual morphs.

The sexual "*Guignardia"* state is represented by *Phyllosticta ampelicida* (Engelm.) Aa (= *Guignardia bidwellii* (Ellis) Viala & Ravaz) and causes leaf spot on grape vines in the USA. Other important species are *Phyllosticta citricarpa* (McAlpine) Aa which causes black spot of citrus and is of quarantine concern (Wulandari et al., 2009; Wong et al., 2012) and *P. citriasiana* Wulandari, Crous and Gruyter which causes tan spot of pomelo. Freckle disease of banana is caused by a complex of species of *Phyllosticta* (Wong et al., 2012). *Phyllosticta capitalensis* is a weak pathogen and appears to be a ubiquitous endophyte. Below we choose this species to illustrate the genus with both sexual and asexual morphs (Figure 2.6).

Generic type: *Phyllosticta convallariae* Pers.

Phyllosticta capitalensis Henn., Hedwigia 48: 13 (1908), (Figure 2.6)

Mycobank: MB168326

Endophytic or *pathogenic* on leaves of a wide range of hosts. *Ascomata* 65–153 µm long, 64–130 diam ($\bar{x}$ = 112.5 × 90.5, n=15), on the upper leaf surface, brown to black, gregarious, unilocular, circular, coriaceous, with a central ostiole, when mature, up to 230 µm. *Asci* 54–60 × 11–13 µm ($\bar{x}$ = 57.5 × 12, n=10), (6–)8-spored, bitunicate, fissitunicate, attached on the basal peridium, clavate, with a gelatinous pedicel and ocular chamber. *Ascospores* 10–15 × 4–6 µm ($\bar{x}$ = 13 × 5,

n=15), irregularly biseriate, hyaline, aseptate, unicellular, ellipsoid to broadly fusoid, but much wider in the middle, smooth, thick-walled, with mucilaginous pads at each end. *Pycnidia* 65–153 µm long, 64–130 µm diam ($\bar{x}$ = 113 × 90.5, n=15), on the upper leaf surface, gregarious, circular, brown to black, coriaceous, with a central ostiole. *Peridium* 7–10 µm ($\bar{x}$ = 8 µm, n=10) thick, comprising brown cells of *textura angularis*. *Conidiogenous cells* lining wall of pycnidium, phialidic, hyaline, cylindrical. *Conidia* 9–11.5 × 5.5–6.5 µm ($\bar{x}$ = 10 × 6.5, n=15), ellipsoidal, hyaline, aseptate, smooth-walled, surrounded by a mucilaginous sheath 0.7–0.9 µm ($\bar{x}$ = 0.83, n=15) thick, bearing a single apical appendage, usually 2−5 µm long ($\bar{x}$ = 4.5, n=15).

Culture characteristic: On OA, Colonies appeared flat with an irregular margin, initially hyaline with abundant mycelium, gradually becoming greenish after 3–4 d. *Conidiophores* produced conidial masses on media. On MEA, colonies appeared woolly, puffy, flat, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 d and white hyphae on the undulate margin, eventually turning black; reverse dark green to black. At 27ºC, in the dark, mycelium reached the edge of the Petri-dish in 20 d with a growth rate of 0.45 cm per day. On PDA, colonies appeared woolly, rather fast growing, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 d and white hyphae on the undulate margin, eventually turning dark green to black; reverse black. After 15 days in the dark at 27ºC, mycelium reached the edge of the Petri-dish with a growth rate of 0.60 cm per day.

Notes. a. Disease symptoms on living leaves of *Crinum* sp. b. Pycnidia and ascostromata developing on host substrate. c−e. Section through pycnidia showing conidiophores, conidia and spermatia. f−h. Asci. i−j Ascospores. k Spermatia state l−q Conidia. Scale bars c = 50 µm, e−d = 10 µm, f−h = 20 µm, i−q = 10 µm.

Figure 2.6 *Phyllosticta capitalensis* on *Crinum* sp. (CPC 20271)

Material examined: THAILAND, Chiang Rai, Muang District, T. Nanglae, Pa Sang Wiwat, on necrotic leaf spot on leaf of *Crinum* sp. July 2011, S. Wikee CPC20271 (MFLUCC 10−0132).

2.3.4 *Saccharata* Denman & Crous, CBS Diversity Ser. 2: 104 (2004)

MycoBank: MB28918

Saprobic on dead leaves. *Ascomata* black, erumpent, solitary, scattered, subglobose to ovoid, rough-walled, papillate. *Papilla* central, with a short neck. *Peridium* composed of brown pseudoparenchymatous cells of *textura globulosa*. *Pseudoparaphyses* hyphae-like, anastomosing mostly above the asci. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical to fusiform, pedicellate, apically rounded with an ocular chamber. *Ascospores* uniseriate, hyaline, aseptate, guttulate, ellipsoidal, clavate, fusiform to broad fusiform, tapering to obtuse ends, smooth-walled. *Conidiomata* pycnidial, dark brown, eustromatic, immersed, subepidermal, separate, uni to multilocular, walls consisting of dark brown *textura angularis*, ostiolate. *Fusicoccum* asexual morph: *Conidiophores* hyaline, smooth, branched, subcylindrical, 1–3-septate, formed from the inner layer of the locule, intermingled with hyaline, septate paraphyses. *Conidiogenous cells* enteroblastic, phialidic, hyaline, smooth, cylindrical, discrete or intergrated. *Conidia* hyaline, aseptate, smooth, clavate, thin-walled, apex subobtuse, base truncate. The *microconidial state* occurs in the same or in separate conidiomata to the *Fusicoccum* asexual morph. *Microconidiophores* hyaline, cylindrical, 1–3-septate, smooth, branched. *Microconidiogenous cells* phialidic, hyaline, smooth, cylindrical, discrete or integrated. *Microconidia* brown, aseptate, subcylindrical to narrowly ellipsoid with rounded ends, thick-walled, finely verruculose, guttulate. The *spermatial state* occurs in conidiomata with the *Fusicoccum* asexual morph, or in separate *spermatogomia*. *Spermatiophores* hyaline, 1–3-septate, cylindrical, smooth, branched. *Spermatiogenous cells* hyaline, cylindrical, discrete or integrated, smooth. *Spermatia* hyaline, aseptate, rod-shape with rounded ends, smooth (asexual morph description follows Denman, Crous & Wingfield, 1999).

Notes: *Saccharata* was introduced as a monotypic genus based on *S. proteae* (≡ *Phyllachora proteae* Wakef) by Crous, Denman, Taylor, Swart and Palm (2004) to accommodate species having unilocular, immersed ascomata, as well as a “Fusicoccum”-

like asexual morph, with a “Diplodia”-like synanamorph with brown, narrowly ellipsoidal, thick-walled, conidia. Doidge (1942) suggested that *Botryosphaeria* would possibly be a better genus to place *Phyllachora proteae* (Wakefield, 1922) based on the ascomatal wall being continuous with, and smaller in structure to the clypeus. Denman et al. (1999) observed a “Fusicoccum”-like asexual morph which was formed in culture and proposed a new combination in *Botryosphaeria proteae* for *Phyllachora proteae* based on its bitunicate asci and ascospore morphology. By employing ITS DNA molecular sequence data, Denman et al. (2000) recognized two correlating clades of *Botryosphaeria*, namely *Diplodia* and *Fusicoccum*. However, *B. proteae* was not congeneric with these two clades. Recent phylogenetic studies using single and combined genes (Crous et al., 2006; Schoch et al., 2009) showed *Saccharata* to be a distinct genus that is basal in the *Botryosphaeriales*. In this study, *Saccharata* clustered together with *Phyllosticta* and formed a clade with *Melanops* at the base of the *Botryosphaeriales*. This basal clade may be a distinct family in *Botryosphaeriales*.

Generic type: *Saccharata proteae* (Wakef.) Denman & Crous

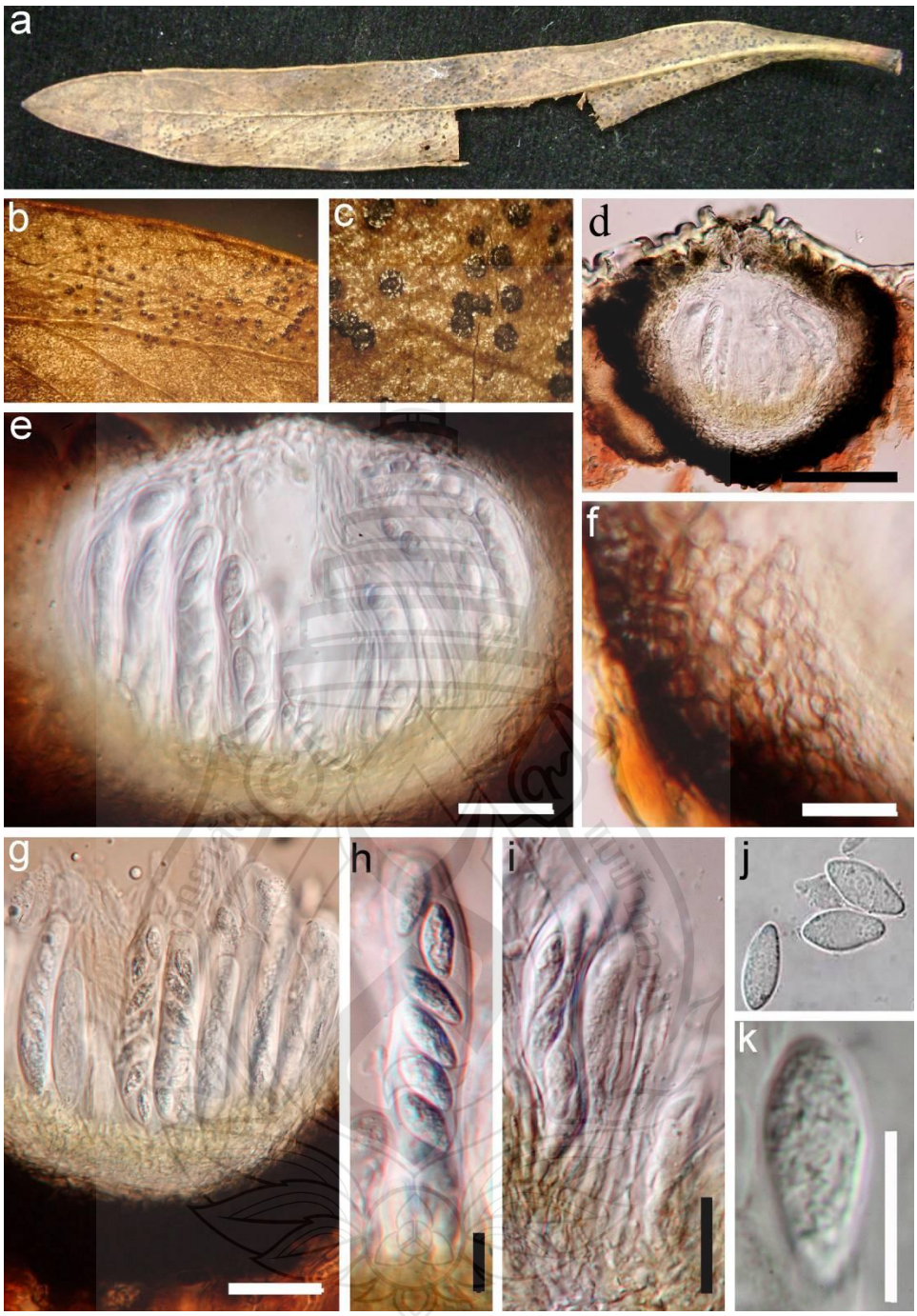
Saccharata proteae (Wakef.) Denman & Crous., CBS Diversity Ser. 2: 104 (2004), (Figure 2.7)

MycoBank: MB370531

≡ *Phyllachora proteae* Wakef., Bull. Misc. Inf., Kew: 164 (1922)

Saprobic on dead leaves. *Ascostromata* black, 190−230 µm high × 240−340 µm diam., immersed, becoming erumpent, but still under host tissue, solitary, scattered, or in small groups of 2−3, subglobose to ovoid, rough-walled, papillate. *Papilla* central, with a short neck, ostiole with a pore, up to 100 µm long. *Peridium* 30−40 µm wide, one-layered, up to 6−23 µm wide, composed of brown pseudoparenchymatous cells of *textura globulosa*, cell wall 2−3 µm thick, near the base composed of hyaline hyphae with numerous asci, up to 20 µm thick. *Pseudoparaphyses* 0.8−1.5 µm broad, hyphae-like, anastomosing mostly above the asci. *Asci* 90−110 × 7.5−10 µm ($\bar{x}$ = 97 × 9 µm, n=10), 8-spored, bitunicate, fissitunicate, cylindrical to fusiform, with a 17.5−27.5 µm long bifurcate pedicel, apically rounded with a large ocular chamber up to 2.5 µm wide × 4 µm high. *Ascospores* 14−15.5 × (5.5-)6−7.5 µm ($\bar{x}$ = 7 × 14.5 µm, n=10), uniseriate, hyaline, aseptate, ellipsoidal, clavate, fusiform to broad fusiform, tapering to obtuse ends, guttulate, smooth-

walled.*Conidiomata* pycnidial, dark brown, eustromatic, to 450µm diam, immersed, subepidermal, separate, uni- to multilocular, walls consisting of dark brown *textura angularis*, ostiolate. *Fusicoccum* asexual morph: *Conidiophores* 20−40 × 3−4.5µm, hyaline, subcylindrical, 1−3 septate, smooth, branched, formed from the inner layer of the locule, intermingled with hyaline, septate paraphyses. *Conidiogenous cells* 20−30 × 2.5−3.5µm enteroblastic, phialidic, hyaline, cylindrical, discrete or intergrated, smooth. *Conidia* (20−)22−25(−30) ×(4.5−)5−6 µm, hyaline, aseptate, clavate, smooth, thin-walled, widest in the middle or upper third of the conidium, apex subobtuse, base truncate.The *microconidial state* occurs in the same or in separate conidiomata to the *Fusicoccum* asexual morph. *Microconidiophores* 15−25 × 2−3 µm, hyaline, cylindrical, 1−3 septate, smooth, branched.*Microconidiogenous cells* 6−10 × 2−3µm, phialidic, hyaline, cylindrical, smooth, discrete or integrated. *Microconidia* (7−)8−11(−14) long with 2.5−3.5 µm wide, brown, aseptate, subcylindrical to narrowly ellipsoid with rounded ends, thick-walled, finely verruculose, guttulate. The *spermatial state* occurs in conidiomata with the *Fusicoccum* asexual morph, or in separate spermatogomia. *Spermatiophores* 15−20 × 3−4 µm, hyaline, cylindrical, 1−3 septate, smooth, branched. *Spermatiogenous cells* 10−12 × 2−3 µm, hyaline, cylindrical, discrete or integrated. *Spermatia* 5−7 × 1.5−2 µm, hyaline, aseptate, rod-shape with rounded ends, smooth.



Note. a–c. Habitat, ascostromata on the host substrate. d–e. Section through of ascostroma. e, g–i. Asci. f. Peridium. j–k. Ascospores. Scale bars d. = 50 µm, e–g. = 20 µm, f. = 10 µm, h–I, k = 10 µm

Figure 2.7 *Saccharata proteae* (PREM 32915, holotype)

Material examined: SOUTH AFRICA, Western Cape Province, Klapmuts, on dead leaves of *Protea repens* (as *P. mellifera*), 5 June 1997, P. Van Der Bijl. No. 357 (PREM 32915, holotype).

2.3.5 ***Sivanesania*** W.H. Hsieh & Chi Y. Chen, Mycol. Res. 100: 1106 (1996)

MycoBank: MB26498

Pathogenic on stems and petioles of *Rubi kawakamii*. *Ascostromata* immersed, erumpent, becoming superficial, scattered, multilocular, subcuticular to subepidermal, pulvinate, cells of ascostromata of brown-walled cell of *textura globulosa to angularis*. *Locules* numerous, globose to compressed, forming in a single layer. *Ostioles* inconspicuous. *Peridium* composed of dark brown cells. *Pseudoparaphyses* hyphae-like, septate, branched. *Asci* 8-spored, bitunicate, fissitunicate, clavate, short pedicellate, apically rounded and thickened, with an inconspicuous ocular chamber. *Ascospores* hyaline to brown when old, ovoid, with a hyaline, filiform, simple appendage. *Asexual state* not established.

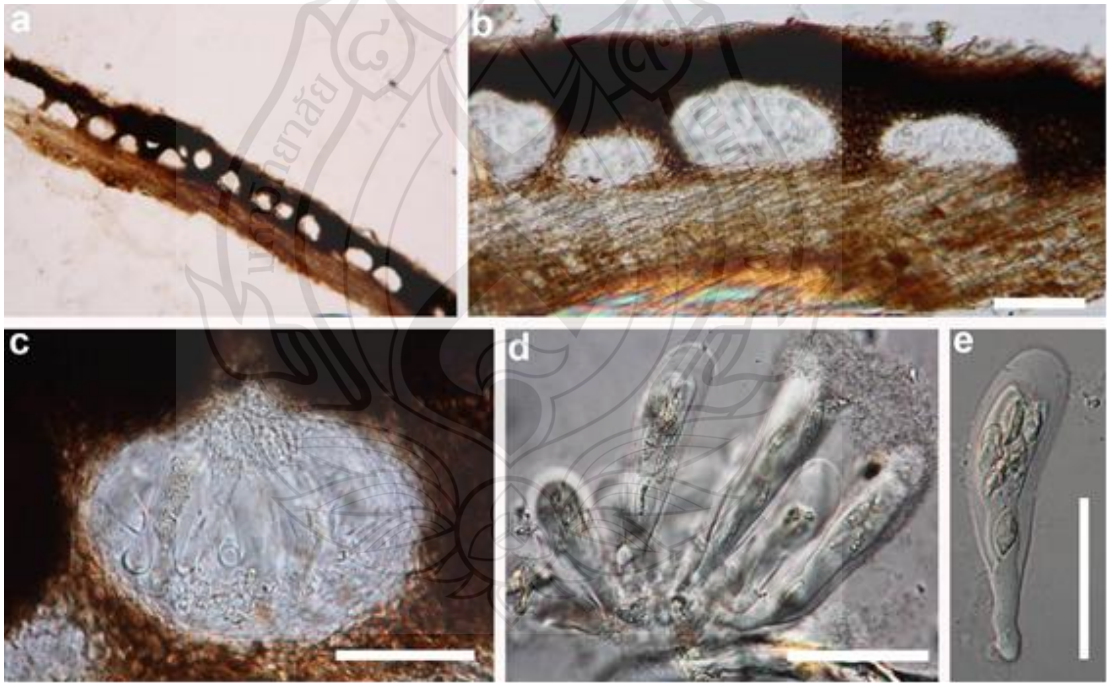
Notes: *Sivanesania* was invalidly introduced as a monotypic genus by Hsieh and Chen (1994) based on *Sivanesania rubi* W.H. Hsieh & Chi Y. Chen which is pathogenic on stems and petioles of *Rubus kawakamii*. The morphological characters of the fungus such as immersed, erumpent, multilocular ascostromata, hyaline, septate pseudoparaphyses and hyaline to brown, aseptate ascospores with an appendage fit well with *Botryosphaeriaceae*. It is most similar to *Botryosphaeria*, *Phyllosticta* and *Saccharata*, but differs by its multilocular ascostromata (*Phyllosticta* and *Saccharata* have uni-locular ascostromata) and aseptate ascospores with a basal filiform, hyaline, simple appendage. No asexual morph has been reported for this genus. No molecular sequence data is available, and therefore fresh collections are needed to confirm the phylogeny. In this study, we accept this genus in *Botryosphaeriaceae* based on morphology.

Generic type: *Sivanesania rubi* W.H. Hsieh & Chi Y. Chen

Sivanesania rubi W.H. Hsieh & Chi Y. Chen, Mycol. Res. 100: 1106 (1996), (Figure 2.8)

MycoBank: MB415938

Pathogenic on stems and petioles of *Rubus kawakamii*. *Ascostromata* immersed, erumpent, becoming superficial, scattered, multilocular, subcuticular to subepidemal, slightly convex, hyphae penetrating the underlying plant host tissue beneath the ascostromata, cells of ascostromata of brown-walled cell of *textura globulosa to angularis*. *Locules* numerous, formed in a single layer, globose to compressed globose, up to 190 µm wide. *Ostiole* central, inconspicuous. *Peridium* of locule a single thin layer, 100−120µm wide. *Pseudoparaphyses* hyphae-like, septate, branched. *Asci* 85−110 × 17−22 µm, 8-spored, bitunicate, fissitunicate, clavate, with a short pedicel, apically rounded and thickened, with an inconspicuous ocular chamber. *Ascospores* 16−25 × 8−11 µm, irregularly biseriate in the ascus, hyaline to brown when old, ovoid to nongranulose, with a basal cellular, hyaline, simple, filiform appendage. *Asexual state* not established.



Note. a−c. Sections of ascostromata. d−e. Asci. Scale bars: b−e. = 50 µm

Figure 2.8 *Sivanesania rubi* (IM1356634, holotype)

Material examined: TAIWAN, Hsianyang, Taitung Hsien, pathogenic on petiole of *Rubi kawakamii* (*Rosaceae*), 10 May 1991, C.Y. Chen, NCHUPP 2234 (IM1356634, holotype).

2.4 Discussion and Conclusion

Specimens of type material in *Botryosphaeria*ceae were studied and are described and illustrated. Generally the sexual morphs of *Botryospharia* are uni to multiloculate ascostromata. Asci are bitunicate, fissitunicate, with a thick endotunica, clavate to cylindro-clavate and apically round with an ocular chamber. Ascospores are hyaline to brown, smooth to verrucose, thin–walled, aseptate to septate, with or without sheath (Liu et al., 2012). Some of asexual morphs of *Botryosphaeriaceae* include species with brown, unicellular or bi-celled conidia (*Neoscytalidium* and *Lasiodiplodia*) and species with hyaline conidia (*Leptoguignardia*, *Phyllosticta*; in this study). The detailed studies of the type material allowed discussion of the genera in *Botryosphearia*les in Liu et al. (2012) which is also supported in some cases by molecular data. It is evident that several groups of *Botryosphaeriaceous* taxa are species complexes and these need to be resolved using multi-gene sequence analysis. Cryptic species have also been resolved in several other pathogenic genera using multigene analysis including *Colletotrichum*, *Fusarium* and *Phyllosticta* (Hyde, Abd Elsalam & Cai, 2010; Summerell, Laurence, Liew & Leslie, 2010; Summerell & Leslie, 2011; Summerell et al., 2011; Cai et al., 2011; Ko-Ko, Stephenson, Bahkali & Hyde, 2011; Wikee, Udayanga et al., 2011; Wikee, Wulandari et al., 2011; Damm, Cannon, Woudenberg & Crous, 2012; Damm , Cannon, Woudenberg, Johnston et al., 2012).

CHAPTER 3

Phyllosticta capitalensis, A WIDESPREAD ENDOPHYTE OF PLANTS

3.1 Introduction

Species in the genus *Phyllosticta* are mostly plant pathogens of a wide range of hosts and are responsible for diseases including leaf spots and black spots on fruits (Baayen et al., 2001; Wulandari et al., 2009; Glienke et al., 2011; Wang et al., 2012). There are about 3200 names listed for the genus *Phyllosticta* in Index Fungorum (http://www.indexfungorum.org/; accessed February 2013) and 3340 names in MycoBank (http://www.mycobank.org/; accessed February 2013). The USDA Fungal Database lists 78 *Phyllosticta* records associated with plant hosts (http://nt.ars-grin.gov/fungaldatabases/; accessed February 2013).

Phyllosticta species may be associated with a "*Guignardia*-like" sexual state (van der Aa, 1973; Wikee et al., 2011). For example, the sexual state of *Phyllosticta ampelicida* (Engelm.) Aa, the black rot pathogen of grapevine is *Guignardia bidwellii* (Ellis) Viala & Ravaz (van der Aa, 1973; Ullrich, Kleespies, Enders & Koch, 2009). Leaf spots on *Morinda citrifolia* (*Rubiaceae*) commonly have both ascomata and pycnidia of *P. morindae* (Petr. & Syd.) Aa (Wulandari, To-Anun & Hyde, 2010). Likewise, both ascomata and pycnidia of *P. maculata* M.H. Wong & Crous are present on banana leaves with freckle disease (Wong et al., 2012). *Guignardia citricarpa* Kiely (synonym of *P. citricarpa* (McAlpine) Aa), which causes black spot of citrus (e.g. oranges), is of quarantine concern in Europe (Baayen et al., 2002; Agostini, Peres, Mackenzie, Adaskaveg & Timmer, 2006), but *P. citriasiana* Wulandari, Crous & Gruyter, which causes brown spot of pomelo fruit (*Citrus maxima*) is not of quarantine concern as this fruit is not grown in Europe (Wulandari

et al., 2009). A few species have also been reported as endophytes and saprobes (van der Aa et al., 2002; Baayen et al., 2002; Glienke et al., 2011). *Phyllosticta maculata* the cause of banana leaf freckle has also been isolated as an endophyte from healthy grapevine leaves (Kuo & Hoch, 1996). *Phyllosticta capitalensis* Henn. is commonly isolated as an endophyte and is a widespread species (Glienke-Blanco, Aguilar-Vildoso, Vieira, Barroso & Azevedo, 2002; Silva & Pereira, 2007; Silva et al., 2008)

Phyllosticta capitalensis was described by Hennings (1908) who found it associated with necrotic leaves of *Stanhopea* sp. (*Orchidaceae*) collected in Brazil. The supposed sexual morph, *G. mangiferae* A.J. Roy was later described from *Mangifera indica* L. (*Anacardiaceae*) in India (Roy 1968). However, there has been confusion with the identification and naming of the *P. capitalensi*s sexual morph. Okane et al. (2003) stated that the teleomorph of *P. capitalensis* differed morphologically from *G. mangiferae* and that it was, in fact, *G. endophyllicola* Okane, Nakagiri & Tad. Ito. The latter fungus was described as a pathogen of several ericaceous plants by Okane et al. (2001). In the past there has also been confusion between *G. endophyllicola* and *G. citricarpa*. Both sexual names have been used for this fungus, for example, *G. endophyllicola* (Okane et al., 2003; Pandey et al., 2003) and *G. mangiferae* (Baayen et al., 2002; Glienke-Blanco et al., 2002; Guo et al., 2003; Suryanarayanan, Ravishankar, Venkatesan & Murali, 2004; Devarajan & Suryanarayanan et al., 2004; Shaw, Carroll & Hoch, 2006). However, *G. citricarpa* is a distinct species and the cause of citrus black spot (Paul et al., 2005; Baayen et al., 2002).

Fungal endophytes colonise healthy plant host tissues but may become pathogenic when the plant host is stressed through environmental or biological factors (Petrini, 1991; Hyde and Soytong, 2008; Purahong and Hyde, 2011) that induce the fungus to change from one life mode to another (Fisher & Petrini, 1992). As with *Phyllosticta*, some species of other common genera such as *Bipolaris*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Diaporthe*, *Fusarium*, *Pestalotiopsis*, *Phoma* and *Verticillium* have been isolated as endophytes (Photita, Lamyong, Lamyoung & Hyde, 2001; Photita, Lamyong S, Lamyoung P, McKenzie & Hyde, 2004; Anderson et al., 2011; Bensch, Braun, Groenewald & Crous, 2012; Damm, Cannon, Woudenberg & Crous, 2012; Damm, Cannon, Woudenberg & Johnston et al., 2012; Gruyter et al.,

2013; Lima et al., 2012; Orlandelli, Alberto, Rubin Filho & Pamphile, 2012), and some of these are also serious pathogens (Photita et al., 2004; Slipper & Wingfield, 2007).

The present study provides an overview of the distribution and host range of *P. capitalensis* worldwide, through the application of multi-gene phylogeny to illustrate its widespread nature. Generally, *Phyllosticta* species are considered plant pathogens but it is still unclear whether they are generalists or host-specific. The distinction between a pathogen and a latent pathogen with endophytic nature is also unclear. In this study we isolated *Phyllosticta* species from northern Thailand, both as endophytes and as pathogens associated with leaf spots of various hosts. We also obtained a range of geographically diverse isolates of *P. capitalensis* from the CBS-KNAW Fungal Biodiversity Centre. All isolates were sequenced compared with sequences downloaded from GenBank.

3.2 Materials and Methods

3.2.1 Fungal Isolates

Twenty three strains of *Phyllosticta* were isolated from leaf spots or as endophytes from healthy leaves of ornamental plants (Table 3.1). If pycnidia were present on diseased tissue then a single spore isolation procedure as described by Chomnunti et al. (2011) was used to obtain cultures. To obtain isolates of *Phyllosticta* from diseased leaves of host plants when fruit bodies were not present, the leaf was surface disinfected by wiping with 70% ethanol. Small pieces of leaf were then cut from the interface between healthy and diseased tissue. These were surface sterilised in 70% ethanol, and plated onto ½ strength potato dextrose agar (½PDA) (Crous Verkleij & Groenewald, 2009). For isolation of endophytes, healthy leaves were washed in tap water and surface disinfected with 70% ethanol. They were then cut into small pieces (about 1 × 1 cm), suspended in 70% ethanol (3 times for 15 minutes each) and washed in distilled water (3 times) before placing on ½PDA. All dishes were incubated at 27ºC for one week and observed daily. The growing tips of hyphae of *Phyllosticta* colonies that developed were cut out and transferred to fresh PDA

dishes. Isolates are deposited in Mae Fah Luang University Culture Collection (MFLUCC) and in the working collection of Pedro Crous (CPC) housed at the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (CBS). Other fungal isolates of representative *Phyllosticta* spp. were obtained from the CBS (Table 3.1).

3.2.2 Morphology

Growth rates, cultural characteristics and morphology of the isolates were determined on culture media prepared according to Crous et al. (2009). All isolates were grown at 27 °C. To induce sporulation, isolates were grown on pine needle agar (PNA) and synthetic nutrient-poor agar (SNA), and incubated under near UV-light. Colony colour and growth rate were established on PDA, malt extract agar (MEA) and oatmeal agar (OA). Culture characteristics were assessed, and the colour of upper and lower surface of cultures was recorded after 14 days in the dark at 27 °C. Colony colour on MEA, OA and PDA were determined using the colour charts of Rayner (1970).

3.2.3 Phylogenetic Analysis

Strains were grown on MEA at room temperature for 2–3 days, after which the mycelium was harvested. DNA was isolated using Ultraclean™ Microbial DNA kit (Mo Bio, Calsbad, CA, USA) following the manufacturer's protocol. Transcribed spacer-polymerase chain reaction (ITS-PCR) was performed with primers V9G (De Hoog & Gerrits Van Den Ende, 1998) and ITS4 as described by White, Bruns, Lee and Taylor (1990). Part of elongation factor 1-α gene (TEF) was amplified with forward primer EF1 and reverse primer EF2 (O'Donnell, Kistler, Cigelnik & Ploetz, 1998). The primers ACT-512F and ACT-783R were used to amplify part of the actin gene (ACT) (Carbone & Kohn, 1999). Cycle sequencing of PCR products were performed in PCR condition. PCR products were separated by gel electrophoresis at 130 volt for 20 min in 1% agarose gel in 1× TAE running buffer and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK). Purified PCR products were sequenced using both PCR primers with a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster

City, CA, USA) containing AmpliTag DNA Polymerase. The amplified products were analyzed on an automatic DNA sequencer (Perkin-Elmer, Norwalk, CN) and aligned using MEGA v5 software (Kumar et al., 2008). Phylogenetic analyzing was executed by Phylogenetic analyses using parsimony; PAUP v4.0b10 (Swafford, 2002) and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003) for Bayesian analyses. *Guignardia bidwellii* was chosen as outgroup for the phylogenetic tree. Representative sequences were deposited in GenBank.

3.2.3 Pathogenicity Testing

Attached, young healthy leaves of five plant species (*Cordyline fruticosa*, *Dendrobium lindleyi*, *Ficus* sp., *Ophiopogon japonica*, *Punica granatum*) were washed with distilled water, wiped with 70% ethanol and dried with sterile tissue paper. To complete the Koch's postulated the inoculation methods followed Than et al. (2008). Two to five leaves of each plant were wounded with a total of ten wounds. The leaves were wounded by pricking them with a pin. Both wounded and unwounded leaves were inoculated with plugs (0.7 mm diam) taken from the edge of 14 day-old colonies of test fungi growing on PDA; sterile agar plugs served as a control. All leaves were kept individually in moist chambers for 1 week and observed for symptom expression every other day. After 7 days, if positive, the fungus was reisolated from any tissue showing lesions and this isolate was considered to be pathogenic; absence of symptoms on leaves classified the isolate as non-pathogenic.

Table 3.1 Isolates of *Guignardia* and *Phyllosticta* Used in the Phylogenetic Study

Strains	Code	Hosts	Mode*	Country	Gene and GenBank Accession No.		
					ITS	TEF1	ACT
G. bidwellii	CBS 111645	*Parthenocissus quinquefolia*	P	USA	JN692542	EU683653	JN692518
G. mangiferae	IMI 260576	*Mangifera indica*	E	India	JF261459	JF261501	JF343641
P. brazilianiae	LGMF 333	*Mangifera indica*	E	Brazil	JF343574	JF343595	JF343658
P. brazilianiae	LGMF 334	*Mangifera indica*	E	Brazil	JF343566	JF343587	JF343650
P. brazilianiae (ex-type)	LGMF 330 CBS 126270	*Mangifera indica*	E	Brazil	JF343572	JF343593	JF343656
P. capitalensis	CPC 20251	wild plant	P	Thailand	KC291333	KC342553	KC342530
P. capitalensis	CPC 20252	*Punica granatum*	P	Thailand	KC291334	KC342554	KC342531
P. capitalensis	CPC 20254	*Saccharum officinarum*	E	Thailand	KC291335	KC342555	KC342532
P. capitalensis	CPC 20255	*Arecaceae*	P	Thailand	KC291336	KC342556	KC342533
P. capitalensis	CPC 20256	*Ophiopogon japonicus*	P	Thailand	KC291337	KC342557	KC342534
P. capitalensis	CPC 20257	*Ficus benjamina*	P	Thailand	KC291338	KC342558	KC342535
P. capitalensis	CPC 20258	*Ophiopogon japonicus*	P	Thailand	KC291339	KC342559	KC342536
P. capitalensis	CPC 20259	*Orchidaceae*	P	Thailand	KC291340	KC342560	KC342537
P. capitalensis	CPC 20263	*Magnoliaceae*	E	Thailand	KC291341	KC342561	KC342538
P. capitalensis	CPC 20266	*Polyscias* sp.	E	Thailand	KC291342	KC342562	KC342539
P. capitalensis	CPC 20268	*Hibiscus syriacus*	E	Thailand	KC291343	KC342563	KC342540
P. capitalensis	CPC 20269	*Ophiopogon japonicus*	E	Thailand	KC291344	KC342564	KC342541

Note. P = pathogen, E=endophyte

Table 3.1 (continued)

Strains	Code	Hosts	Mode*	Country	Gene and GenBank Accession No.		
					ITS	TEF1	ACT
P. capitalensis	CPC 20270	*Tectona grandis*	E	Thailand	KC291345	KC342565	KC342542
P. capitalensis	CPC 20272	*Orchidaceae*	P	Thailand	KC291346	KC342566	KC342543
P. capitalensis	CPC 20275	*Polyalthia longifolia*	E	Thailand	KC291347	KC342567	KC342544
P. capitalensis	CPC 20270	*Tectona grandis*	E	Thailand	KC291345	KC342565	KC342542
P. capitalensis	CPC 20270	*Tectona grandis*	E	Thailand	KC291345	KC342565	KC342542
P. capitalensis	CPC 20278	*Euphorbia milii*	E	Thailand	KC291348	KC342568	KC342545
P. capitalensis	CPC 20423	*Philodendron* 'Xanadu'	P	Thailand	KC291349	KC342569	KC342546
P. capitalensis	LC 0002	*Alocasia* sp.	E	Thailand	KC291350	KC342570	KC342547
P. capitalensis	CPC 20278	*Euphorbia milii*	E	Thailand	KC291348	KC342568	KC342545
P. capitalensis	CPC 20423	*Philodendron* 'Xanadu'	P	Thailand	KC291349	KC342569	KC342546
P. capitalensis	LC 0006	*Dieffenbachia* sp.	E	Thailand	KC291351	KC342571	KC342548
P. capitalensis	LC 0008	*Anthurium* sp.	E	Thailand	KC291352	KC342572	KC342549
P. capitalensis	LC 0009	*Sansevieria hyacinthoides*	E	Thailand	KC291353	KC342573	KC342550
P. capitalensis	LC 0010	*Tinospora craspa*	E	Thailand	KC291354	KC342574	KC342551
P. capitalensis	LC 0025	*Calophyllum* sp.	E	Thailand	KC291355	KC342575	KC342552
P. capitalensis	LC 0008	*Anthurium* sp.	E	Thailand	KC291352	KC342572	KC342549
P. capitalensis	LC 0009	*Sansevieria hyacinthoides*	E	Thailand	KC291353	KC342573	KC342550

Note. P = pathogen; E=endophyte

Table 3.1 (continued)

Strains	Code	Hosts	Mode*	Country	Gene and GenBank Accession No.		
					ITS	TEF1	ACT
P. capitalensis	CBS 100175	*Citrus* sp.	E	Brazil	FJ538320	FJ538378	FJ538436
P. capitalensis	CBS 114751	*Vaccinium* sp.	P	New Zealand	EU167584	FJ538407	FJ538465
P. capitalensis	CBS 115046	*Myracrodruon urundeuva*	E	Brazil	FJ538322	FJ538380	FJ538438
P. capitalensis	CBS 115047	*Aspidosperma polyneuron*	E	Brazil	FJ538323	FJ538381	FJ538439
P. capitalensis	CBS 115049	*Bowdichia nitida*	E	Brazil	FJ538324	FJ538382	FJ538440
P. capitalensis	CBS 123373	*Musa paradisiacal*	E	Thailand	FJ538341	FJ538399	FJ538457
P. capitalensis	CBS 123404	*Musa paradisiacal*	E	Thailand	FJ538333	FJ538391	FJ538449
P. capitalensis	CBS 226.77	*Paphiopedilum callosum*	P	Germany	FJ538336	FJ538394	FJ538452
P. capitalensis	LGMF 03	*Citrus lalifolia*	P	Brazil	JF261452	JF261494	JF343634
P. capitalensis	LGMF 181	*Citrus reticulate*	P	Brazil	JF261447	JF261489	JF343629
P. capitalensis	LGMF 219	*Citrus sinensis*	E	Brazil	JF261448	JF261490	JF343630
P. capitalensis	LGMF 240	*Citrus sinensis*	E	Brazil	JF261443	JF261485	JF343625
P. capitalensis	LGMF 222	*Citrus sinensis*	E	Brazil	JF261450	JF261492	JF343632
P. capitalensis	LGMF 220	*Citrus sinensis*	E	Brazil	JF261446	JF261488	JF343628
P. capitalensis	LGMF 358	*Mangifera indica*	E	Brazil	JF261449	JF261491	JF343631
P. capitalensis (ex-epitype)	CPC18848	*Stanhopea graveolens*	P	Brazil	JF261465	JF261507	JF343647
P. capitalensis	LGMF 222	*Citrus sinensis*	E	Brazil	JF261450	JF261492	JF343632

Note. P= pathogen, E=endophyte

Table 3.1 (continued)

Strains	Code	Hosts	Mode*	Country	Gene and GenBank Accession No.		
					ITS	TEF1	ACT
P. citriasiana (ex-type)	CBS 120486	*Citrus maxima*	P	Thailand	FJ538360	FJ538418	FJ538476
P. citriasiana	CBS 123370	*Citrus maxima*	P	Vietnam	FJ538355	FJ538413	FJ538471
P. citriasiana	CBS 123371	*Citrus maxima*	P	Vietnam	FJ538356	FJ538414	FJ538472
P. citriasiana	CBS 123372	*Citrus maxima*	P	Vietnam	FJ538357	FJ538415	FJ538473
P. citribraziliensis (ex-type)	CBS 100098	*Citrus* sp.	H	Brazil	FJ538352	FJ538410	FJ538468
P. citribraziliensis	LGMF09	*Citrus* sp.	H	Brazil	JF261436	JF261478	JF343618
P. citricarpa	CBS 102374	*Citrus aurantium*	P	Brazil	FJ538313	GU349053	FJ538429
P. citricarpa	CBS 120489	*Citrus sinensis*	P	Zimbabwe	FJ538315	FJ538373	FJ538431
P. citricarpa (ex-epitype)	CBS 127454	*Citrus limon*	P	Australia	JF343583	JF343604	JF343667
P. citricarpa	CBS 127452	*Citrus reticulate*	P	Australia	JF343581	JF343602	JF343665
P. citricarpa	CBS 127455	*Citrus sinensis*	P	Australia	JF343584	JF343605	JF343668
P. citrichinaensis	ZJUCC 200956	*Citrus reticulate*	P	China	JN791664	JN791515	JN791589
P. citriasiana (ex-type)	CBS 120486	*Citrus maxima*	P	Thailand	FJ538360	FJ538418	FJ538476
P. citriasiana	CBS 123370	*Citrus maxima*	P	Vietnam	FJ538355	FJ538413	FJ538471
P. citrichinaensis	ZJUCC 200964	*Citrus maxima*	P	China	JN791662	JN791514	JN791582
P. citrichinaensis	ZJUCC 2010150	*Citrus maxima*	P	China	JN791620	JN791459	JN791533

Note. P= pathogen, E=endophyte

Table 3.1 (continued)

Strains	Code	Hosts	Mode*	Country	Gene and GenBank Accession No.		
					ITS	TEF1	ACT
P. citrichinaensis	ZJUCC 2010152	*Citrus sinensis*	P	China	JN791611	JN791461	JN791535
P. kerriae (ex-holotype)	MUCC 17	*Kerria japonica*	P	Japan	AB454266	KC342576	AB704209
P. hypoglossi	CBS 101.72	*Ruscus aculeatus*	P	Italy	FJ538365	FJ538423	FJ538481
P. hypoglossi	CBS 434.92	*Ruscus aculeatus*	P	Italy	FJ538367	FJ538425	FJ538483
P. hypoglossi	CBS 167.85	*Ruscus hypoglossum*	P	Italy	FJ538366	FJ538424	FJ538482
P. owaniana	CBS 776.97	*Brabejum stellatifolium*	P	South Africa	FJ538368	FJ538426	FJ538484
P. owaniana	CPC 14901	*Brabejum stellatifolium*	P	South Africa	JF261462	JF261504	JF343644
P. spinarum	CBS 292.90	*Chamaecyparis pisifera*	P	France	JF343585	JF343606	JF343669
P. podocarpi	CBS 111646	*Podocarpus falcatus*	P	South Africa	AF312013	KC357671	KC357670
P. citrichinaensis	ZJUCC 200964	*Citrus maxima*	P	China	JN791662	JN791514	JN791582
P. citrichinaensis	ZJUCC 2010150	*Citrus maxima*	P	China	JN791620	JN791459	JN791533
P. citrichinaensis	ZJUCC 2010152	*Citrus sinensis*	P	China	JN791611	JN791461	JN791535

Note. P = pathogen, E = endophyte

[1]CBS: CBS-KNAW Fungal Biodiverstiy Centre, Utrecht, The Netherlands; CPC: working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, LC: culture collection of [1]CBS: CBS-KNAW Fungal Biodiverstiy Centre, Utrecht, The Netherlands; CPC: working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, LC: culture collection of Nilam F. Wulandari, Chiangmai, Thailand. LGMF: culture

collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil, ZJUCC: Zhejiang University Culture Collection, Zhejiang, China. [1]CBS: CBS-KNAW Fungal Biodiverstiy Centre, Utrecht, The Netherlands; CPC: working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, NFW: culture collection of Nilam F. Wulandari, Chiangmai, Thailand. LGMF: culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil, ZJUCC: Zhejiang University Culture Collection, Zhejiang, China.

3.3 Results

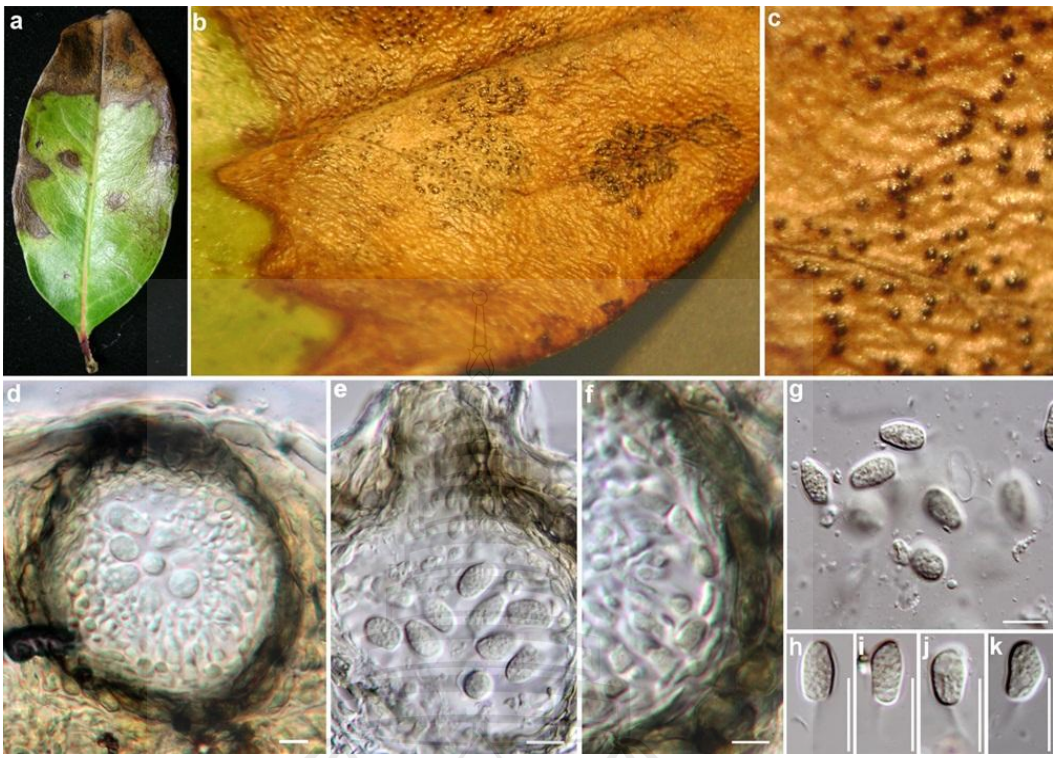
3.3.1 Collection of *Phyllosticta* Species

Twenty three strains of *Phyllosticta capitalensis* were isolated from 20 host plants growing in the north of Thailand (Table 3.1, 3.2 and Figure 2).

3.3.2 Morphological Description of *Phyllosticta capitalensis*

On Punica granatum: *Pycnidia* epiphyllous, globose, brown or black, 120–125 µm high, 135–140 µm wide, wall 12–15 µm thick. *Conidiogenous cells* lining wall of pycnidium, phialidic, cylindrical, hyaline, 2–2.2 × 2.2–3 µm. *Conidia* ellipsoidal, hyaline, 1-celled, smooth-walled, 8–11 × 5–6 µm, surrounded by a mucilaginous sheath, bearing a single apical appendage, usually 5–8 µm long.

In culture: On SNA, *ascomata* forming on and under media in 3 days, black, globose, 69–74 × 104–119 µm ($\bar{x}$ = 73 ± 2 × 109 ± 5; n=10), wall composed of a single layer, 7–9 µm thick ($\bar{x}$ = 8 ± 1 ; n=10), brown. *Asci* bitunicate, containing 6–8 ascospores, irregularly biseriate, clavate, 36–80 × 7–15 µm ($\bar{x}$ = 51 ± 1 × 11 ± 2, n=10). *Ascospores* ellipsoid to broadly fusoid, widest in the middle, hyaline, smooth, thin-walled, 12–22 × 5–10 µm ($\bar{x}$ = 16 ± 2 × 7 ± 1, n=50), 1-celled, surrounded by mucilaginous sheath. On OA, colonies appear flat with an irregular margin, initially hyaline with abundant mycelium, gradually becoming greenish after 3–4 days. On MEA, colonies appear woolly, flat, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days with white hyphae on the undulate margin, eventually turning black; reverse dark green to black. At 27 ºC, in the dark, mycelium reached the edge of the Petri-dish in 20 days with a growth rate of 0.4 cm per day. On PDA, colonies appear woolly, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days with white hyphae on the undulate margin, eventually turning dark green to black; reverse black. After 10 days in the dark at 27 ºC, mycelium reached the edge of the Petri-dish with a growth rate of 0.9 cm per day.

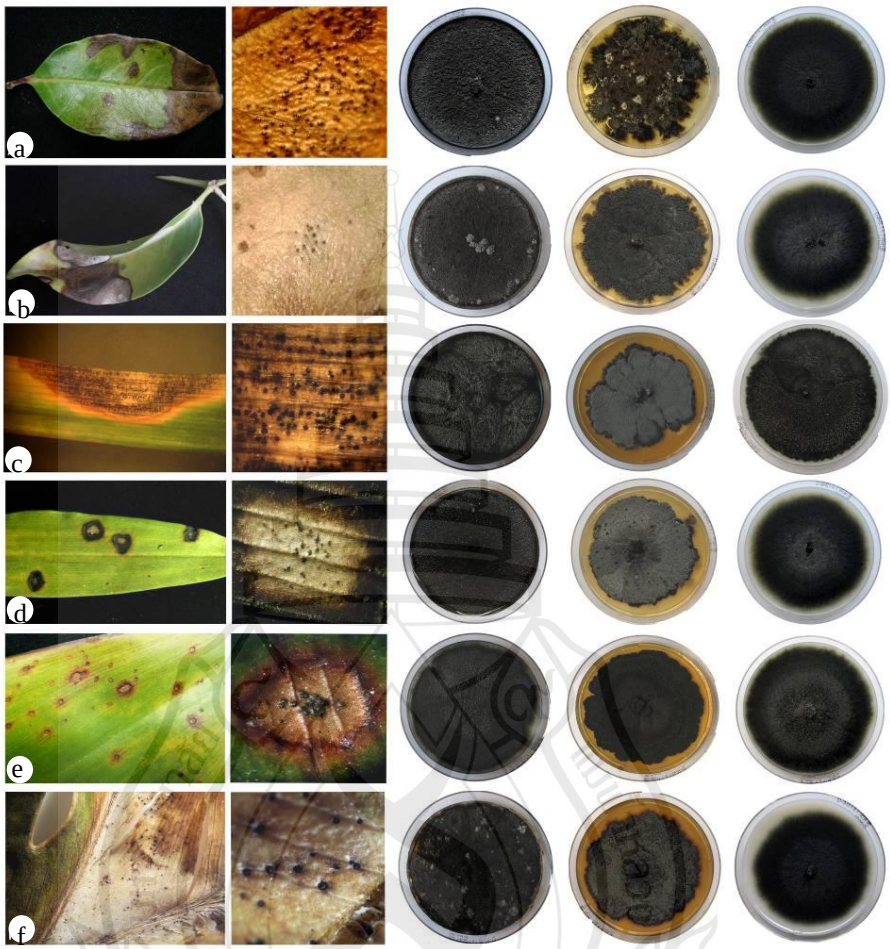


Notes. a-c. Leaf spots on host plant d-f. Vertical section through pycnidia showing developing conidia g-k. Conidia (d, bar = 20 µm, g-k bars = 10 µm).

Figure 3.1 *Phyllosticta capitalensis* on *Punica granatum* (CPC 20252)

Material examined: All CPC collected by Saowanee Wikee and LC by Nilam F. Wulandari, from June 2010 to November 2011, Chiang Rai, Thailand. From leaf spots of unknown wild plant CPC 20251; from leaf spots *Punica granatum*, CPC 20252; from healthy leaf of *Saccharum officinarum* CPC 20254; from leaf spots of *Arecaceae* CPC 20255; from leaf spots of *Ophiopogon japonica* CPC 20256, CPC 20258 and CPC 20269; from leaf spots of *Ficus benjamina* CPC 20257; from leaf spots of *Orchidaceae* CPC 20259 and CPC 20272; from healthy leaf of *Magnoliaceae* CPC 20263; from healthy leaf of *Polyscias* sp. CPC 20266; from healthy leaf of *Hibiscus syriacus* CPC 20268; from healthy leaf of *Tectona grandis* CPC 20270; from healthy leaf of *Poloalthia longifolia* CPC 20275; from healthy leaf of *Euphorbia milli* CPC 20278; from healthy leaf of *Philodendron* sp. CPC 20423; from healthy leaf of

Alocasia sp. LC 0002; from healthy leaf of *Dieffenbachia* sp. LC 0006; from healthy leaf of *Anthurium* sp. LC 0008; from healthy leaf of *Sansevieria hyacinthodes* LC 0009; from healthy leaf of *Tinospora craspa* LC0010; from healthy leaf of *Calophyllum* sp. LC 0025.



Notes. a. *Punica granatum* (CPC20252; MFLUCC11-0053) b. *Ficus* sp. (CPC20257; MFLUCC11-0058) c. *Ophiopogon japonica* (CPC20258; MFLUCC11-0059) d. *Dendrobium lindleyi* (CPC20259; MFLUCC11-0064) e. *Cordyline fruticosa* (CPC20273; MFLUCC10-0135) f. *Philodendron* 'Xanadu' (CPC20423; MFLUCC-12-0232).

Figure 3.2 Leaf Spot Symptoms on Living Leaves of Hosts and Cultures Characteristic of *Phyllosticta capitalensis* on PDA (left), MEA (middle) and OA (right)

3.3.3 Phylogenetic Analysis

Phylogenetic relationships among the *Phyllosticta capitalensis* isolates from various hosts and locations were investigated in this study using MP and Bayesian phylogenetic analyses. The analysis of combined ITS, TEF and ACT genes of the *Phyllosticta* strains newly sequenced in this study and 67 strains of *Phyllosticta* obtained from GenBank and Mei University, Japan (Table 3.1) were aligned and used to construct their phylogeny. The combined dataset of 67 strains (including the out-group) consisted of 974 characters, of which 483 characters were constant, and 148 characters were variable and parsimony-uninformative. Parsimony analysis generated 48 trees, of which the best one is shown in Fig. 3.3 (TL=873, CI=0.804, RI=0.963, RC=0.774). In the parsimony tree (Fig. 3.3) bootstrap values and Bayesian analysis of combined data are given at the nodes.

In the phylogenetic tree 13 clades representing various *Phyllosticta* species are evident. *Guignardia bidwellii* was chosen as outgroup. The representative strain of *G. mangiferae* (IMI 260576) fell outside the *P. capitalensis s. str.* clade. The isolates in the *P. capitalensis s. str.* clade were from different hosts and different continents. *Phyllosticta brazilianiae* was isolated from an orchid in Brazil; *P. citricarpa* was isolated from *Citrus* sp. and *P. citriasiana* was isolated from *Citrus maxima*, Vietnam; *P. spinarum* was isolated from *Chamaecyparis pisifera,* France; *P. kerriae* was isolated from *Kerria japonica,* Japan; *P. citribraziliensis* was isolated from citrus, Brazil; *P. hypoglossi* was isolated from *Ruscus aculeatus,* Italy; *P. citrichinaensis* was isolated from *Citrus maxima,* China; *P. podocarpi* was isolated from *Podocarpus falcatus*, South Africa and *P. owaniana* from *Brabejum stellatifolium*, South Africa.

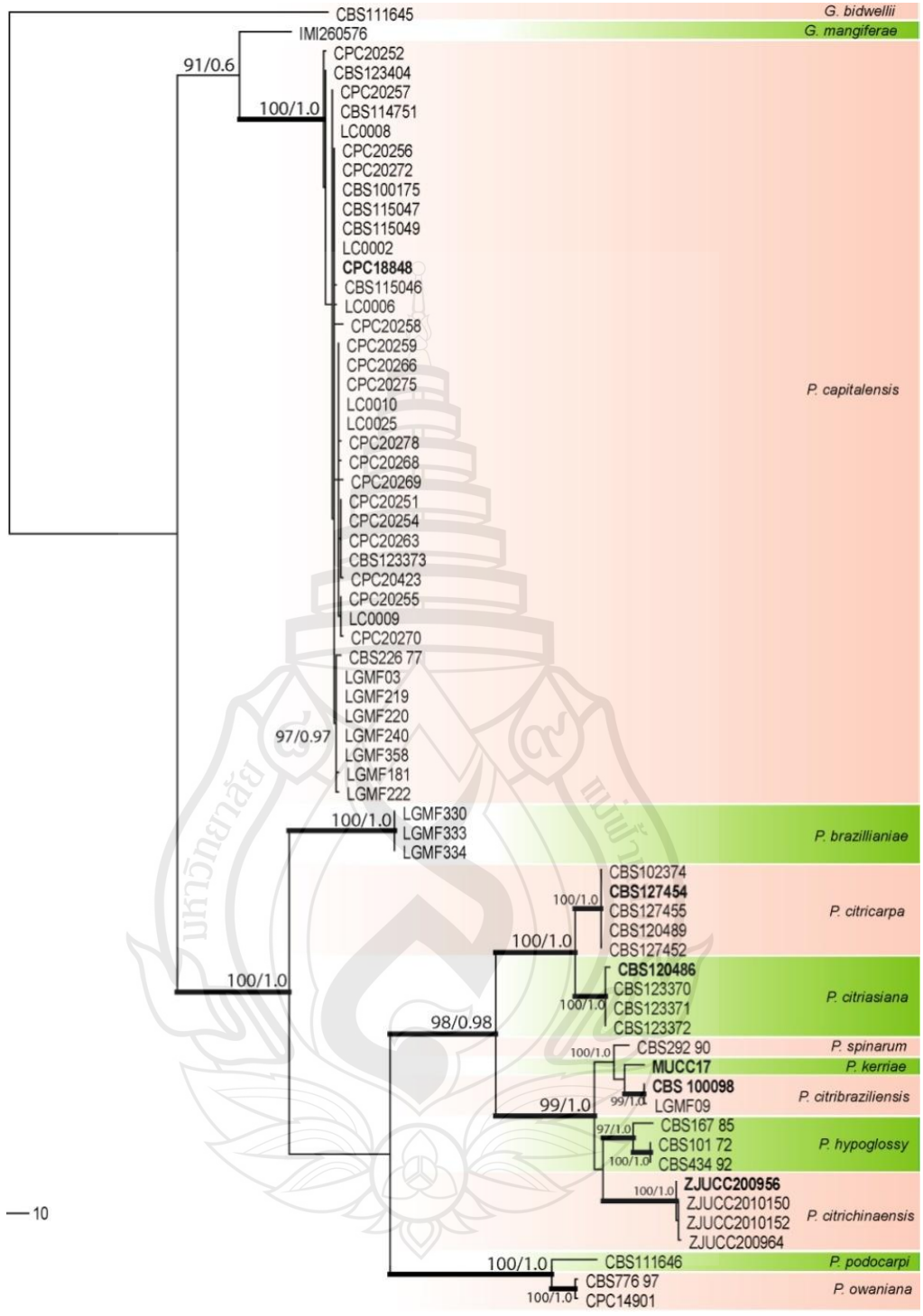


Figure 3.3 Phylogenetic Tree of *Phyllosticta* Generated from 1000 Replicates Bootstrap Values Parsimony Analysis/ Bayesian Analysis Based on combined ITS rDNA, TEF1 and ACT sequence data. The Tree is Rooted with *Guignardia bidwellii* (CBS 111645)

3.3.4 Pathogenicity Testing with *Phyllosticta capitalensis*

The ability of *Phyllosticta capitalensis* strains isolated from leaf spots of five hosts in Thailand to induce leaf spot symptoms on these host species was tested through inoculating mycelium plugs onto attached wounded and unwounded living leaves. In all cases there was no infection of the young healthy plant leaves.

3.3.5 Host Range

Table 3.2 Hosts and Countries from Which *Phyllosticta capitalensis* has been Isolated, Usually as an Endophyte, Rarely as a Pathogen (P) (see also Figure 2)

Plant family	Plant genus	Country	References
Acanthaceae	*Mackaya*	South Africa	Carroll (1990)
Anacardiaceae	*Anacardium*	Brazil	Glienke et al. (2011)
	Comocladia	Puerto Rico	Carroll (1990)
	Loxostylis	South Africa	Carroll (1990)
	Mangifera	Brazil	Carroll (1990)
		Ghana	Baayen et al. (2002)
	Myracrodruon	Brazil	Glienke et al. (2011)
	Rhus	South Africa	Baayen et al. (2002)
	Sclerocarya	South Africa	Carroll (1990)
	Spondias	Brazil	Carroll (1990)
Annonaceae	*Monanathotaxis*	South Africa	Carroll (1990)
	Polyalthia	Thailand	Present study
Apocynaceae	*Aspidosperma*	Brazil	Glienke et al. (2011)
	Secamone	South Africa	Carroll (1990)
	Cerbera	Japan	Okane et al. (2003)
	Nerium	Japan	Motohashi et al. (2009)
Aquifoliaceae	*Ilex*	USA	Carroll (1990)
Aquifoliaceae	*Cerbera*	Japan	Okane et al. (2003)
Araliaceae	*Cussonia*	South Africa	Carroll (1990)
	Hedera	South Africa	Carroll (1990)
	Polyscias	Puerto Rico	Carroll (1990)
	Schefflera	Costa Rica	Baayen et al. (2002)
	Polyscias	Thailand	Present study

Table 3.2 (continued)

Plant family	Plant genus	Country	References
Araceae	*Alocasia*	Thailand	Present study
	Anthurium	Thailand	Present study
	Dieffenbachia	Thailand	Present study
	Livistona	Thailand	Present study
	Spathiphyllum	Japan	Motohashi et al. (2009)
	Philodendron	Thailand	Present study
Asparagaceae	*Sansevieria*	Thailand	Present study
	Ophiopogon (P)*	Thailand	Present study
Boraginaceae	*Cordia*	South Africa	Carroll (1990)
Calophyllaceae	*Calophyllum*	Thailand	Present study
Capparaceae	*Maerua*	South Africa	Carroll (1990)
Chrysobalanaceae	*Parinari*	South Africa	Carroll (1990)
Combretaceae	*Combretum*	South Africa	Carroll (1990)
Convolvulaceae	*Ipomoea*	Malaysia	Present study
Cornaceae (Nyssaceae)	*Curtisia*	South Africa	Baayen et al. (2002)
	Davidia	Japan	Motohashi et al. (2009)
Celastraceae	*Putterlickia*	South Africa	Baayen et al. (2002)
Cercidiphyllaceae	*Cercidiphyllum*	Japan	Motohashi et al. (2009)
Ebenaceae	*Diospyros*	South Africa	Carroll (1990)
	Euclea	South Africa	Carroll (1990)
Ericaceae	*Rhododendron*	Japan	Okane et al. (2003)
	Enkianthus	Japan	Okane et al. (2001)
	Vaccinium	New Zealand	Glienke et al. (2011)
Fabaceae	*Bowdichia*	Brazil	Glienke et al. (2011)
	Cercis	Japan	Motohashi et al. (2009)
Fagaceae	*Lithocarpus*	Japan	Motohashi et al. (2009)
Ginkgoaceae	*Ginkgo*	Japan	Motohashi et al. (2009)
Lamiaceae	*Vitex*	Malaysia	Present study
Lauraceae	*Cinnamomum*	Japan	Okane et al. (2003)
	Ocotea	South Africa	Carroll (1990)
Lecythidaceae	*Barringtonia*	South Africa	Baayen et al. (2002)
Leguminosae	*Caesalpinia*	Japan	Okane et al. (2003)
Loganiaceae	*Stychnos*	South Africa	Carroll (1990)
	Anthocleista	South Africa	Carroll (1990)

Note. (P) = Leaf Sport

Table 3.2 (continued)

Plant family	Plant genus	Country	References
Lythraceae	*Punica* (P)	Thailand	Present study
Malvaceae	*Hibiscus*	Thailand	Present study
Meliaceae	*Ekebergia*	South Africa	Carroll (1990)
	Trichilia	South Africa	Baayen et al. (2002)
Menispermaceae	*Cocculus*	USA	Carroll (1990)
Moraceae	*Artocarpus*	Thailand	Baayen et al. (2002)
	Ficus (P)	Thailand	Present study
	Morus	Thailand	Stringari, Glienke, Christo, Maccheroni and Azevedo, (2009)
Magnoliaceae	*Michelia*	Thailand	Present study
	Magnolia	Thailand	Glienke et al. (2011)
		USA	Carroll (1990)
Menispermaceae	*Tinospora*	Thailand	Present study
Euphorbiaceae	*Clutia*	South Africa	Baayen et al. (2002)
	Croton	South Africa	Carroll (1990)
	Codiaeum	Thailand	Present study
	Ctenomeria	South Africa	Carroll (1990)
	Euphorbia	Thailand	Present study
Musaceae	*Musa*	Thailand	Okane et al. (2003)
		Indonesia, USA	Glienke et al. (2011)
Myrtaceae	*Eucalyptus*	Brazil, South Africa	Carroll (1990), Glienke et al. (2011)
	Psidium	Brazil	Baayen et al. (2002)
Oleaceae	*Ligustrum*	Japan	Motohashi et al. (2009)
	Schrebera	South Africa	Carroll (1990)
Ophioglossaceae	*Botrychium*	USA	Carroll (1990)
Orchidaceae	*Arundina*	Japan	Okane et al. (2003)
Orchidaceae	*Coelogyne*	Thailand	Carroll (1990)
	Dendrobium	Thailand	Present study
Orchidaceae	*Paphiopedilium*	Germany	Okane et al. (2001)
Orchidaceae	*Rhynchostylis* sp.	Malaysia	William & Liu (1976), Singh (1980)
	Stanhopea	Brazil	Glienke et al. (2011)
Pittosporaceae	*Pittosporum*	Hawaii	Baayen et al. (2002)
Poaceae	*Saccharum*	Thailand	Present study
Podocarpaceae	*Podocarpus*	South Africa	Carroll (1990)

Note. (P) = Leaf Sport

Table 3.2 (continued)

Plant family	Plant genus	Country	References
Proteaceae	*Leucospermum*	Hawaii	Carroll (1990)
	Protea	Hawaii	Carroll (1990)
	Telopea	Australia	Carroll (1990)
Pittosporaceae	*Pittosporum*	Japan	Motohashi et al. (2009)
Orchidaceae	*Paphiopedilium*	Germany	Okane et al. (2001)
Pteridophta	*Pteridophytes*	Japan	Okane et al. (2003)
Rhamanaceae	*Scutia*	South Africa	Carroll (1990)
	Zizyphus	South Africa	Carroll (1990)
Rhizophoraceae	*Kandelia*	Japan	Okane et al. (2003)
Rosaceae	*Cliffortia*	South Africa	Carroll (1990)
	Rubus	Japan	Okane et al. (2003)
	Prunus	Japan	Okane et al. (2003)
	Eriobotrya	Japan	Motohashi et al. (2009)
Rubiaceae	*Canthium*	South Africa	Carroll (1990)
	Coprosma	Hawaii	Baayen et al. (2002)
	Gardenia	South Africa	Carroll (1990)
	Pavetta	South Africa	Carroll (1990)
	Rauvolfia	South Africa	Carroll (1990)
	Rothmannia	South Africa	Carroll (1990)
Rutaceae	*Zanthoxylum*	Japan	Okane et al. (2003)
Rutaceae	*Citrus* (P)	Argentina, Australia, Brazil, China, Hong Kong, New Zealand, South Africa, Taiwan, Thailand, USA	Glienke et al. (2011); Wang et al. (2012)
Rutaceae	*Fortunella*	USA	Carroll (1990)
	Vitex	South Africa	Carroll (1990)
	Zanthoxylum	Pueto Rico	Baayen et al. (2002)
Sapindaceae	*Allophylus*	South Africa	Carroll (1990)
	Dodonaea	Hawaii	Carroll (1990)
	Litchi	South Africa	Carroll (1990)
	Nephelium	USA	Glienke et al. (2011)
	Paullinia cupana	Brazil	Baayen et al. (2002)
Smilacaceae	*Smilax*	South Africa	Glienke et al. (2011)

Note. (P) = Leaf Sport

Table 3.2 (continued)

Plant family	Plant genus	Country	References
Stangeriaceae	*Stangeria*	South Africa	Baayen et al. (2002)
Rutaceae	*Fortunella*	USA	Carroll (1990)
Sterculiaceae	*Sterculia*	Puerto Rico	Carroll (1990)
Theaceae	*Camellia*	USA	Baayen et al. (2002)
Tiliaceae	*Grewia*	South Africa	Carroll (1990)
Trimeniaceae	*Xymalos*	South Africa	Carroll (1990)
Ulmaceae	*Trema*	South Africa	Carroll (1990)
Veronicaceae	*Hebe (Veronica)*	South Africa	Carroll (1990)
Viscaceae	*Viscum*	South Africa	Stringari et al. (2009)
Vitaceae	*Ampelopsis*	USA	Baayen et al. (2002)
	Cryphostemma	South Africa	Carroll (1990)
	Rhoicissus	South Africa	Carroll (1990)
Zamiaceae	*Encephalartos*	South Africa	Carroll (1990)
Zingiberaceae	*Amomum*	Thailand	Okane et al. (2003)
	Zingiber	Thailand	Okane et al. (2003)

Note. (P) = Leaf Sport

3.4 Discussion

This study reviews previous data on *Phyllosticta capitalensis* and provides additional data on host infection and distribution in Thailand. Many factors such as environmental conditions, host and non-host organisms, and plant defence mechanisms (e.g. secondary metabolite, specific and non-specific protein expression and hydrogen peroxide residue) play an important role in response to microbial infection.

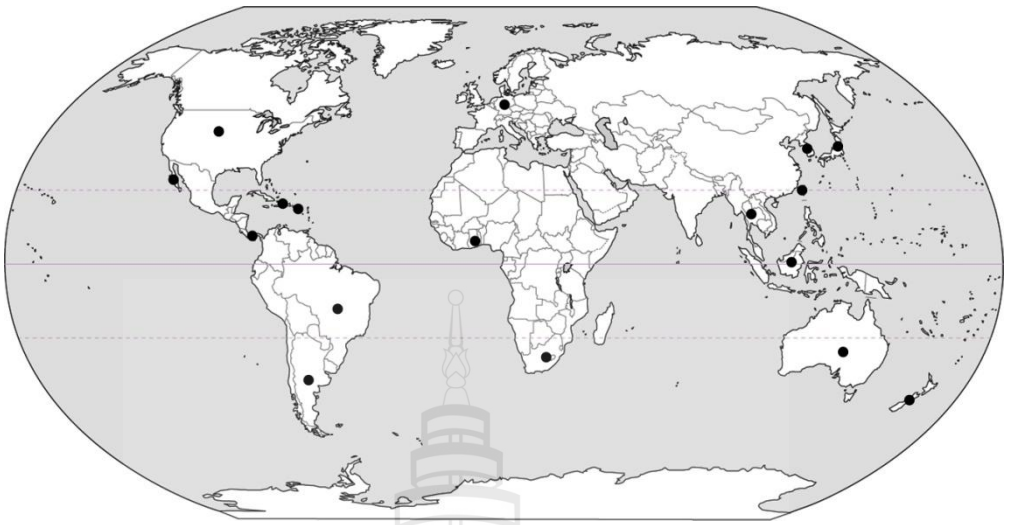


Figure 3.4 World Distribution of *Phyllosticta capitalensis* (the dots represent countries)

Phyllosticta capitalensis has been repeatedly isolated worldwide from healthy plant tissues as an endophyte and rarely from leaf spots as a pathogen, and has been recorded from almost 70 plant families (Baayen et al., 2002; Okane et al., 2003; Motohashi et al., 2009; Table 3.1, Figure 3.4). The fact that it is isolated so often as an endophyte has important implications to studies of fungal biology including plant pathology methodology, ecological results of endophyte studies and screening for novel compounds from endophytes.

3.4.1 Implications to Plant Pathology Methodology

A standard protocol used for isolating plant pathogens involves cutting segments from the leading edge of lesions, which are then surface sterilized and plated onto media (Crous et al., 2009). The rationale is that the causative agent grows out from the lesions and can be isolated as a pure culture. Testing can then be undertaken to establish pathogenicity, while the colony can be identified using morphology. This standard methodology (Koch's postulate) has been long used by plant pathologists to determine the identity of non-sporulating pathogens ad infinitum (Phoulivong et al., 2010; Thomson et al., 2010; Wikee et al., 2011).

Recent studies on *Phyllosticta* causing freckle disease of banana and disease of other hosts have shown that extreme caution must be applied when using the above standard plant pathology approach (Wong et al., 2012). Conidia of *Phyllosticta* rarely germinate in culture and thus with many species it is impossible to obtain single spore cultures (Chomnunti et al., 2011). If freckle infected banana tissues are surface sterilized and plated on agar, *P. capitalensis* invariably grows out and, therefore, is concluded to be the pathogen, which is not the case. If these strains of *P. capitalensis* are used in pathogenicity testing they may also be weak pathogens and thus "substantiate" the record as the causal agent. However, Wong et al. (2012) carefully dissected whole ascomata from freckle diseased banana tissues. They then surface sterilized the ascomata and plated them out to obtain "single ascomata cultures". In this way they were able to establish that freckle disease was caused by more than one species of *Phyllosticta* and discerned the causal agent of freckle in Queensland as *P. cavendishii* M.H. Wong & Crous (Wong et al., 2012). *Phyllosticta citricarpa*, which causes citrus black spot (CBS) is widespread in some citrus-producing countries but is absent from EU and USA, where it is a regulated pathogen. CBS has been often misdiagnosed on citrus fruit and many of the lesions are, in fact, colonised by *P. capitalensis*. Traditional methods of diagnosis are time consuming and involve incubation of infected material, morphological examination of the fungus, and perhaps dissecting and plating of lesion pieces. Misdiagnosis of CBS may result in significant financial loss to farmers and exporters. An acurate and less time consuming method to verify and identify *Phyllosticta* species on citrus fruit is essential for both the producer and regulatory authorities (Meyer, Jacobs, Kotzé, Truter & Korsten, 2012).

Further careful research of this type in other banana growing regions is likely to reveal other species causing freckle disease. The above example serves to illustrate how a Koch's postulate can result in incorrect data concerning the identity of causal agents of disease, particularly with *Phyllosticta* species. Besides banana disease we suspect that many diseases caused by *Phyllosticta* (and "*Guignardia*"), unless directly identified via sporulating structures, e.g. *Guignardia candeloflamma* K.D. Hyde, on a species of *Pinanga* in north Queensland, Australia and an unidentified palm in Irian Jaya (Fröhlich & Hyde, 1995), may be wrongly attributed to *P. capitalensis*. Future studies must take this problem of protocol into account. Whether this phenomenon applies to other fungal genera needs future investigation.

3.4.2 Endophyte Study Protocols

There are many definitions of an endophyte and these have been summarized by Hyde and Soytong (2008). A standard definition is "organisms that colonize plant organs in some period of time in plant life cycle without causing obvious harm on the host" (Petrini 1984, 1991). The standard methodology for isolating endophytes has been reviewed in numerous instances (e.g. Guo, Hyde & Liew., 1998, 2001, Photita, et al., 2004; Photita, Taylor, Ford, Hyde & Lamyong, 2005) and has been criticised for being biased towards fast growing fungal strains (Hyde & Soytong, 2007). However, in principle the method is the same as that used by plant pathologists for isolating pathogens from diseased tissue, albeit that endophyte researchers use healthy leaves. The problem with the protocol mentioned above concerning the isolation of *P. capitalensis* rather than the *Phyllosticta* causal agent may also occur in endophyte studies. *Phyllosticta capitalensis* is a quick growing species; in culture, the colony covered 9 cm Petri-dish in 10 days. Other species grow more slowly, e.g. *P. yuccae* reaches 3–5 cm diam in 15 days (Bissett, 1986), while growth of *P. vaccinii* can be as low as 0.4 cm/day (Weidemann et al., 1982). Four species of *Phyllosticta* (*P. citriasiana*, *P. capitalensis*, *P. citricarpa* and *P. citrichinaensis*) were recently isolated from *Citrus* in China (Wang et al., 2012) and *P. citrichinaensis* grew at 3.8 ± 0.34 mm per day at 24°C on PDA. Therefore, it is highly likely that *P. capitalensis* will be isolated in endophyte studies, while others species which are probably also endophytes, will not be isolated. This will skew the results considerably and the resulting endophyte lists, percentages and statistics may have little scientific meaning.

If this phenomenon of isolating *P. capitalensis* for the reasons mentioned above is happening in the case of *Phyllosticta* it may also be happening in other genera such as *Colletotrichum*, *Diaporthe*, *Fusarium* or *Pestalotiopsis* (Promputtha, Jeewon, Lamyoung, McKenzie & Hyde, 2005; Udayanga et al., 2011; Summerell et al., 2010; Maharachchikumbura, Guo, Chukeatirote, Bahkali & Hyde, 2011; Damm, Cannon, Woudenberg, Johnston et al., 2012). To determine this fact we took the common ubiquitous endophytes *Colletotrichum siamense* Prihastuti, L. Cai & K.D. Hyde, *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., and *Pestalotiopsis adusta* (Ellis & Everh) Steyaert and blasted the ITS sequence data from the epitype strains against GenBank accessions and established the percentage of them that were isolated as

endophytes. Twelve strains of *Colletotrichum* in GenBank had 100% similarity with the ITS sequence data of *C. siamense* (Prihastuti, Cai, Chen, McKenZie & Hyde, 2009) and 50% of these strains were isolated as endophytes. The ITS sequence of ex-isotype of *D. phoenicicola* (CBS161.64, Udayanga et al., 2012) was subjected to a standard BLAST search in GenBank to analyze the homology of sequences. Among the first 10 results of highly similar sequences (100 or 99% similarity) of retrieved data, eight were isolated as endophytes from a wide range of hosts. This is not surprising as *Diaporthe* is a commonly isolated genus of fungal endophytes (Botella & Diez, 2011; Sun, Gou & Hyde, 2011; Hofstetter et al., 2012). Eleven strains of *Pestalotiopsis* in GenBank had 100% similarity with the ITS sequence data of *P. adusta* (Maharachchikumbura et al., 2012) and 73% were endophytes. Again this is not surprising as *Pestalotiopsis* species are often isolated as endophytes (Aly, Debbab, Kjer & Proksh, 2010; Debbab, Aly & Proksch, 2011, 2012; Maharachchikumbura et al., 2011). Therefore, it seems that certain taxa in these genera are widespread endophytes and this needs further study.

3.4.3 Screening of Endophytes for Novel Compounds

It has been common practice to isolate endophytes from medicinal plants using the premise that strains will be isolated that can produce bioactive compounds similar to those produced by the plant (Krohn et al., 2007; Huang et al., 2008; Kumaran et al., 2008a, 2008b; Xu et al., 2010; Zhao et al., 2010). The fungi are thought to have obtained the mechanisms of production of natural products from the plant by so called horizontal gene transfer (Strobel, Daisy, Castillo & Harper, 2004), whether this premise is correct or pure speculation is open to debate (Schulz, Boyle, Draeger & Römmert, 2002; Selim, El-Beih, Abdel-Rahman & El-Diwany, 2012) and in fact may be false (Heinig, Scholz & Jennewein, 2013). The isolation of endophytes may provide a large diversity of highly creative fungi for screening (Aly et al., 2010; Xu et al., 2010; Debbab et al., 2011, 2012). The findings of the present study indicate that there are problems with the above approach. It is clear in the case of *Phyllosticta* that *P. capitalensis* will probably be the only endophyte species isolated. Therefore, we recommend that researchers screening for novel compounds should study the saprobes and pathogens as well as the endophytes. This will give a higher fungal

diversity and higher likelihood of isolating rare and unusual species, and thus a higher likelihood of discovering greater chemical diversity.

3.5 Conclusion

Phyllosticta is an important plant pathogenic genus known to cause leaf spots and various fruit diseases worldwide on a large range of hosts. Species recognition in *Phyllosticta* has historically been based on morphology, culture characters and host association. Although there have been several taxonomic revisions and enumerations of species, there is still considerable confusion when identifying taxa. Recent studies based on molecular data have resolved some cryptic species and some novel taxa have been discovered. However, compared to the wide species diversity and taxonomic records, there is a lack of molecular studies to resolve current names in the genus. In this present study a phylogenetic tree is generated by combined gene analysis (ITS, partial actin and partial elongation factor 1α) using a selected set of taxa including type-derived sequences available in GenBank. Based on the phylogenetic tree, it is still likely that all *P. capitalensis* isolates used in this study formed a monophyletic clade. The data obtained also showed that *P. capitalensis* species are worldwide distributed (i.e., Brazil, Germany, Ghana, Hawaii, Japan, New Zealand, South Africa, and USA); the species are present in more than 60 plant hosts.

CHAPTER 4

A PHYLOGENETIC RE-EVALUATION OF *Phyllosticta, Botryosphaeriales*

4.1 Introduction

The genus *Phyllosticta* was introduced by Persoon (1818) with *P. convallariae* designated as the type species (Donk, 1968), which is a synonym of *P. cruenta* (Van der Aa, 1973). Species of *Phyllosticta* are mostly plant pathogens covering a broad range of hosts, and responsible for numerous diseases including leaf and fruit spots. Some of these are of huge economic importance, namely *P. citricarpa*, the cause of black spot on citrus, which is regarded as a quarantine pest in Europe and the USA (Baayen et al., 2002; Glienke et al., 2011). Other economically important plant pathogenic species include the *P. amplicida* species complex that causes black rot disease on grapevines (Wicht, Petrin, Jermini, Gessler & Broggini, 2012), and the *P. musarum* species complex that causes banana freckle disease (Pu et al., 2008; Wong et al., 2012). Several members of the genus have also been isolated as endophytes, often occurring on a wide range of hosts, e.g. *P. capitalensis*, while others are again regarded as saprobes, e.g. *P. carpogena* and *P. ericae* (van der Aa, 1973; Baayen et al., 2002; van der Aa et al., 2002; Glienke et al., 2011; Wikee et al., 2011). Presently there (http://www.indexfungorum. are approximately 3,200 epithets known for *Phyllosticta* org/ Names/Names.asp; accessed March 2013), but many of these reflect old conceptsof the genus, and have since been accommodated elsewhere (van der Aa et al., 2002). Many species also produce spermatial states, and in some cases these have also been named in *Leptodothiorella* (van der Aa, 1973)

4.1.1 History of the Genus

For many years researchers have confused the generic circumscription of *Phoma* and *Phyllosticta*. Both genera were recognised as pycnidial fungi forming unicellular, hyaline conidia. Allescher (1898) separated the two genera based on the plant part being attacked, with *Phyllosticta* occurring on leaves, and *Phoma* occurring on other plant parts. This concept was further refined by Grove (1935) who regarded *Phyllosticta* as a parasite and *Phoma* as saprobe or wound parasite. As was common at the time for many fungi, Seaver (1922) and Grove (1935) separated "*Phyllosticta*" species based on host preference. Seaver described 300 species, and Grove approximately 150 species. In both cases the host plant was the main criterion on which species were separated. Indeed, Seaver's book was largely characterised based on spore size on host plants, while Grove's book was arranged in alphabetical order of the host genus. Many *Phyllosticta* species were given specific epithets that are based on the host family, genus or species. For example, *P. iridis* on *Iris versicolor* (*Iridaceae*), *P. eugeniae* on *Eugenia buxifolia* (*Myrtaceae*), *P. monor* on *Vinca monor* (*Apocynaceae*), etc. (Seaver, 1922). For the plant pathogenic *Phyllosticta* species, separation based on host species (or sometimes genus) has in general proven to be a reliable method to distinguish species, but certainly does not hold true for the endophytic or saprobic species.

4.1.2 Sexual Morph

Viala & Ravaz (1892) introduced *Guignardia* as a replacement name for *Laestadia* Auersw. (1869), which was a later homonym of *Laestadia* Kunth ex Lessing (1832). Viala & Ravaz applied the name only to *Sphaeria bidwellii* (≡ *G. bidwellii*), a species that is different from *L. alnea*, the type species of *Laestadia* Auersw. (Bissett, 1986). Petrak (1958) concluded that *G. bidwellii* and related species could be accommodated in *Botryosphaeria*, and Barr (1970, 1972) agreed with Petrak and placed *Guignardia* and *Phyllosticta* in *Botryosphaeria*, and other related species in *Discosphaerina*.

Punithalingam (1974) suggested that the genus *Guignardia* must be confined to only those taxa with *Phyllosticta* morphs as typified by *G. bidwellii*. He stated that *Botryosphaeria* usually has larger ascomata and ascospores, and also a multilocular

stroma, features that distinguish it from *Guignardia*. van der Aa (1973) also pointed out that these two genera had different growth characteristics in culture. Following molecular studies, Schoch et al. (2006) placed *Phyllosticta* in the *Botryosphaeriales.* Since *Botryosphaeria* has been shown to be poly- and paraphyletic, numerous genera have been distinguished in the *Botryosphaeriaceae* (Crous et al., 2006; Phillips et al., 2008; Liu et al., 2012). With the increasing use of molecular data to link asexual and sexual morphs, and the end of dual nomenclature for fungi (Hawksworth, 2011; Wingfield et al., 2012), the oldest, more important and commonly used name, *Phyllosticta*, was chosen over that of *Guignardia* (Glienke et al., 2011; Wikee et al., 2011, 2013; Wong et al., 2012).

4.1.3 Morphology

The principal character by which a fungus is recognised as a species of *Phyllosticta* is by the production of pycnidia containing aseptate, hyaline conidia that are usually covered by a mucoid layer and bearing a single apical appendage (van der Aa, 1973). However, the mucoid layer and appendage is not necessarily a universal feature, and some species such as *P. colocasiicola*, *P. minima*, and *P. sphaeropsoidea* lack these features. Furthermore, mucoid appendages formed on agar media could also disappear with age, or vary in size and shape when the same isolate is compared on different media such as pine needle agar, oatmeal agar, or potato dextrose agar. Presently *Phyllosticta* is circumscribed by producing pycnidia that are usually globose to subglobose, flattened above, and closely connected with the subepidermal pseudostroma. They are mostly unilocular but occasionally may be multilocular. The conidia are commonly hyaline, aseptate, ovoid, obovoid to ellipsoid, or short cylindrical, seldomly pyriform, globose or subglobose, and usually covered by a mucoid layer and bearing a single apical appendage (van der Aa, 1973). The sexual morph is characterised by erumpent ascomata that are globose to pyriform in section, often irregularly shaped, unilocular, and with a central ostiole. The peridium is thin, comprising a few layers of angular cells. Asci are 8-spored, bitunicate, clavate to broadly ellipsoid, with a wide, obtusely rounded or slightly square apex, tapering gradually to a small pedicel, and with a well-developed ocular chamber. Ascospores are ellipsoid to limoniform, sometimes slightly elongated, aseptate, hyaline, often multiguttulate or with a large central guttule, and some have mucilaginous polar

appendages or a sheath. A spermatial state readily forms in culture. Spermatia are hyaline, aseptate, cylindrical to dumbbell-shaped with guttules at each end (van der Aa, 1973; Wulandari et al., 2011).

Phyllosticta s. str. was first monographed by van der Aa (1973), who described and illustrated 46 species, and listed the sexual morphs for 12 species, and the spermatial morphs for 17 species. This study was largely based on material collected in Europe and North America. More recently van der Aa et al., (2002) revised all species names described in *Phyllosticta*, and provided a list of 190 accepted epithets, and a second list of excluded names, indicating their current disposition where known.

4.1.4 Molecular Approach and Current Taxonomic Situation

In recent years DNA sequencing of conserved loci has vastly improved our knowledge of fungal phylogeny. Several studies have shown that phylogenetic analysis can resolve the taxonomy and identification of *Phyllosticta* spp. (Baayen et al., 2002; Wulandari et al., 2009; Glienke et al., 2011; Wikee et al., 2011). Indeed, new species of *Phyllosticta* are increasingly being described based on molecular results (Crous et al., 2012; Wang et al., 2012; Su & Cai, 2012; Wong et al., 2012; Zhang et al., 2012).

Phyllosticta was placed in the order *Botryosphaeriales* by Schoch et al. (2006), who proposed that the *Botryosphaeriaceae* contained both *Botryosphaeria* and *Phyllosticta*, though no support was obtained for this relationship. Crous et al. (2006) also classified *Phyllosticta* in the *Botryosphaeriaceae*, as did Liu et al. (2012). In both studies it was however noted that *Phyllosticta* was distinct from other genera in the *Botryosphaeriaceae*, and that they expected it to eventually be allocated elsewhere. Seaver (1922) used the order *Phyllostictales* and family *Phyllostictaceae* for the genus *Phyllosticta*. The family name *Phyllostictaceae* (as Phyllostictei) was first proposed by Fries (1849) and accepted by Hawksworth and David (1989). This family name is still available for use, and therefore we suggest that *Phyllosticta* again be placed in this family, which is sister to the *Botryosphaeriaceae* in the *Botryosphaeriales*.

4.1.5 Objectives of This Chapter

Although phylogenetic analysis has become a standard approach in fungal identification, phylogenetic studies should combine both molecular and morphological data to help discriminate among taxa (Crous & Groenewald, 2005; Hyde, Abd-Elsalam et al., 2010). Suitable type material that can be sequenced is not available for many species of fungi and, hence, epitypification is required in order to obtain a clear understanding of the relationship between species. The objectives of this study are: (1) to clarify relationships among species of *Phyllosticta* using multi-gene sequence data (internal transcribed spacer region (ITS), translation elongation factor 1-α gene (TEF1), actin gene (ACT), 28S rRNA gene (LSU) and glyceraldehydes-3-phophate dehydrogenase gene (GPDH) combined with morphological characteristics; (2) to provide a phylogenetic backbone for the genus *Phyllosticta*, and (3) to designate epitype specimens for fungal isolates that correlate well with original type material, thereby fixing the genetic application of these names.

4.2 Material and Methods

4.2.1 Isolates

A global collection of 169 strains of *Phyllosticta* associated with both leaf spot diseases and healthy leaves of various host plants were studied (Table 4.1). All isolates were sequenced and analysed along with analysis of sequences downloaded from GenBank. If fruit bodies were present on diseased tissue then a single spore isolation procedure as described by Chomnunti et al. (2011) was used to obtain cultures. To obtain isolates of *Phyllosticta* from diseased leaves of host plants when fruit bodies were not present, the leaf surface was cleaned by wiping with 70 % ethanol. Small pieces of leaf were then cut from the interface between healthy and diseased tissue. These were surface sterilised in 70 % ethanol, washed and plated onto ½ strength potato dextrose agar (½ PDA). For isolation of endophytic species, healthy leaves were washed in tap water and wiped with 70 % ethanol. They were then cut into small pieces (about 1 × 1 cm), suspended in 70 % ethanol for 15 min (3 times) and washed in distilled water (3 times) before placing on ½ PDA. All plates were

incubated at 27 ºC for 1 wk and observed daily. The growing tips of hyphae of *Phyllosticta* colonies that developed were cut out and transferred to fresh PDA plates. Isolates are deposited in Mae Fah Luang University Culture Collection (MFLUCC) and in the working collection of Pedro Crous housed at CBS in Utrecht, The Netherlands (CPC). Other fungal isolates of representative *Phyllosticta* spp. were obtained from CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands.

4.2.1.1 Morphology

Growth rates, culture characteristics, and morphology of the isolates were determined at 27 °C. To induce sporulation, isolates were grown on pine needle agar (PNA) (Smith, Wingfield, Crous & Coutinho, 1996) and synthetic nutrient-poor agar (SNA) under near UV-light. Colony colour and growth rate were established on PDA, malt extract agar (MEA) and oatmeal agar (OA) according to Crous et al., (2009). Culture characteristics were assessed, and the colour of upper and lower sides of cultures was determined after 14 d in the dark at 27 °C. Colony colour on MEA, OA and PDA were determined using the colour charts of Rayner (1970). Details of nomenclatural novelties and descriptions were deposited in MycoBank (www. Myco-bank.org; Crous et al., 2004).

4.2.1.2 DNA Extraction, Amplification, and Sequencing

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

primers (Myllys, Stenroos & Thell, 2002). *P. citricarpa* isolates were amplified by specific primer Gpd1 (Guerber, Lui, Correll & Johnston, 2003) and GPDHR2 (5′-CTCRGMRGCRGCCTTG ATGG-3′) as developed by Glienke et al. (2011). Cycle sequencing of PCR products was performed in PCR conditions. PCR products were separated by gel electophoresis at 130 V for 20 min in 1 % agarose gel in 1× TAE running buffer and visualized under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK). Purified PCR was sequenced using both PCR primers with a BigDay Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) containing AmpliTag DNA Polymerase. The amplified products were analysed on an automatic DNA sequencer (Perkin-Elmer, Norwalk, CN). Sequences generated were automatically aligned using MAFFT v. 6 (http://mafft.cbrc.jp/alignment/server/). The sequences were corrected and manually aligned using MEGA v. 5.05 software (Tamura et al., 2011).

4.2.1.3 Molecular Phylogeny

Phylogenetic analysis was done using parsimony (PAUP v. 4.0b10; Swofford, 2002). For parsimony analysis, alignment gaps were treated as a fifth character state and all character were unordered and given equal weight. *Botryosphaeria obtusa* was used as outgroup in the phylogenetic tree. The confidence limit of the resulting tree was estimated by bootstrap analysis with 1000 replications (Hillis & Bull, 1993) and the tree was prepared in Adobe Illustrator CS3. Tree length (TL), consistency (CI), retention index (RI) and rescaled consistency index (RC) were calculated. Novel and representative sequences were deposited in GenBank.

4.3 Result

The phylogenetic relationship was determined of the internal transcribed spacer (ITS) and actin gene (ACT) sequences of 160 *Phyllosticta* strains (including one outgroup). The combined partial dataset of *Phyllosticta* comprised 883 characters (including gaps), of which 341 characters are constant, and 150 characters are variable and parsimony-uninformative. Parsimony analysis generated 1000 trees, one of the trees with similar with bootstrap values was chosen (number of bootstrap replicated =

1,000), as shown in Figure 4.1 (TL = 2099, CI = 0.481, RI = 0.898, RC = 0.432, HI = 0.519). The phylogenetic tree of the ITS and ACT region resolved 46 clades (see Table 4.1 for details).

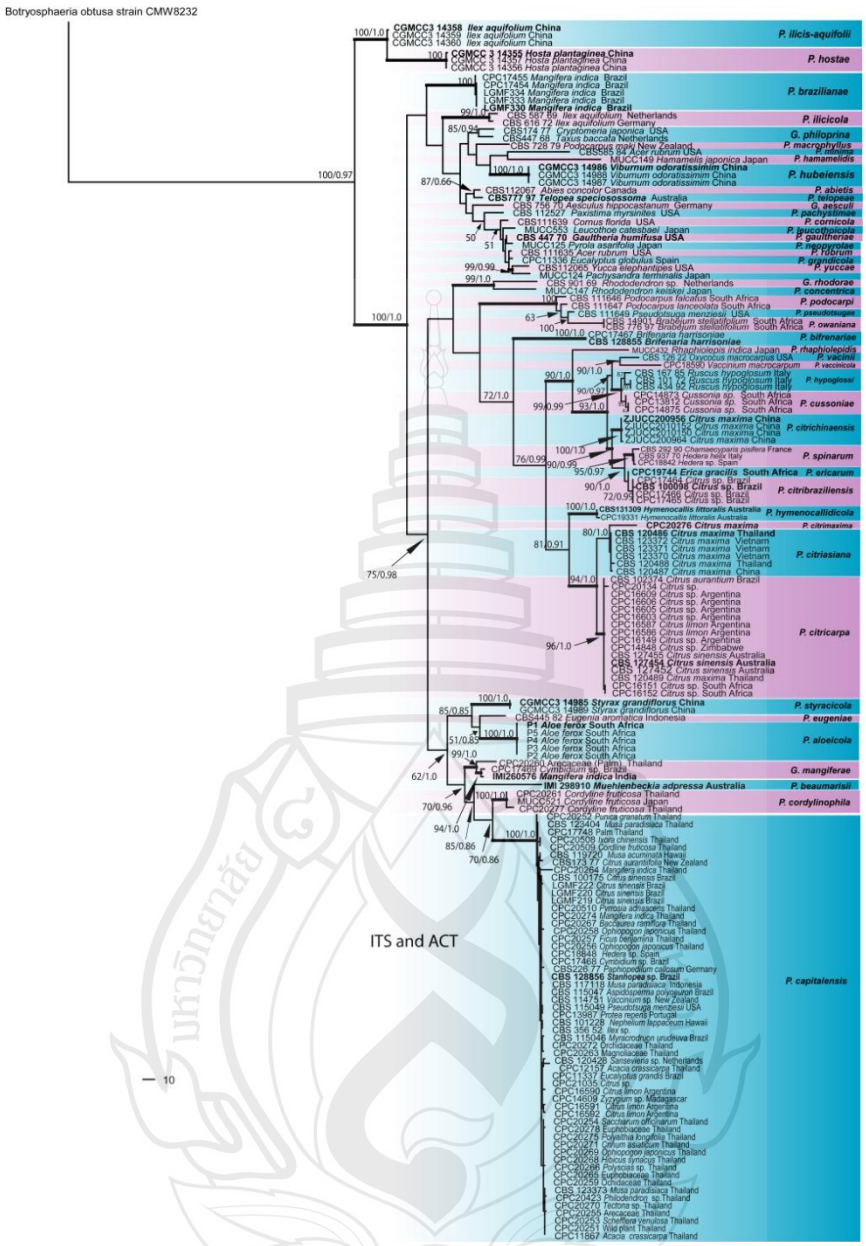


Figure 4.1 Phylogenetic Tree of *Phyllosticta* Generated from a Maximum Parsimony Analysis Vased on the ITS and ACT Sequence Alignment. Names in Bold are Represented Types and Ex-Types. Values on the Branches Represent Parsimony Bootstrap Support Value (> 50%). Thickend Branches Represent Significant Bayesian Analysis Value (≥ 90%) and the Scale Bar Indicates 10 Changes. *Botryosphaeria obtusa* Represent Out Group

A second analysis including all isolates available to us (129 strains including the outgroup) was run based on the internal transcribed spacer (ITS), 28s rRNA gene region (LSU), actin gene (ACT), translation elongation factor 1-α gene (TEF1) and glyceraldehyde-3- phosphate dehydrogenase (GPDH) sequences of) (Table 4.1). The combined partial dataset of *Phyllosticta* comprised 2,577 characters (including gaps), of which 1,547 characters are constant, 296 characters are variable and parsimony-uninformative. Parsimony analysis generated 1 000 trees, of which one tree with similar bootstrap values was chosen (number of bootstrap replicates = 1,000) and is shown in Figure 4.2 (TL = 3173, CI = 0.517, RI = 0.906, RC = 0.468, HI = 0.483). The phylogenetic tree using combined multi-gene data resolved 33 clades (see Table 4.1 for details).

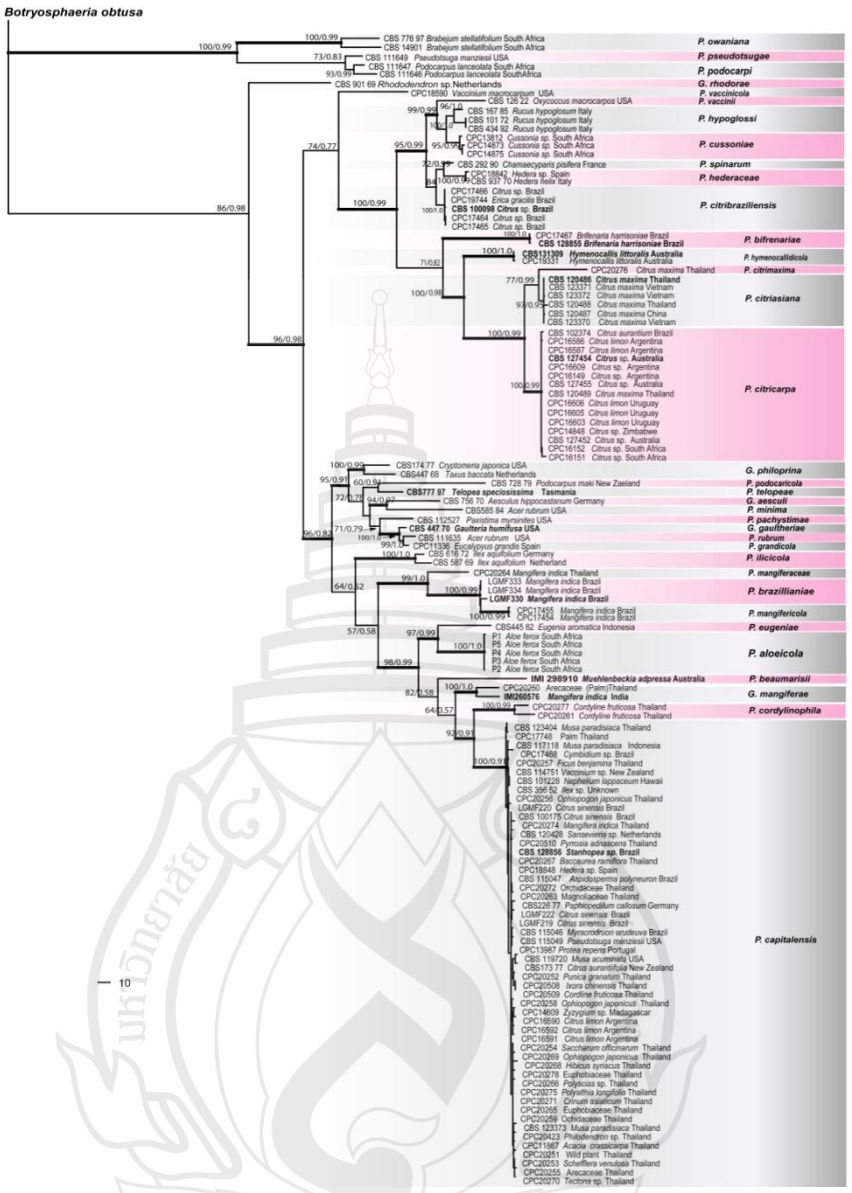


Figure 4.2 Phylogenetic Tree of *Phyllosticta* Generated from a Maximum Parsimony Analysis Based on the ITS, LAU, ACT, TEF and GPDH Sequence Alignment. Names in Bold are Represented Types and Ex-Types. Values on the Branches Represent Parsimony Bootstrap Support Value (>50%). Thickend Branches Represent Significant Bayesian Analysis Value (≥ 90%) and the Scale Bar Indicates 10 Changes. *Botryosphaeria obtusa* Represent Out Group

4.3.1 Taxonomy

Phyllosticta is quite distinct from members of the *Botryosphaeriaceae* in culture characteristics (slow growing, black erumpent colonies vs. grey, fluffy, fast-growing colonies). Furthermore, morphologically it is also quite distinct, as conidia are encased in a mucoid sheath, and frequently with apical appendage, and the sexual morph differs from those in the *Botryosphaeriaceae* (ascomata unilocular, ascospores frequently with mucoid caps, and hamathecial tissue disintegrating at maturity). As *Phyllosticta* is also phylogenetically supported as distinct from members of the *Botryosphaeriaceae*

Table 4.1 Isolates of *Guignardia* and *Phyllosticta* Used in the Phylogenetic Study

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
1	*G. aesculi*	CBS 756.70	*Aesculus hippocastanum*	Germany	AY042934	KF206294	KF289202	KF289253	KF289133
2	*G. gaultheriae*	CBS 447.70	*Gaultheria humifusa*	USA	JN692543	KF206298	JN692531	KF289248	JN692508
3	*G. mangiferae*	CPC 17469	*Cymbidium sp.*	Brazil	KF206189	-	-	KF289285	-
4	*G. mangiferae*	IMI 260576	*Mangifera indica*	India	JF261459	KF206222	JF261501	JF343641	JF343748
5	*G. mangiferae*	CPC 20260	*Arecaceae*	Thailand	KF206193	KF206243	KF289187	KF289294	KF289114
6	*P. abietis*	CBS 112067	*Abies concolor*	Canada	KF206208	EU754193	-	KF289238	-
7	*P. beaumarisii*	IMI 298910	*Muehlenbekia appressa*	Australia	AY042927	KF306229	KF289170	KF306232	KF289074
8	*P. citriasiana*	CBS 123370	*Citrus maxima*	Vietnam	FJ538355	KF206310	FJ538413	FJ538471	JF343689
9	*P. citriasiana*	CBS 120487	*Citrus maxima*	China	FJ538361	KF206313	FJ538419	FJ538477	JF343687
10	*P. bifrenariae*	VIC30556; CBS 128855	*Bifrenaria harrisoniae*	Brazil	JF343565	KF206209	JF343586	JF343649	JF343744
11	*P. bifrenariae*	CPC 17467	*Bifrenaria harrisoniae*	Brazil	KF170299	KF206260	KF289207	KF289283	KF289138
12	*P. brazilianiae*	LGMF330 CBS 126270	*Mangifera indica*	Brazil	JF343572	KF206217	JF343593	JF343656	JF343758

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
13	*P. brazilianiae*	LGMF 333	*Mangifera indica*	Brazil	JF343574	KF206216	JF343595	JF343658	JF343760
14	*P. brazilianiae*	LGMF 334	*Mangifera indica*	Brazil	JF343566	KF206215	JF343587	JF343650	JF343752
15	*P. mangifericola*	CPC 17454	*Mangifera indica*	Brazil	KF206206	KF206265	KF289192	KF289278	KF289123
16	*P. mangifericola*	CPC 17455	*Mangifera indica*	Brazil	KF206207	KF206264	KF289191	KF289279	KF289122
17	*P. capitalensis*	CPC20251	Wild plant	Thailand	KC291333	KF206252	KC342553	KC342530	KF289101
18	*P. capitalensis*	CPC20252	*Punica granatum*	Thailand	KC291334	KF206251	KC342554	KC342531	KF289097
19	*P. capitalensis*	CPC20254	*Saccharum officinarum*	Thailand	KC291335	KF206249	KC342555	KC342532	KF289103
20	*P. capitalensis*	CPC20255	*Arecaceae*	Thailand	KC291336	KF206248	KC342556	KC342533	KF289115
21	*P. capitalensis*	CPC20256	*Ophiopogon japonicus*	Thailand	KC291337	KF206247	KC342557	KC342534	KF289089
22	*P. capitalensis*	CPC20257	*Ficus benjamina*	Thailand	KC291338	KF206246	KC342558	KC342535	KF289099
23	*P. capitalensis*	CPC20258	*Ophiopogon japonicus*	Thailand	KC291339	KF206245	KC342559	KC342536	KF289094
24	*P. capitalensis*	CPC20259	*Orchidaceae*	Thailand	KC291340	KF206244	KC342560	KC342537	KF289104

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
25	*P. capitalensis*	CPC20263	*Magnoliaceae*	Thailand	KC291341	KF206241	KC342561	KC342538	KF289085
26	*P. capitalensis*	CPC20266	*Polyscias* sp.	Thailand	KC291342	KF206238	KC342562	KC342539	KF289109
27	*P. capitalensis*	CPC20267	*Baccaurea ramiflora*	Thailand	KF206195	KF206237	KF289173	KF306233	KF289078
28	*P. capitalensis*	CPC20268	*Hibiscus syriacus*	Thailand	KC291343	KF206236	KC342563	KC342540	KF289117
29	*P. capitalensis*	CPC20269	*Ophiopogon japonicus*	Thailand	KC291344	KF206235	KC342564	KC342541	KF289118
30	*P. capitalensis*	CPC20270	*Tectona grandis*	Thailand	KC291345	KF206234	KC342565	KC342542	KF289110
31	*P. capitalensis*	CPC20272	*Orchidaceae*	Thailand	KC291346	KF206232	KC342566	KC342543	KF289079
32	*P. capitalensis*	CPC20275	*Polyalthia longifolia*	Thailand	KC291347	KF206230	KC342567	KC342544	KF289107
33	*P. capitalensis*	CPC20278	*Euphorbia milii*	Thailand	KC291348	KF206227	KC342568	KC342545	KF289113
34	*P. capitalensis*	CPC20423	*Philodendron* sp.	Thailand	KC291349	KF206226	KC342569	KC342546	KF289116
35	*P. capitalensis*	CBS 100175	*Citrus* sp.	Brazil	FJ538320	KF206327	FJ538378	FJ538436	JF343699
36	*P. capitalensis*	CBS 114751	*Vaccinium* sp.	New Zealand	EU167584	EU167584	FJ538407	FJ538465	KF289088
37	*P. capitalensis*	CBS 117118	*Musa acuminata*	Indonesia	FJ538339	JQ743603	FJ538455	FJ538397	KF289090

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
38	*P. capitalensis*	CBS 115046	*Myracrodruon urundeuva*	Brazil	FJ538322	KF206319	FJ538380	FJ538438	KF289082
39	*P. capitalensis*	CBS 115047	*Aspidosperma polyneuron*	Brazil	FJ538323	KF206318	FJ538381	FJ538439	KF289077
40	*P. capitalensis*	CPC 20510	*Pyrrosia adnascens*	Thailand	KF206200	KF206223	KF289174	KF289304	KF289080
41	*P. capitalensis*	CBS 120428	*Sensevieria* sp.	Netherlands	JN692544	KF206315	JN692532	JN692520	JN692509
42	*P. capitalensis*	CBS 356.52	*Ilex* sp.	Unknown	FJ538342	KF206300	FJ538400	FJ538458	KF289087
43	*P. capitalensis*	CBS 101228	*Naphelium lappaceum*	Hawaii	FJ538319	KF206325	FJ538377	FJ538435	KF289086
44	*P. capitalensis*	CPC 13987	*Protea repens*	Portugal	KF206183	KF206281	KF289176	KF289263	KF289083
45	*P. capitalensis*	CPC 14609	*Zyzygium* sp.	Madagascar	KF206184	KF206280	KF289175	KF289264	KF289081
46	*P. capitalensis*	CBS 128856	*Stanhopea* sp.	Brazil	JF261465	KF206304	JF261507	JF343647	JF343776
47	*P. capitalensis*	CBS 115049	*Bowdichia nitida*	Brazil	FJ538324	KF206317	FJ538382	FJ538440	KF289084
48	*P. capitalensis*	CBS 123373	*Musa paradisiaca*	Thailand	FJ538341	JQ743604	FJ538399	FJ538457	JF343703
49	*P. capitalensis*	CBS 123404	*Musa paradisiaca*	Thailand	FJ538333	JQ743601	FJ538391	FJ538449	KF289095

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
50	*P. spinarum*	CBS 292.90	*Chamaecyparis pisifera*	France	JF343585	KF206301	JF343606	JF343669	JF343773
51	*P. hederaceae*	CBS 937.70	*Hedera helix*	Spain	FJ538350	KF206291	FJ538408	KF289257	JF411745
52	*P. hederaceae*	CPC 18842	*Hedera* sp.	Italy	KF170310	KF206256	KF289228	KF289288	KF289163
53	*P. capitalensis*	CBS 226.77	*Paphiopedilum callosum*	Germany	FJ538336	KF206289	FJ538394	FJ538452	JF343718
54	*P. capitalensis*	CPC 18848	*Stanhopea graveolens*	Brazil	JF261465	KF206255	JF261507	KF289289	JF343776
55	*P. citriasiana*	CBS 120486	*Citrus maxima*	Thailand	FJ538360	KF206314	FJ538418	FJ538476	JF343686
56	*P. citriasiana*	CBS 120488	*Citrus maxima*	Thailand	JN692545	KF206312	JN692533	JN692521	KF289144
57	*P. citriasiana*	CBS 123371	*Citrus maxima*	Vietnam	FJ538356	KF206309	FJ538414	FJ538472	JF343690
58	*P. citriasiana*	CBS 123372	*Citrus maxima*	Vietnam	FJ538357	KF206308	FJ538415	FJ538473	KF289145
59	*P. citribraziliensis*	CBS100098	*Citrus* sp.	Brazil	FJ538352	KF206221	FJ538410	FJ538468	JF343691
60	*P. citribraziliensis*	CPC 17464	*Citrus* sp.	Brazil	KF170300	KF206263	KF289224	KF289280	KF289159
61	*P. citribraziliensis*	CPC 17465	*Citrus* sp.	Brazil	KF170301	KF206262	KF289225	KF289281	KF289160

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
62	*P. citribraziliensis*	CPC 17466	*Citrus* sp.	Brazil	KF170302	KF206261	KF289226	KF289282	KF289161
63	*P. ericarum*	CPC 19744	*Erica gracilis*	Brazil	KF206170	KF206253	KF289227	KF28291	KF289162
64	*P. citricarpa*	CBS 102374	*Citrus aurantium*	Brazil	FJ538313	KF206324	GU349053	FJ538429	JF343679
65	*P. citricarpa*	CBS 120489	*Citrus sinensis*	Thailand	FJ538315	KF206311	FJ538373	FJ538431	KF289150
66	*P. citricarpa*	CBS127454	*Citrus limon*	Australia	JF343583	KF206306	JF343604	JF343667	JF343771
67	*P. citricarpa*	CBS 127452	*Citrus* sp.	Australia	JF343581	KF206307	JF343602	KF289241	JF343769
68	*P. citricarpa*	CBS 127455	*Citrus sinensis*	Australia	JF343584	KF206305	JF343605	JF343668	JF343772
69	*P. citricarpa*	CPC 16586	*Citrus limon*	Argentina	KF170293	KF206274	KF289220	KF289269	KF289155
70	*P. citricarpa*	CPC 16587	*Citrus limon*	Argentina	KF170294	KF206273	KF289219	KF289270	KF289154
71	*P. citricarpa*	CPC 16603	*Citrus limon*	Uruguay	KF170295	KF206269	KF289213	KF289274	KF289147
72	*P. citricarpa*	CPC 16605	*Citrus limon*	Uruguay	KF170296	KF206268	KF289214	KF289275	KF289148
73	*P. citricarpa*	CPC 16606	*Citrus limon*	Uruguay	KF170297	KF206267	KF289215	KF289276	KF289149
74	*P. citricarpa*	CPC 16609	*Citrus* sp.	*Argentina*	KF170298	KF206266	KF289217	KF289277	KF289152

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
75	*P. citricarpa*	CPC 14848	*Citrus* sp.	Zimbabwe	FJ538317	KF306230	FJ538375	KF289265	KF289146
76	*P. citricarpa*	CPC 16149	*Citrus* sp.	Argentina	KF170290	KF206277	KF289216	KF289266	KF289151
77	*P. citricarpa*	CPC 16151	*Citrus* sp.	South Africa	KF170291	KF206276	KF289221	KF289267	KF289156
78	*P. citricarpa*	CPC 16152	*Citrus* sp.	South Africa	KF170292	KF206275	KF289218	KF289268	KF289153
79	*P. citricarpa*	CPC 20134	*Citrus sp.*	-	KF170298	-	-	KF289292	-
80	*P. citrichinaensis*	ZJUCC 200956	*Citrus reticulata*	China	JN791664	-	JN791515	JN791589	-
81	*P. citrichinaensis*	ZJUCC 200964	*Citrus maxima*	China	JN791662	-	JN791514	JN791582	-
82	*P. citrichinaensis*	ZJUCC 2010150	*Citrus maxima*	China	JN791620	-	JN791459	JN791533	-
83	*P. citrichinaensis*	ZJUCC 2010152	*Citrus sinensis*	China	JN791611	-	JN791461	JN791535	-
84	*P. cordylinophila*	MUCC 521	*Cordyline fruticosa*	Japan	AB454357	-	-	AB704244	-
85	*P. cordylinophila*	CPC 20261	*Cordyline fruticosa*	Thailand	KF170287	KF206242	KF289172	KF289295	KF289076
86	*P. cordylinophila*	CPC 20277	*Cordyline fruticosa*	Thailand	KF170288	KF206228	KF289171	KF289301	KF289075
87	*P. cussonia*	CPC 13812	*Cussonia* sp.	South Africa	KF170311	KF206282	KF289223	KF289262	KF289158

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
88	*P. cussonia*	CPC 14873	*Cussonia* sp.	South Africa	JF343579	KF206279	JF343600	JF343663	JF343764
89	*P. cussonia*	CPC 14875	*Cussonia* sp	South Africa	JF343578	KF206278	JF343599	JF343662	JF343765
90	*P. hypoglossi*	CBS 434.92	*Ruscus aculeatus*	Italy	FJ538367	KF206299	FJ538425	FJ538483	JF343695
91	*P. hypoglossi*	CBS 101.72	*Ruscus aculeatus*	Italy	FJ538365	KF206326	FJ538423	FJ538481	JF343694
92	*P. hypoglossi*	CBS 167.85	*Ruscus hypoglossum*	Italy	FJ538366	KF206302	FJ538424	FJ538482	JF343696
93	*P. ilicis-aquifolii*	CGMCC 3.14358	*Ilex aquifolium*	China	JN692538	-	JN692526	JN692514	-
94	*P. ilicis-aquifolii*	CGMCC 3.14359	*Ilex aquifolium*	China	JN692539	-	JN692527	JN692515	-
95	*P. ilicis-aquifolii*	CGMCC 3.14360	*Ilex aquifolium*	China	JN692540	-	JN692528	JN692516	-
96	*P. hostae*	CGMCC 3.14355	*Hosta plantaginea*	China	JN692535	-	JN692523	JN692511	JN692503
97	*P. hostae*	CGMCC 3.14356	*Hosta plantaginea*	China	JN692536	-	JN692524	JN692512	JN692504
98	*P. hostae*	CGMCC 3.14357	*Hosta plantaginea*	China	JN692537	-	JN692525	JN692513	JN692505
99	*P. owaniana*	CBS 776.97	*Brabejum stellatifolium*	South Africa	FJ538368	KF206293	FJ538426	KF289254	JF343767
100	*P. owaniana*	CPC 14901	*Brabejum stellatifolium*	South Africa	JF261462	KF206303	JF261504	KF289243	JF343766

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
101	*P. podocarpi*	CBS 111646	*Podocarpus falcatus*	South Africa	AF312013	KF206323	KC357671	KC357670	KF289169
102	*P. eugeniae*	CBS 445.82	*Eugenia aromatica*	Indonesia	AY042926	KF206288	KF289208	KF289246	KF289139
103	*P. styracicola*	CGMCC 3.14985	*Styrax gradiflorus*	China	JX052040	-	JX025045	JX025035	JX025030
104	*P. styracicola*	CGMCC 3.14989	*Styrax gradiflorus*	China	JX052041	-	JX025046	JX025036	JX025031
105	*P. vacinii*	CPC 18590	*Vaccinium macrocarpum*	USA	KF170312	KF206257	KF289229	KF289287	KF289165
106	*P. vacinii*	CBS 126.22	*Oxycoccus macrocarpos*	USA	FJ538353	AB095508	FJ538411	FJ538469	KF289164
107	*G. rhodorae*	CBS 901.69	*Rhododendron* sp.	Netherlands	KF206174	KF206292	KF289230	KF289256	KF289166
108	*P. podocarpi*	CBS 111647	*Podocarpus lanceolata*	South Africa	KF154276	KF206322	KF289232	KF289235	KF268168
109	*P. pseudotsugae*	CBS 111649	*Pseudotsuga manziesii*	USA	KF154277	KF206321	KF289231	KF289236	KF289167
110	*P. hymenocallidicola*	CBS 131309	*Hymenocallis littoralis*	Australia	JQ044423	JQ044443	KF289211	KF289242	KF289142
111	*P. hymenocallidicola*	CPC 19331	*Hymenocallis littoralis*	Australia	KF170303	KF206254	KF289212	KF289290	KF289143
112	*P. citrimaxima*	CPC 20276	*Citrus maxima*	Thailand	KF170304	KF206229	KF289222	KF289300	KF289157

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
113	*P. telopeae*	CBS 777.97	*Telopea speciosissima*	Australia	KF206205	KF206285	KF289210	KF289255	KF289141
114	*P. pachystima*	CBS 112527	*Paxistima mysinites*	USA	KF206172	KF206320	KF289209	KF289239	KF289140
115	*P. ilicicola*	CBS 587.69	*Ilex aquifolium*	Netherlands	KF154278	KF206297	KF289206	KF289250	KF289137
116	*P. ilicicola*	CBS 616.72	*Ilex aquifolium*	Netherlands	KF154279	KF206296	KF289205	KF289251	KF289136
117	*G. philoprina*	CBS 174.77	*Cryptomeria japonica*	USA	KF170308	KF206290	KF289200	KF289245	KF289131
118	*G. philoprina*	CBS 447.68	*Taxus baccata*	Netherlands	KF170309	KF206287	KF289201	KF289247	KF289132
119	*P. macrophyllus*	CBS 728.79	*Podocarpus maki*	New Zealand	KF206173	KF206295	KF289203	KF289252	KF289134
120	*P. minima*	CBS 585.84	*Acer rubrum*	USA	KF206176	KF206286	KF289204	KF289249	KF289135
121	*P. grandicola*	CPC 11336	*Eucalyptus grandis*	Spain	KF206177	KF206284	KF289199	KF289258	KF289130
122	*P. mangiferaceae*	CPC20264	*Mangifera indica*	Thailand	KF170305	KF206240	KF289190	KF289296	KF289121
123	*P. aloeicola*	CPC 21020	*Aloe ferox*	South Africa	KF154280	KF206210	KF289193	KF289311	KF289124
124	*P. aloeicola*	CPC 21021	*Aloe ferox*	South Africa	KF154281	KF206211	KF289194	KF289312	KF289125

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
125	*P. aloeicola*	CPC 21022	*Aloe ferox*	South Africa	KF154282	KF206212	KF289195	KF289313	KF289126
126	*P. aloeicola*	CPC 21023	*Aloe ferox*	South Africa	KF154283	KF206213	KF289196	KF289314	KF289127
127	*P. aloeicola*	CPC 21024	*Aloe ferox*	South Africa	KF154284	KF206214	KF289197	KF289315	KF289128
128	*P. capitalensis*	CPC 17748	*Palm*	Thailand	KF206190	KF206258	KF289180	KF289286	KF289096
129	*P. capitalensis*	CPC 17468	*Cymbidium* sp.	Brazil	KF206188	KF206259	KF289189	KF289284	KF289120
130	*P. capitalensis*	LGMF 220	*Citrus sinensis*	Brazil	KF206203	KF206219	JF261488	KF289307	JF343735
131	*P. capitalensis*	CPC 20274	*Mangifera indica*	Thailand	KF206197	KF206231	KF289188	KF289299	KF289119
132	*P. capitalensis*	LGMF 222	*Citrus sinensis*	Brazil	KF206204	KF206218	JF343632	KF289308	JF343739
133	*P. capitalensis*	LGMF 219	*Citrus sinensis*	Brazil	KF206202	KF206220	JF261490	KF289306	JF343737
134	*P. capitalensis*	CBS 119720	*Musa acuminata*	USA	KF206178	KF206316	FJ538398	KF289240	KF289098
135	*P. capitalensis*	CBS 173.77	*Citrus aurantiifolia*	New Zealand	KF206179	KF306231	FJ538393	KF289244	KF289100
136	*P. capitalensis*	CPC 20508	*Ixora chinensis*	Thailand	KF206198	KF206225	KF289185	KF289301	KF289111
137	*P. capitalensis*	CPC 20509	*Cordyline fruticosa*	Thailand	KF206199	KF206224	KF289186	KF289302	KF289112

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
138	*P. capitalensis*	CPC 16590	*Citrus limon*	Argentina	KF206185	KF206272	KF289177	KF289271	KF289091
139	*P. capitalensis*	CPC 16592	*Citrus limon*	Argentina	KF206187	KF206270	KF289178	KF289273	KF289092
140	*P. capitalensis*	CPC 16591	*Citrus limon*	Argentina	KF206186	KF206271	KF289179	KF289272	KF289093
141	*P. capitalensis*	CPC 20271	*Crinum asiaticum*	Thailand	KF206196	KF206233	KF289183	KF289298	KF289106
142	*P. capitalensis*	CPC 20265	*Euphobiaceae*	Thailand	KF206194	KF206239	KF289182	KF289297	KF289105
143	*P. capitalensis*	CPC 11867	*Acacia crassicarpa*	Thailand	KF206181	KF206283	KF289184	KF289260	KF289108
144	*P. capitalensis*	CPC 20253	*Scheffera venulosa*	Thailand	KF206192	KF206250	KF289181	KF289293	KF289102
145	*P. hamamelidis*	MUCC 149	*Hamamelis japonica*	Japan	KF170289	-	-	KF289309	-
146	*P. hubeiensis*	CGMCC3.14986	*Viburnum odoratissimim*	China	JX025037	-	JX025042	JX025032	JX025027
147	*P. hubeiensis*	CGMCC3.14987	*Viburnum odoratissimim*	China	JX025038	-	JX025043	JX025033	JX025028
148	*P. hubeiensis*	CGMCC3.14988	*Viburnum odoratissimim*	China	JX025039	-	JX025044	JX025034	JX025029
149	*P. cornicola*	CBS 111639	*Coemus florida*	USA	KF170307	-	-	KF289234	-
150	*P.leucothoicola*	MUCC553	*Leucothoe catesbaei*	Japan	AB454370	-	-	KF289310	-

Table 4.1 (continued)

No.	Strain	Code	Host name	Country	Gene and GenBank No.				
					ITS	LSU	TEF1	ACT	GPDH
151	*P. neopyrolae*	MUCC125	*Pyrola asarifolia*	Japan	AB454318	-	-	AB704233	-
152	*P. rubrum*	CBS 111635	*Acer rubrum*	USA	KF206171	EU754194	KF289198	KF289233	KF289129
153	*P. yuccae*	CBS 112065	*Yucca elephantipes*	USA	KF206175	-	-	KF289237	-
154	*Phyllosticta*. sp.	MUCC 124	*Pachysandra leminalis*	Japan	AB454317	-	-	AB704232	-
155	*P. concentrica*	MUCC 147	*Rhododendron keiskei*	Japan	AB454319	-	-	AB704234	-
156	*P. rhaphiolepidis*	MUCC 432	*Rhaphiolepis indica*	Japan	DQ632660	-	DQ632724	AB704242	-
157	*P. capitalensis*	CPC 12157	*Acacia crassicarpa*	Thailand	KF206182	-	-	KF289261	-
158	*P. capitalensis*	CPC 11337	*Eucalyptus gradis*	Brazil	KF206180	-	-	KF289259	-
159	*P. capitalensis*	CPC 21035	*Citrus* sp.	Unknown	KF206201	-	-	KF289305	-
160	*Botryosphaeria obtusa*	CMW 8232	*Conifers*	South Africa	AY972105	-	DQ280419	AY972111	-

Note: [1]CBS: CBS-KNAW Fungal Biodiverstiy Centre, Utrecht, The Netherlands; CPC: Working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, NFW: Culture collection of Nilam F. Wulandari, Chiangmai, Thailand. LGMF: Culture collection of Laboratory of Genetics of Mycroorganisms, Federal University of parana, Curitiba, Brazil, ZJUCC: Zhejiang University Culture Collection, Zhejiang, China

Phyllostictaceae **Fr.** (as "Phyllostictei"), Summa veg. Scand., Section Post. (Stockholm): 420. 1849.

Foliicolous, plant pathogenic, endophytic or saprobic. *Ascomata* pseudothecial, separate to gregarious, globose, brown to black, with a central ostiole. *Asci* bitunicate, fissitunicate, clavate to subcylindrical, 8-spored, fasciculate, stipitate, with an ocular chamber. *Ascospores* bi- to triseriate, hyaline, aseptate, ellipsoid-fusoid to limoniform, smooth-walled, usually with mucilaginous caps at ends, or surrounded by a mucilaginous sheath. *Asexual morph*: *Conidiomata* pycnidial globose, dark brown, separate to aggregated, with a central ostiole; wall of 3–6 layers of brown *textura angularis*. *Conidiogenous cells* lining the inner wall, hyaline, smooth, subcylindrical to ampulliform or doliiform, proliferating percurrently near apex, frequently covered in mucilaginous sheath. *Conidia* hyaline, smooth, ellipsoid-fusoid to obovoid or ovoid, aseptate, smooth-walled, guttulate or granular, frequently surrounded by a mucilaginous sheath, and bearing a single mucilaginous apical appendage.

Type genus: *Phyllosticta* Pers.

Phyllosticta Pers., Traité sur les Champignons Comestibles (Paris): 55. 147. 1818.

Conidiomata pycnidial, immersed, subepidermal to erumpent, unilocular, rarely multilocular, glabrous, ostiolate, dark brown to black; ostiole circular to oval; wall of thick-walled, dark brown *textura angularis*, with inner layers of hyaline to pale brown, thin-walled *textura prismatica* to *angularis*. *Conidiophores* lining the cavity of the conidioma, reduced to conidiogenous cells, invested in mucus. *Conidiogenous cells* discrete, producing macroconidia and spermatia (also produced in separate spermatogonia); ampulliform, lageniform, doliiform to subcylindrical, hyaline, smooth, proliferating several times percurrently near apex, invested in a mucoid layer. *Spermatogenous cells* ampulliform to lageniform or subcylindrical, hyaline smooth, phialidic. *Conidia* ellipsoid-fusoid to obovoid, ovoid, rarely subcylindrical, aseptate, broadly rounded at the apex, often tapering strongly toward the base, unicellular, hyaline, smooth-walled, guttulate to granular, often enclosed in a persistent mucilaginous sheath, and bearing an unbranched, tapering, straight to curved, mucoid apical appendage *Spermatia* hyaline, smooth, granular, subcylindrical or dumbbell-shaped, with rounded or blunt ends. *Ascomata* pseudothecial, separate to gregarious, globose to subglobose,

brown to black, unilocular with a central ostiole. *Asci* bitunicate, fissitunicate, clavate to subcylindrical, 8-spored, fasciculate, stipitate, with an ocular chamber. *Ascospores* bi- to triseriate, hyaline, guttulate to granular, aseptate, ellipsoid, ellipsoid-fusoid to limoniform, smooth-walled, usually with mucilaginous caps at ends, or surrounded by a mucilaginous sheath.

Type species: *P. convallariae* Pers.

Other species

Note that the new species presented below are not formally published. They will not be considered as published until they are placed in an international publication with Mycobank number.

4.3.1.1 *Phyllosticta abieticola* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Abies*.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 250 µm diam, elongated in culture on PNA; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 15 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, that can be branched at the base, 10–25 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 7–15 × 3–5 µm; proliferating several times percurrently near apex. *Conidia* (11–)13–16(–18) × (7–)8 µm, solitary, hyaline, aseptate, thin and smooth-walled, granular, or with a single large central guttule, fusoid-ellipsoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin persistent mucoid sheath, 3–4 µm thick, and bearing a hyaline, apical mucoid appendage, (15–)20–25(–30) × 1.5(–2) µm, flexible, unbranched, tapering towards an acutely rounded tip. *Ascomata* similar to conidiomata in general anatomy. *Asci* bitunicate, hyaline, clavate to broadly fusoid-ellipsoid, with visible apical chamber, 2 µm diam, 65–120 × 12–15 µm. *Ascospores* bi- to multiseriate, hyaline, smooth, granular to guttulate, aseptate, straight, rarely curved, widest in the middle, limoniform with obtuse ends, (15–)16–18(–20) × (6–)7 µm.

Culture characteristics: Colonies erumpent, spreading with moderate aerial

mycelium, covering dish after 1 mo at 25 ºC. On OA surface iron-grey. On PDA and MEA surface grey-olivaceous, reverse iron-grey.

Specimen examined. Canada, on living leaf of *Abies concolor*, Jan. 2001, M. Forve (ex-type culture CBS 112067).

Notes: The present isolate of *P. abieticola* was originally identified as *P. abietis*. However, *P. abietis* has smaller conidia (7–12 × 6.5–9 µm), and a sheath up to 1.5 µm wide, with apical appendages being up to 2.5 µm long when they are present (Bissett & Palm, 1989), thus clearly being distinct.

4.3.1.2. *Phyllosticta aloeicola* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Aloe*.

Associated with leaf tip blight. *Conidiomata* pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 250 µm diam; pycnidial wall of several layers of textura angularis, up to 40 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 20 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 5–13 × 3–4 µm; proliferating several times percurrently near apex. *Conidia* (8–)14–18(–27) × (6.5–) 7–8(–9) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, ellipsoid to obovoid or subcylindrical, tapering towards a narrow truncate base, 3–5 µm diam, enclosed in a thin, persistent mucoid sheath, 1–2 µm thick, and bearing a hyaline, apical mucoid appendage, (7–)15–20(–23) × 2–3 (–3.5) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies erumpent, spreading, with sparse aerial mycelium and feathery margins, covering the dish in 1 mo. On MEA surface olivaceous-grey, reverse iron-grey; on OA and PDA iron-grey on surface and reverse.

Specimen examined: South Africa, Free State Province, Bloemfontein Botanical Garden, Bloemfontein, on living leaf of *Aloe ferox*, 7 May 2012, P.W. Crous W.J. Swart, (culture ex-type CPC 20677).

Notes: *Phyllosticta aloeicola* needs to be compared to *P. aloës*, which was described from *Aloë latifolia*, also collected in South Africa. Van der Aa Aa & Vanev (2002) examined the type specimen (deposited in B), and concluded that it represents

either a *Phoma* or *Asteromella* sp., and not a *Phyllosticta*, thus being distinct from *P. aloeicola.*

4.3.1.3. *Phyllosticta citrimaxima* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

MycoBank MB803675

Etymology: Named after this host on which it occurs, *Citrus maxima*.

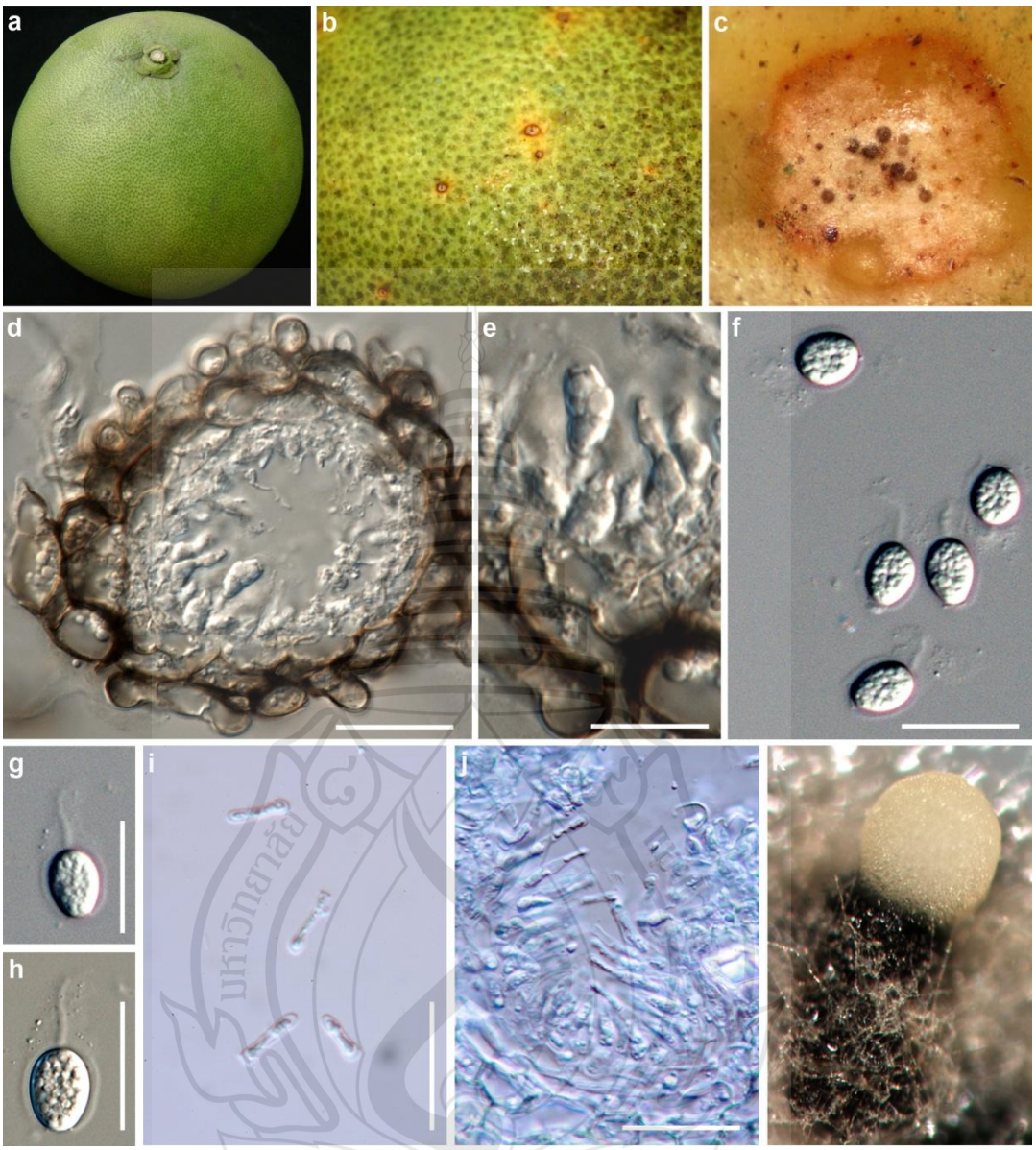
Conidiomata pycnidial (on PNA), forming after 4 d of incubation, black, superficial, globose, 150–160 × 120–130 µm; wall 1–3 layers, 20–30 µm thick. *Conidiogenous cells* developed after 5 d, lining wall of pycnidium, phialidic, cylindrical, hyaline, 3–5 × 1–2 µm. *Conidia* ellipsoidal, hyaline, 1-celled, smooth, 5(–8) × (3–)4(–7) µm, surrounded by mucilaginous sheath, 1 µm thick, bearing a single, apical appendage, 2–16 µm long.

Culture characteristics: On OA, colonies flat, with irregular margin, initially hyaline with abundant mycelium, gradually becoming greenish after 2–3 day. On MEA colonies woolly, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 day with white hyphae on the undulate margin, eventually turning black; reverse dark green to black. After 25 d in the dark at 27 ºC the colony covered the whole plate. On PDA, colonies were flat, rather fast growing, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 day, with white hyphae at the margin, eventually turning black; reverse black and after 14 d in the dark at 27 ºC colony covered whole plate.

Specimen examined: Thailand, Chiangrai, Weing Khaen, on fruit peel of *Citrus maxima*, June 2011, S. Wikee (holotype MFUCC 13-0001, ex-type culture CPC 20276 = MFLUCC10-0137).

Notes: *Phyllosticta citrimaxima* was isolated from tan spots on the fruit surface of *Citrus maxima*, which is commonly grown as an economically important crop in Thailand and Asia. Recently, *P. citriasiana*, and *P. citrichinaensis* have been described from *Citrus maxima* in Vietnam and China (Wulandari et al., 2009; Wang et al., 2012), and *P. citribraziliensis* from Brazil (Glienke et al., 2011). Phylogenetically *P. citrimaxima* is well supported (Figure. 4.3). Wang et al., (2012) provided a table in which they compared the morphology of five *Phyllosticta* species associated with citrus:

P. citricarpa, *P. citriasiana*, *P. capitalensis*, *P. citribraziliensis* and *P. citrichinaensis*. *Phyllosticta citrimaxima* produces smaller conidia (5–8 × 3–7 µm) than *P. citricarpa* (11–12 × 6–8 µm), *P. citriasiana* (12–14 × 6–7 µm), *P. capitalensis* (11–12 × 6–7 µm), *P. citribraziliensis* (10–12 × 6–7 µm) and *P. citrichinaensis* (8–12 × 6–9 µm), and has longer apical appendages (2–16 µm) than any of these four species, except *P. citrichinaensis* (14–26 µm).



Notes. a-c. Symtoms on host. d-e. Cross section of pycnidia showing developing conidia. f-h. conidia. i-j. *Leptodothiorella* state, spermogonium. k conidia produced on OA. Scale bars d = 30µm; e-j = 10µm.

Figure 4.3 *Phyllosticta citrimaxima*

4.3.1.4 *Phyllosticta concentrica* Sacc., Fungi venet. nov. vel. Crit., Sér. 5: 203. 1878.

Etymology: Named after the host genus on which it occurs, *Hedera*.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 400 µm diam, elongated in culture on PNA; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 25 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, that gives rise to 1–2 conidiogenous cells, 12–20 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 7–10 × 3–6 µm; proliferating several times percurrently near apex. *Conidia* (10–)11–13(–14) × (6–)8(–9) µm, solitary, hyaline, aseptate, thin and smooth-walled, granular, or with a single large central guttule, ellipsoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin persistent mucoid sheath, 1–2 µm thick, and bearing a hyaline, apical mucoid appendage, (5–)8–12(–15) × (1–)1.5 µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies flat, spreading with sparse aerial mycelium, and feathery, lobate margins, reaching 30 mm after 2 wk at 25 ºC. On PDA surface greenish black, reverse iron-grey; on OA surface iron-grey; on MEA surface olivaceous-grey in centre, pale olivaceous-grey in outer region, olivaceous-grey underneath.

Specimen examined. Italy, Padua, on withering leaves of *Hedera helix*, July 1875, syntype (L); Sardegna, Cologne near Oleina, leaf litter of *Hedera helix*, 31 Aug. 1970, W. Gams (epitype designated here CBS H-16992, culture ex-epitype CBS 937.70). Spain, on living leaf of *Hedera* sp., 10 July 2010, U. Damm, culture CPC 18842 = CBS xxx.

Notes: *Phyllosticta concentrica*, and its purported sexual state, *Guignardia philoprina*, represent different taxa, with each name representing a species complex for which numerous old names are available. *Phyllosticta concentrica* was originally introduced by Saccardo for a species occurring on *Hedera helix* in Italy, but which appears to be common in Europe on this host. The present collection closely matches the original description, for which an epitype is designated.

4.3.1.5 *Phyllosticta cordylinophila* P.A. Young, Bulletin of the Bernice P. Bishop Museum, Honolulu, Hawaii 19: 133. 1925.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless to opaque conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of 3–6 layers of textura angularis, up to 40 µm thick; inner wall of hyaline textura angularis. *Ostiole* central, up to 20 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, at times branched at base, 10–20 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 10–17 × 3–6 µm; proliferating several times percurrently near apex. *Conidia* (10–)11–13(–15) × 7–8(–11) µm, solitary, hyaline, aseptate, thin and smooth walled, coarsely guttulate, or with a single large central guttule, ellipsoid to obovoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin, persistent mucoid sheath, 1–2 µm thick, and bearing a hyaline, apical mucoid appendage, (10–)20–35(–40) × 2(–3) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies spreading, erumpent, with sparse aerial mycelium and even, smooth margins. On MEA surface pale olivaceous-grey in centre, dirty white in outer region, reverse iron-grey; on OA olivaceous-grey; on PDA olivaceous-grey on surface and reverse.

Specimens examined: Thailand, Chiangrai, Nang lae, Pasang, on leaf spot of *Cordyline fruticosa*, Nov. 2011, S. Wikee (neotype designated here, ex-neotype culture CPC 20261 = WK024). Japan, Kagoshima, Amami-Ohshima, Amagi, on *C. fruticosa*, 22 Oct. 2003, Y. Ono & T. Kobayashi, culture ex-type MUCC 521 = CPC 21880 = CBS xxxx.

Notes: Van der Aa Aa (1973) did not manage to locate any type material, and the material studied by Petrak & Sydow (1927) was depauperate. As the present collections match the morphology of the original species description [conidia ellipsoid to ovoid, 7–12(–15) × 5–7.5(–8) µm], we herewith designate one specimen as neotype.

4.3.1.6 *Phyllosticta cornicola* (DC.) Rabenh., Klotzschii Herb. Viv. Mycol., Edn 2: no. 454. 1857.

≡ *Sphaeria lichenoides* var. *cornicola* DC., in de Candolle & Lamarck, *Fl. franç*., Edn 3 (Paris) 6: 148. 1815.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 10 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, that can be branched at the base, 10–20 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 7–12 × 2.5–4 µm; proliferating several times percurrently near apex. *Conidia* (6–)7–8 × (5.5–)6(–7) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, ellipsoid to obovoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin persistent mucoid sheath, 1 µm thick, and bearing a hyaline, apical mucoid appendage, (3–)4–5 (–7) × 1(–1.5) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies erumpent, spreading with moderate aerial mycelium and feathery, lobate margins, covering dish after 1 mo at 25 ºC. On OA, MEA and PDA surface olivaceous-grey, reverse iron-grey.

Specimen examined. USA, on living leaf of *Cornus florida*, July 1999, G. Carroll, CBS H-xxx, culture CBS 111639.

Notes: The name *P. cornicola* is based on European collections (*Cornus sanguinea*, Czech Republic), and until fresh material has been collected, we cannot be sure that the name is authentic for this taxon.

4.3.1.7 *Phyllosticta cussoniae* Cejp, Bothalia 10: 341. 1971.

Leaf spots amphigenous, subcircular, pale to medium brown, 0.5–1 cm diam, frequently surrounded by a red-purple margin. *Conidiomata* pycnidial, solitary, black, erumpent, globose, exuding colourless to opaque conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of textura angularis; inner wall of hyaline textura angularis. *Ostiole* central, up to 20 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, branched at base, 10–25 × 3–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 5–10 × 3–4 µm; proliferating several times percurrently near

apex. *Conidia* (10–)12–15 (–17) × (6–) 7(–8) µm, solitary, hyaline, aseptate, thin and smooth walled, coarsely guttulate, or with a single large central guttule, ellipsoid ot obovoid, tapering towards a narrow truncate base, 3–4 µm diam, enclosed in a thin, persistent mucoid sheath, 2–4 µm thick, and bearing a hyaline, apical mucoid appendage, (8–)10–12(–13) × 2(–3) µm, flexible, unbranched, tapering towards an acutely rounded tip. Spermatia occurring in same conidioma with conidia, hyaline, smooth, guttulate to granular, bacilliform, 7–10 × 2–3 µm.

Culture characteristics: Colonies erumpent, spreading, with sparse aerial mycelium and feathery margins, covering the dish in 1 mo. On MEA surface olivaceous-grey, reverse iron-grey; on OA iron-grey; on PDA iron-grey on surface and reverse.

Specimen examined. South Africa, Mpumalanga, Schagen, Nelspruit, on *Cussonia umbellifera*, 25 Dec. 1933, L.C.C. Liebenberg, holotype PREM 32871; Eastern Cape, Graaff Reinet, Valley of Desolation, on leaf spot of *Cussonia* sp., 9 Jan. 2008, P.W. Crous (epitype designated here CBS H-xxx, culture ex-epitype CPC 14873–14875); Gauteng, Walter Sisulu National Botanical Garden, on leaves of *Cussonia* sp., 2 Mar. 2007, P.W. Crous, cultures CPC 13812–13815 = CBS 125996.

Note: *Phyllosticta cussoniae* occurs commonly on various *Cussonia* spp. throughout South Africa, where it causes a prominent leaf spot disease.

4.3.1.8 *Phyllosticta foliorum* (Sacc.) Wikee, Crous, K.D. Hyde & McKenzie, comb. nov.

≡ *Physalospora gregaria* var. *foliorum* Sacc., Syll. fung. (Abellini) 1: 435. 1882.

= *Pyreniella foliorum* (Sacc.) Theiss., Annls mycol. 14(6): 411. 1917 (1916).

= *Melanops foliorum* (Sacc.) Petr. (as “foliicola”), Kryptogamenflora Forsch. Bayer. Bot. Ges. Erforsch Leim. Flora 2(2): 165. 1931.

= *Botryosphaeria foliorum* (Sacc.) Arx & E. Müll., Beitr. Kryptfl. Schweiz 11(no. 1): 42. 1954.

Conidiomata pycnidial, solitary, black, erumpent, globose or with elongated body, exuding colourless to opaque conidial masses; *pycnidia* up to 400 µm diam; pycnidial wall of several layers of textura angularis; inner wall of hyaline

textura angularis. *Ostiole* central, up to 40 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, branched at base or not, 10–25 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 8–20 × 3–4 µm; proliferating several times percurrently near apex. *Conidia* (12–)13–14(–15) × (9–)10(–11) µm, solitary, hyaline, aseptate, thin and smooth walled, coarsely guttulate, or with a single large central guttule, broadly ellipsoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin, persistent mucoid sheath, 2–3 µm thick, and bearing a hyaline, apical mucoid appendage, (10–)12–15(–20) × 1.5(–2) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies erumpent, spreading, with sparse aerial mycelium and feathery margins, covering the dish in 1 mo. On MEA surface olivaceous-grey, reverse iron-grey; on OA iron-grey; on PDA iron-grey on surface and reverse.

Specimens examined. Italy, on fallen leaves of *Taxus baccata*, type of *Physalospora gregaria* var. *foliorum*, Herb. P.A. Saccardo, PAD. Netherlands, Baarn, Maarschalksbos, on dead twigs and needles of *Taxus baccata*, Sep. 1969, H.A. van der Aa Aa (neotype designated here CBS H-9495, culture ex-neotype CBS 447.68). USA, from bonsai tree of *Cryptomeria japonica*, 25 Feb. 1977, G.H. Boerema, specimens CBS H-13111, CBS H-619, culture CBS 174.77.

Notes: *Guignardia philoprina* (from *Ilex*) is a species complex with numerous old names. The oldest name linked to European specimens from *Taxus* appears to be *Physalospora gregaria* var. *foliorum*, which we recombine in *Phyllosticta*. As the holotype specimen in PAD only contains immature ascomata and spermatia, a neotype is herewith designated.

4.3.1.9 *Phyllosticta grandicola* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Specimen examined: Spain, on living leaf of *Eucalyptus globulus*, 4 Jan. 2004, M.J. Wingfield (holotype, culture ex-type CPC 11336).

Notes: Two species of *Phyllosticta* are known from *Eucalyptus*. van der Aa (2002) treated *P. eucalyptorum* (on *E. grandis* from Brazil, conidia (7.5–)11–20 × [5–6

(–6.5) µm; Crous, 1993] as synonymous with *P. eucalyptina* (on *E. globulus*, Tunisia, conidia 18–20 × 5–6 µm). Although no cultures are available for *P. eucalyptina*, *P. eucalyptorum* has been shown to be a synonym of *P. capitalensis* (Figure. 1).

4.3.1.10 *Phyllosticta hypoglossi* (Mont.) Allesch., Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1(6): 163. 1898.

≡ *Sphaeropsis hypoglossi* Mont., Annls Sci. Nat., Bot., sér. 3 12: 307. 1849.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 15 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, that can be branched at the base, 15–25 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 10–15 × 3–5 µm; proliferating several times percurrently near apex. *Conidia* (10–)11–12(–14) × (9–)10(–11) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, broadly ellipsoid to obovoid or globose, tapering towards a narrow truncate base, 3–4 µm diam, enclosed in a thin, mucoid sheath, 1–3 µm thick, mostly not persistent, and bearing a hyaline, apical mucoid appendage, (8–)10–12(–15) × 1.5(–2) µm, flexible, unbranched, tapering towards an acute tip.

Culture characteristics: Colonies flat, spreading with sparse aerial mycelium and feathery, lobate margins, reaching 25 mm diam on MEA, 30 mm diam on PDA and 35 mm diam on OA after 2 wk at 25 ºC. On OA centre olivaceous-grey, outer zone with diffuse pale yellow pigment in agar. On PDA surface olivaceous-grey, reverse iron-grey. On MEA surface iron-grey in centre, pale grey-olivaceous in outer region, iron-grey in reverse.

Specimens examined: France, near Marseille, on cladodes of *Ruscus hypoglossum*, 1845, J.L.M. Castagne, (type not found, presumably missing). Italy, Prov. Napoli, Cratere degli Astroni, on dead cladodes of *Ruscus aculeatus*, May 1992, W. Gams (neotype designated here CBS H-5331; ex-neotype culture CBS 434.92).

Note: *Phyllosticta hypoglossi* is a common European species on cladodes of *Ruscus hypoglossum*

4.3.1.11 *Phyllosticta leucothoicola* Wikee, C. Nakash., Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Leucothoe.*

Leaf spots purple-brown, scattered, enlarged and becoming confluent, subcircular to oblong, with brown to dark brown border (Takeuchi & Horie, 1998).

Anamorphic state: *Conidiomata* scattered or clustered. *Pycnidia* immersed, brown to dark brown, subglobular to oblate, 110–275 x 145–253 um (95–285 x 130–260 um on PDA); picnidial wall 2–6 cells, 9–15 um thick. *Conidiophores* short. *Conidia* sporulated phialidic, hyaline, aseptate, ovoid, ellipsoid to subglobose, 7.5–12.9 x 5.9–8.8 um (7–13.5 x 5–9.5 um on PDA), with an apical appendage, 2.5–6.5 um long (2–7.5 um long on PDA).

Teleomorphic state: *Fruitbodies* scattered or clustered. *Ascomata* immersed, subglobose, 195–305 x 190–285 um (205–295 x 200–280 um on PDA); *Peridium* 2–7 cells (3–7 cells on PDA), 14–28 um thick (15–28 um thick on PDA). *Asci* 8-spored, clavate, bitunicate, 90–140 x 8.8–15 um (95–140 x 9.5–15 um on PDA). *Ascospore* hyaline, aseptate, ellipsoid, swollen in the center, 15–21 x 5.5–7.5 um (14–20 x 5–8 um on PDA) with polar muciginous appendage at both ends.

Culture characteristics: Colonies erumpent, spreading, with sparse aerial mycelium and feathery margins, covering the dish in 1 mo. On MEA surface olivaceous-grey, reverse iron-grey; on OA iron-grey; on PDA iron-grey on surface and reverse.

Specimen examined: Japan, Tokyo, on living leaf of *Leucothoe catesbaei*, May 1996, J. Takeuchi (culture ex-type MUCC553).

Notes: *Phyllosticta leucothoës* has previously been described from *Leucothoe acuminata*, though van der Aa et al. (2002) transferred this species to *Fusicoccum* based on an examination of type material. *Phyllosticta leucothoicola* clearly represents a distinct taxon on *L. catesbaei*, corroborating earlier findings of Motohashi et al. (2009).

Takeuchi and Horie (1998) First Occurrence of Leaf Spot of English Ivy and Dog-hobble Caused by Guignardia sp. in Japan. Proceedings of the Kanto-Tosan Plant Protection Society 45: 139–142. (In Japanese)

4.3.1.12 *Phyllosticta macrophyllus* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov. MycoBank MB314758

Etymology: Named after the host genus on which it occurs.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless to opaque conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of 3–6 layers of brown textura angularis; inner wall of hyaline textura angularis. *Ostiole* central, up to 20 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, at times branched at base, 10–25 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical to doliiform, hyaline, smooth, coated in a mucoid layer, 10–17 × 4–6 µm; proliferating several times percurrently near apex. *Conidia* 12–13(–16) × 8–9(–9.5) µm, solitary, hyaline, aseptate, thin and smooth walled, coarsely guttulate, or with a single large central guttule, broadly ellipsoid, tapering towards a narrow truncate base, 2–5 µm diam, enclosed in a thin, persistent mucoid sheath, 3–4 µm thick, and bearing a hyaline, apical mucoid appendage, (25–)30–45(–55) × 3–4(–5) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies after 3 wk reaching 30 mm diam on MEA, 60 mm on PDA and OA. Colonies flattened, spreading, with sparse aerial mycelium and feathery margins. On MEA surface and reverse olivaceous-grey; on OA olivaceous-grey; on PDA iron-grey on surface and reverse.

Specimen examined: USA, Florida, on seed of *Podocarpus maki* (intercepted in New Zealand)), Sep. 1979, G. Laundon (holotype CBS H-13109; ex-type culture CBS 728.79).

Note: *Phyllosticta macrophyllus* was originally treated as part of the *G. philoprina* species complex, from which it is phylogenetically distinct (Figure xx). It is also distinct from *Phyllosticta podocarpi*, which was originally described from *Podocarpus elongatus* leaf litter collected in South Africa [conidia (10–)14(–17) × (8–) 9(–10) µm, appendages 10–40 × 1.5–2 µm; Crous, Seifert, Castaneda & Ruiz, 1996].

4.3.1.13 *Phyllosticta mangiferaceae* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

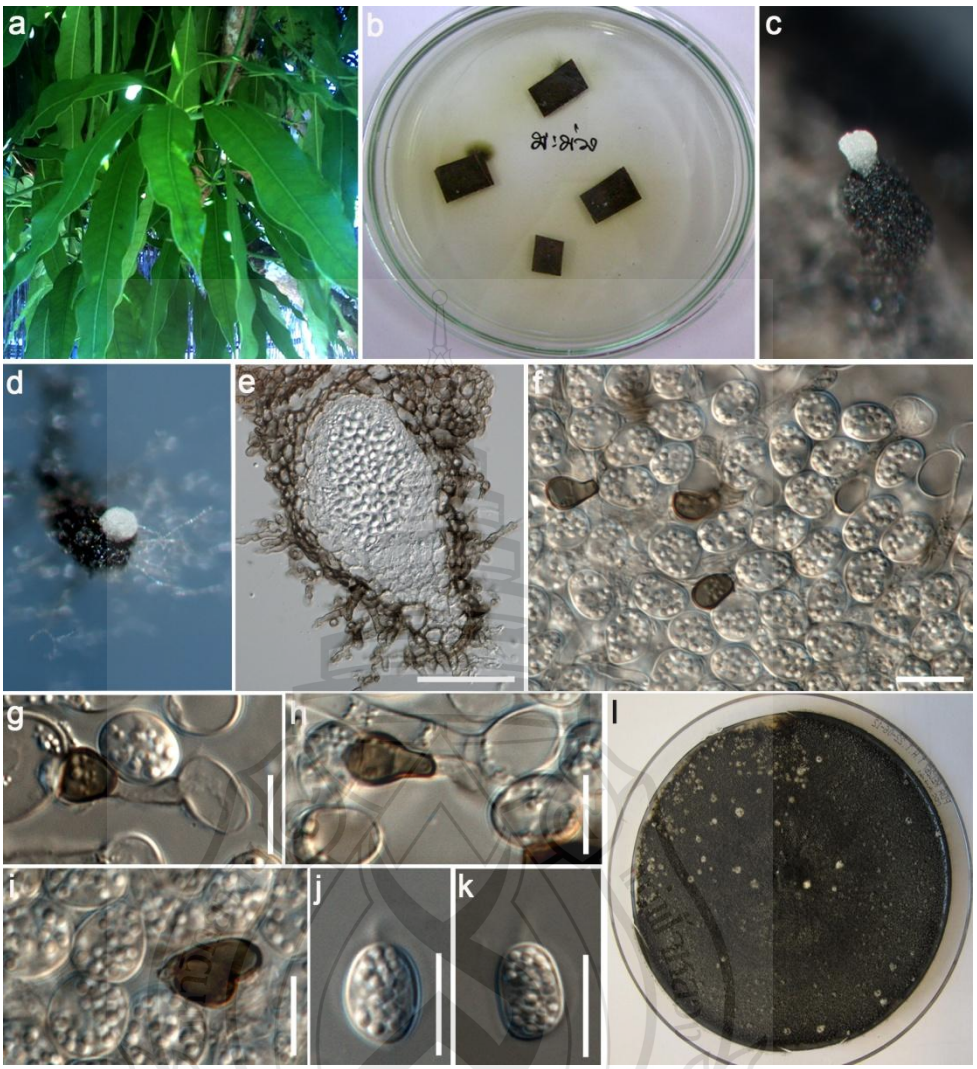
Etymology: Named after the host genus on which it occurs, *Mangifera*.

Conidiomata pycnidial (on PNA), initially forming after 4 d of incubation, black, superficial, subglobose or ellipsoidal, 220–300 × 160–180 µm; wall of 1–3 layers of brown *textura angularis*, 20–30 µm thick. *Conidiogenous cells* lining the inner wall, phialidic, cylindrical, hyaline, 3–5 × 3–4 µm. *Conidia* ellipsoidal, hyaline, aseptate, smooth, (6–)9(–13) × (4–)5(–6) µm, surrounded by mucilaginous sheath, 0.5–2 µm thick, bearing single apical appendage, 3–14 µm long.

Culture characteristics: On OA colonies appeared flat, with irregular margins, initially hyaline with abundant mycelium, gradually becoming greenish after 2–3 days on MEA, colonies woolly, irregular, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days, with white hyphae at the undulate margin, eventually turning black; reverse dark green to black. After 25 days in the dark at 27 ºC colony covering the whole plate. On PDA colonies flat, rather fast growing, initially white with abundant mycelium, gradually becoming greenish to dark green after 2–3 days with white hyphae at the margin, eventually turning black; reverse black and after 14 days in the dark at 27 ºC colony covering the whole plate.

Specimen examined: Thailand, Chiangrai, Nanglae, on healthy leaf of *Mangifera indica*, July 2011, S. Wikee (ex-type culture CPC 20264 = MFLUCC10-0029).

Notes: *Phyllosticta mangiferaceae* was isolated as an endophyte from a healthy leaf of *Mangifera indica*. Several species have been reported as pathogens on *M. indiga* including *G. mangiferae* and *P. brazilianiae* (Glienke et al., 2011). *Phyllosticta mangiferaceae* produced many conidia on OA and formed appressoria within 2 days Morphologically, it is distinct from *P. capitalensis* (conidia 8–11 × 5–6 µm) in producing larger conidia (conidia 6–13 × 4–6), and represents a distinct linage with 99% bootstrap support. It is phylogenetically distinct from *P. mangiferae*, and appears most closely related to *P. brazilianiae.*



Notes. a. Healthy leaf of *Mangifera indica*. b. isolation on WA. c. Conidia developed on OA. d. Conidia developed on SNA. e. Cross section of pycnidium shows developing conidia. f–i. Appressoria. j–k. Conidia**.** l. Culture on MEA. Scale bars e = 100µm, f–k = 10 µm.

Figure 4.4 *Phyllosticta mangiferaceae*

4.3.1.14 *Phyllosticta mangifericola* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Specimens examined: Brazil, São Paulo, Pompeia, on living leaf of *Mangifera indica*, 14 May 2007, C. Glienke & D. Stringari (ex-type culture CPC 17454); *ibid*., CPC 17455

4.3.1.15 *Phyllosticta minima* (Berk. & M.A. Curtis) Underw. & Earle, Bull. Alabama Agricultural Experiment Station 80: 168. 1897.

≡ *Sphaeropsis minima* Berk. & M.A. Curtis, N. Amer. Fungi: no. 418. 1874.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 180 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 15 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, that can be branched at the base, 15–50 × 5–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 8–20 × 3–4 µm; proliferating several times percurrently near apex. *Conidia* (9–)10–11(–12) × (6–) 7(–8) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, broadly ellipsoid to obovoid or globose, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin mucoid sheath, absent at maturity or 1 µm thick, and bearing a hyaline, apical mucoid appendage, 6–7(–10) × 1.5(–2) µm, flexible, unbranched, tapering towards an acute tip.

Culture characteristics: Colonies flat, spreading with sparse aerial mycelium and feathery, lobate margins, reaching 15 mm diam on MEA, 40 mm diam on PDA and 8 mm diam on OA after 2 week at 25 ºC. On OA surface olivaceous-grey. On PDA surface and reverse iron-grey. On MEA surface olivaceous-grey with patches of pale luteus.

Specimens examined: USA, North Dakota, New England, on *Acer rubrum*, R. Sprague 5314; Tennessee, Gatlinburg, Great Smoky Mountains National Park, on leaf spot of *Acer rubrum*, June 1984, D.H. Defoe (epitype designated here CBS H-17023; ex-epitype culture CBS 585.84 = IFO 32917).

Note: This taxon appears to be common on *Acer* spp. in the USA, where it is associated with leaf spots.

4.3.1.16 *Phyllosticta neopyrolae* Wikee, C. Nakash., Crous, K.D. Hyde & McKenzie, sp. nov.

MycoBank: MB803676.

Etymology: Named after the host genus on which it occurs, *Pyrola.*

Leaf spots orbicular to ellipsoid, black. *Conidiomata* pycnidial, epiphyllous, sparse, solitary or aggregated, immersed at first, then erumpent breaking through the epidermis, brown to dark brown, subglobose, 60–100 × 60–113 µm; pycnidial wall composed of the depressed or irregular cells in 1–4 layers, brown to dark brown, hyaline or paler toward the inside, with a central ostiole, up to 10 µm diam. *Conidiophores* subcylindrical, reduced to conidiogenous cells, or with 1–2 supporting cells, branched at the base, 15–20 × 2–3 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 8–15 × 2–3 µm; proliferating several times percurrently near apex. *Conidia* (6–)7(–8) × (5–)6(–7) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, broadly ellipsoid to globose, mucoid sheath and appendage lacking.

Culture characteristics: Colonies erumpent, spreading, with sparse aerial mycelium and feathery margins, covering the dish in 1 mo. On MEA surface olivaceous-grey, reverse iron-grey; on OA iron-grey; on PDA iron-grey on surface and reverse.

Specimen examined. Japan, Nagano, Sugadaira, on living leaf of *Pyrola asarifolia* subsp. *incarnata*, 17 June 2006, T. Hosoya (holotype TFM:FPH 7887, ex-type culture MUCC125 = CPC 21881 = CBS 134750).

Notes: Two species of *Phyllosticta* are known from *Pyrola* spp., namely *P. pyrolae* Ellis & Everh. and *P. pyrolae* (Ehrenb.: Fr) Allesch. Of these, the latter species is an illegitimate homonym. Furthermore, its morphological characteristics (conidia 3–4 µm), indicate that it should be excluded from *Phyllosticta s.str*. (van der Aa et al., 2002). The second species, *P. pyrolae* Ellis & Everh. (conidia ovoid to globose, 4.5–7.5 × 4–9 µm, with mucoid layer and an apical appendage) resembles *P. neopyrolae*. These species are, however, distinguishable, as conidia of *P. neopyrolae* lack a mucoid sheath and apical appendage.

4.3.1.17 *Phyllosticta owaniana* G. Winter, Hedwigia 24: 31. 1885. Figure XX.

Leaf spots amphigenous, irregular to subcircular, pale to medium brown, turning greyish with age, surrounded by a broad purplish border, and chlorotic margin. *Conidiomata* pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 300 µm diam, frequently with elongated neck on OA and MEA; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 10 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1–2 supporting cells, that can be branched at the base, 10–30 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 10–25 × 3–4.5 µm; proliferating several times percurrently near apex. *Conidia* (10–)11–12(–13) × (7–)8(–9) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, ellipsoid to obovoid, tapering towards a bluntly obtuse or narrow truncate base, 2–3 µm diam, enclosed in a thin persistent mucoid sheath, 1–2 µm thick, and bearing a hyaline, apical mucoid appendage, (5–)8 –12(–15) × (1–)1.5 µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies flat, spreading with sparse aerial mycelium and feathery, lobate margins, reaching 30 mm diam on MEA, 40 mm diam on PDA and 25 mm diam on OA after 2 week at 25 ºC. On OA surface iron-grey. On PDA surface and reverse iron-grey. On MEA surface and reverse
iron-grey.

Specimens examined: South Africa, Western Cape Province, Cape Town, Table Mountain, on leaves of *Brabejum stellatifolium*, 1884, P. McOwan, holotype in B; Western Cape Province, Jonkershoek Nature Reserve, on leaf spot of *Brabejum stellatifolium*, 3 Jan. 1995, A. den Breeÿen, (epitype designated here, ex-epitype culture CPC 1009 = CBS 776.97).

Note: *Phyllosticta owaniana* causes a serious leaf spot disease on *Brabejum stellatifolium*, and is generally found wherever this host occurs.

4.3.1.18 *Phyllosticta pachysandriicola* Wikee, C. Nakash., Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Pachysandra*.

Leaf spots circular to ellipsoid, pale brown to brown, often extend with concentric rings, 6–16 mm diam, surrounded by a dark brown border. *Conidiomata* pycnidial, amphiphyllous, sparse, solitary or aggregated, immersed at first, then erumpent breaking through the epidermis, brown to dark brown, subglobular, 25–80 × 90–140 µm, with central ostiole; pycnidial wall composed of depressed or irregular cells with 1–4 layers, brown to dark brown, hyaline or paler toward the inside. *Conidiogenous cells* integrated, lining the innermost layer of the pycnidial wall, cylindrical, straight or slightly curved, hyaline, proliferating percurrently at least once, with minute periclinal thickenings, 5–12 × 2–2.5 µm. *Conidia* sporulating holoblastically, solitary, unicellular, spherical, elliptical to obovoid, 4.5–7.5 × 5.5–8.5 µm, truncate at the base or rounded at both ends, containing numerous greenish guttulae, surrounded by a mucous sheath, rarely with a short apical appendage.

Specimen examined: Japan, Hokkaido, Asahikawa, on *Pachysandra terminalis*, K. Motohashi, C. Nakashima & T. Akashi, 7 June 2006 (holotype TFM:FPH7877, isotype MUMH10488, ex-holotype culture MUCC 124 = NBRC102276).

Notes: Although *P. pachysandrae* has previously been described from *Pachysandra*, van der Aa et al. (2002) excluded it from *Phyllsoticta s. str*. based on its conidia, which are asteromella-like (unicellular, oblong, 4.5–6 × 1 µm). The Japanese collection is thus described as a new species, in accordance to the observations made by Motohashi et al. (2009).

4.3.1.19 *Phyllosticta paxistimae* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Paxistima*.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 10 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, 15–30 × 4–6 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 10–20 × 4–5 µm; proliferating

several times percurrently near apex. *Conidia* (10–)12–14(–16) × 6–7(–8) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, broadly ellipsoid, tapering towards a narrow truncate base, 2–3 µm diam, enclosed in a thin persistent mucoid sheath, 1 µm thick, and bearing a hyaline, apical mucoid appendage, (5–)9–11(–13) × 1.5(–2) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies erumpent, spreading with moderate aerial mycelium and feathery, lobate margins, reaching 60 mm diam after 1 mo at 25 ºC. On OA surface iron-grey with patches of olivaceous-grey. On PDA surface and reverse iron-grey. On MEA surface dirty white with patches of iron-grey, reverse iron-grey.

Specimens examined. USA, Oregon, on living leaf of *Paxistima myrsinites*, 1994, G. Carroll (holotype, ex-type culture CBS 112527).

Notes: We have been unable to trace the holotype of *Phyllosticta pachystimae* (USA, Wyoming, Hoback Canyon, near Granite Creek, on *Paxistima myrsinites*, 1 Aug. 1940, L.E. Wehmeyer No 1198). However, conidia of *Phyllosticta pachystimae* (9–14 × 4–5 µm) are much narrower than those of *P. paxistimae*.

4.3.1.20 *Phyllosticta philoprina* (Berk. & M.A. Curtis) Wikee, Crous, K.D. Hyde & McKenzie, comb. nov.

≡ *Sphaeria philoprina* Berk. & M.A. Curtis, Grevillea 4(no. 32): 154. 1876.

≡ *Guignardia philoprina* (Berk. & M.A. Curtis) Aa, Stud. Mycol. 5: 44. 1973. For additional synonyms see van der Aa (1973).

Specimens examined: Spain, on living leaf of *Ilex aquifolium*, July 1970, H.A. van der Aa, specimen CBS H-13113, culture CBS 587.69. Germany, on *Ilex aquifolium*, Aug. 1972, R. Schneider, CBS 616.72.

Notes: The oldest name for the taxon occurring on *Ilex* is *Sphaeria philoprina*. However, this name was based on material collected in the USA, and the present isolates were derived from European collections, so this needs to be further corroborated. If material from the USA were shown to be distinct, a nom. et comb. nov. will have to be introduced for *Sphaeropsis ilicicola* Cooke & Ellis in *Phyllostica*, as *P. ilicicola* (Cooke & Ellis) Ellis & Everh. is illegitimate, being a homonym of *P. ilicicola*

Pass. *Sphaeropsis ilicicola* is, however, based on European collections.

4.3.1.21 *Phyllosticta rhaphiolepidis* Wikee, C. Nakash., Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology: Named after the host genus from which it was collected, *Rhaphiolepis.*

Leaf spots irregular, pale brown. *Conidiomata* pycnidial, amphiphyllous, immersed, subglobose to globose, composed of depressed or irregular cells in 2–3 layers, dark brown to black, hyaline or paler toward the inside, 85–175 × 100–110 µm diam, with central ostiole, 10–13 µm diam. *Conidiogenous cells* integrated, lining the inner layer of pycnidia, hyaline, lageniform, cylindrical or conical, 3–4 × 3–4 µm, proliferating percurrently near apex. *Conidia* unicellular, spherical, ellipsoid to obovoid, truncate at base, later rounded at both ends, surrounded by a mucoid layer, containing numerous minute guttules, 7.5–10 × 4.6–6 µm, with a slender and short apical appendage 4–6 × 1–2 µm.

4.3.1.22 *Specimen examined*: Japan, Kagoshima, Tokunoshima Is., on living leaf of *Rhaphiolepis indica* var. *umbellata*, T. Kobayashi & Y. Ono, 22 Oct. 2003 (ex-type culture MUCC432).

Notes: No species of *Phyllosticta* have thus far been described from *Rhaphiolepis. Phyllosticta rhaphiolepidis* is also phylogenetically distinct from other species of *Phyllosticta* that have been deposited in GenBank

4.3.1.23 *Phyllosticta rubrum* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Etymology. Named after the host species from which it was collected, *Acer rubrum.*

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 15 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, that can be branched at the base, 7–10 × 2–3 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 3–8 × 2–3 µm; proliferating several times percurrently near apex. *Conidia* (6–)6.5–7(–8) × (4–)5(–5.5) µm, solitary, hyaline, aseptate, thin and

smooth walled, granular, or with a single large central guttule, ellipsoid to obovoid, tapering towards a narrow truncate base, 1.5–2 µm diam, enclosed in a thin persistent mucoid sheath, 1–1.5 µm thick, and bearing a hyaline, apical mucoid appendage, (5–) 6–7(–9) × (1–)1.5 µm, flexible, unbranched, tapering towards an acutely rounded tip. *Ascomata* similar to conidiomata in general anatomy. *Asci* bitunicate, hyaline, clavate to broadly fusoid-ellipsoid, with visible apical chamber, 1 µm diam, 30–50 × 10–12 µm. *Ascospores* bi- to triseriate, hyaline, smooth, granular to guttulate, aseptate, straight, rarely curved, widest in the upper third, limoniform, (8–)9–10(–12) × (4–)5 µm.

Culture characteristics: Colonies erumpent, spreading with moderate aerial mycelium, covering dish after 1 mo at 25 C. On OA surface iron-grey. On PDA and MEA surface olivaceous-grey, to iron-grey, reverse iron-grey.

Specimen examined: USA, Missouri, on *Acer rubrum*, July 1999, G. Carroll, (holotype, culture ex-type CBS 111635).

Notes: The present isolate of *P. rubrum* was originally identified as *P. minima*, which appears to be a species complex. *Phyllosticta minima* has larger conidia (10 µm long), and two synonyms, namely *P. arida* (on *Acer negundo*, conidia 8–10 × 6–7 µm), and *P. acericola* (on *Acer rubrum*, conidia 5–8 × 3–3.5 µm), thus distinct from *P. rubrum.*

4.3.1.24 *Phyllosticta spinarum* (Died.) Nag Raj & M. Morelet, Bull. Soc. Sci. nat. Arch. Toulon et du Var 34(219): 12. 1978.

≡ *Phoma spinarum* Died., Krypt.-Fl. Brandenburg (Leipzig) 9: 148. 1912.

Conidiomata pycnidial, solitary, black, erumpent, globose, exuding colourless conidial masses; *pycnidia* up to 200 µm diam; pycnidial wall of several layers of *textura angularis*, up to 30 µm thick; inner wall of hyaline *textura angularis*. *Ostiole* central, up to 15 µm diam. *Conidiophores* subcylindrical to ampulliform, reduced to conidiogenous cells, or with 1 supporting cell, that can be branched at the base, 10–15 × 4–5 µm. *Conidiogenous cells* terminal, subcylindrical, hyaline, smooth, coated in a mucoid layer, 5–12 × 3–5 µm; proliferating several times percurrently near apex. *Conidia* (10–)12–14 (–15) × (7–)7.5(–8) µm, solitary, hyaline, aseptate, thin and smooth walled, granular, or with a single large central guttule, ellipsoid to obovoid,

tapering towards a narrow truncate base, 3–4 µm diam, enclosed in a thin persistent mucoid sheath, 1–2 µm thick, and bearing a hyaline, apical mucoid appendage, (7–)8–12(–20) × (2–)2.5(–3) µm, flexible, unbranched, tapering towards an acutely rounded tip.

Culture characteristics: Colonies flat, spreading with sparse aerial mycelium and feathery, lobate margins, reaching 70 mm diam after 1 mo at 25 ºC. On OA surface olivaceous-grey. On PDA surface olivaceous-grey, reverse iron-grey. On MEA surface pale olivaceous-grey in outer region, olivaceous-grey in centre; in reverse iron-grey in centre, smoke-grey in outer region.

Specimens examined: Germany, Nieder Lauslitz: Colbus, on *Juniperus* sp., 4 July 1910, Diedicke, holotype in B. France, St. Denis en Val, on living leaf of *Chamaecyparis pisifera*, 1970, M. Morelet (epitype designated here CBS H-17034, ex-epitype culture CBS 292.90).

Note: Nag Raj and Morelet (1979) provide a detailed description of the type specimen.

4.3.1.25 *Phyllosticta vaccinicola* Wikee, Crous, K.D. Hyde & McKenzie, sp. nov.

Specimen examined: USA, on living leaf of *Vaccinium macrocarpum*, Mariusz Tadych, (holotype CBS H-xxxx, ex-type culture CPC 18590 = CBS xxx).

4.4 Discussion

The resurrection of the *Phyllostictaceae*, and the separation from the *Botryosphaeriaceae* is justified based on morphology and DNA phylogeny (Crous et al., 2006; Liu et al., 2012). *Phyllosticta* is now a well-established genus, quite distinct from genera in the *Phoma* complex (Aveskamp et al., 2010; Gruyter et al., 2010, 2013), while the *Botryosphaeria* complex has also been shown to represent numerous genera (Crous et al., 2006; Phillips et al., 2008; Liu et al., 2012), and even families.

Traditionally species of *Phyllosticta* have been chiefly identified based on their host substrate. Several recent papers have shown, however, that many traditional morphological species were in fact representative of species complexes, e.g.

P. citricarpa on citrus, and *P. musarum* on banana (Glienke et al., 2011; Wang et al., 2012).

Freckle disease of banana was usually referred to in literature under its sexual name, *Guignardia musae*, or that of its purported asexual morph, *Phyllosticta musarum*. By employing multigene DNA analysis combined with a morphological comparison, Wong et al. (2012) demonstrated that these two names were not conspecific, and that the organism occurring in Southeast Asia and Oceania was in fact *P. maculata*, while *P. musarum* was confirmed from India and Thailand, and that the common species occurring on Cavendish banana (common eating banana) was in fact a novel taxon, which they described as *P. cavendishii*. The most recent studies focusing on the taxonomy of *Phyllosticta* species associated with citrus black spot is that of Glienke et al. (2011) and Wang et al. (2012). Surprisingly, several species of *Phyllosticta* were shown to be able to cause these symptoms on citrus, though there was a difference in their host range or preference. The citrus black spot pathogen which is presently subjected to phytosanitary legislation in the EU and United States, *P. citricarpa*, was isolated from lemons, mandarins and oranges in China, though Wang et al. (2012) did define two subclades, one from mandarins, and another from oranges and lemons. *Phyllosticta citriasiana* was newly described on *Citrus maxima* in Asia by Wulandari et al. (2009), while Glienke et al. (2011) described *P. citribraziliensis* on *Citrus limon* from Brazil. Wang et al. (2012) again described *P. citrichinaensis* on *C. maxima* and *C. reticulata* from China. The present study adds yet a fifth species to this complex, namely *P. citrimaxima*, which is associated with tan spots on the fruit surface of *Citrus maxima* in Thailand. When considering that *P. capitalensis* can still co-occur as endophyte in fruit or leaf lesions caused by these five species, it is obvious that these taxa are best distinguished based on DNA sequence data in future studies, and at the respective ports of entry into various countries.

Guignardia philoprina (asexual morph *P. concentrica*) has been known as the taxon occurring on hosts such as *Rhododendron*, *Hedera*, *Ilex*, *Magnolia*, and *Taxus* (von Arx & Müller, 1954). Not surprisingly, this turned out to represent a species complex, with numerous names available for consideration under the sexual and asexual morph. Although some of these names have been resurrected and applied in the present study, e.g. *P. concentrica* on *Hedera helix*, *P. foliorum* on *Taxus* and

P. philoprina on *Ilex*, many taxa still need to be recollected to resolve their phylogeny and correct taxonomy.

One aim of the present paper was thus to employ multigene DNA sequence analysis to discriminate among all species of *Phyllosticta* that were available to us from the CBS culture collection, supplemented by our own working collections, which resulted in a total of 170 strains. Other than dealing with old synonymies that represented names that now again had to be resurrected, a further challenge has been to also merge *Phyllosticta* and *Guignardia* epithets, to derive the best possible unit nomenclature for these species (Wingfield et al., 2012). In the present study we described 17 novel species, and designated a further xx epitype or neotype specimens. From results obtained here, it is clear that in the case of epitypification, epitypes need to be designated based on the same host, recollected in the same geographic region (Cannon et al., 2012). This is extremely difficult, as American names are commonly used for European of Asian taxa, and also vice versa [see the same situation in *Cercospora* and *Pseudocercospora*]. In these cases the application of names to collections from other countries that appear morphologically similar, can at best be regarded as tentative, pending further collections.

Obviously a multi-gene approach works well for distinguishing these taxa. In this study the intron dominated genes (ITS, ACT, TEF), and highly conserved gene coding regions (LSU, GPDH) were used. However, the result from the five gene analysis vs the two gene analysis tended to be the similar (Figure 4.1, 4.2), suggesting that for *Phyllosticta* a phylogeny derived from the ITS and ACT gene loci is sufficiently robust to distinguish most taxa. The biggest challenge, however, will still be to recollect specimens representative of the more than 3000 names that exist in this complex.

CHAPTER 5

CONCLUSION

Phyllosticta species are plant endophytes and weak plant pathogens with a worldwide distribution. Recent studies based on molecular data have resolved some cryptic species and some novel taxa have been discovered (Su & Cai 2012; Wang et al., 2012; Zhang et al., 2013). Study of the relationship by phylogenetic analysis of ITS, LSU, ACT, TEF and GPDH gene regions could distinguish the species within the genera of *Phyllosticta* and solve these cryptic species.

5.1 Widespread of Endophytic *Phyllosticta capitalensis*

Phyllosticta capitalensis is an endophyte and weak plant pathogen with a worldwide distribution presently known from 70 plant families. This study was undertaken to isolate *P. capitalensis* from different host plants in northern Thailand, and to establish their different life modes. Twenty three strains of *P. capitalensis* were isolated from 20 hosts, and subjected to phylogenetic analysis. An additional 16 strains of *P. capitalensis* from other hosts and geographic locations were also obtained from the official culture collections (i.e., CBS and MFLUCC). The fact that *P. capitalensis* is often isolated as an endophyte has important implications for studies in fungal biology and plant health. Although *P. capitalensis* is commonly found associated with lesion of plants, this species is not considered as a serious pathogen and thus not include in the fungal quarantine list. The problem is that *P. capitalensis* is often misidentified as *P. citricarpa* (the serious pathogen causing citrus black spot). Due to the species similarity, it is therefore important to establish a rapid and accurate

technology to detect the species identity (Meyer et al., 2001; Stringari et al., 2009; Meyer et al., 2012).

This study confirmed the distinct characteristics between *P. capitalensis* and *P. citricarpa* using multigene analysis. These approaches if properly developed and established would help plant pathologist for coping with the problem.

5.2 Multigene of Taxonomy of *Phyllosticta* Species

Based on morphology, *Phyllosticta* type specimens differ from the fungal species in *Botryosphearіaceae*. In the present study, we introduce multigene analysis which can resolve and reveal some novel taxa. The gene sequences used in this study were ITS, LSU, ACT, TEF and GPDH regions which are now considered as a standard marker gene for fungal identification (Glienke et al., 2011). A combination of two (ITS and ACT), three (ITS, ACT and TEF) and five genes (ITS, LSU, ACT, TEF and GPDH) were determined and proved that they could be used in *Phyllosticta* identification at species level. Fifteen new species were proposed in this Thesis: *Phyllosticta abieticola, P. aloeicola, P. citrimaxima, P. foliorum, P. grandicola P. leucothoicola, P. macrophyllus, P. mangiferaceae, P. neopyrolae, P. pachysandriicola, P. paxistimae, P. philoprina, P. rhaphiolepidis*, *P. rubrum*, *P. vaccinicola* and seven epitypes were designated for *P. cordylinophila*, *P. cornicola*, *P. cussoniae*, *P. hypoglossi*, *P. minima*, *P. owaniana*, and *P. spinarum*. However, we suggested that *Phyllosticta* should be annotated within *Phyllostictaceae*. Sequences of genes used in this study are now available in GenBank.

5.3 Significance and Publications from this Thesis

During this study, 37 *Phyllosticta* strains were obtained; they were isolated from plant specimens northern Thailand. Of 37 isolaltes, 33 strains were identified as *Phyllosticta capitalensis*, two as *P. cordinopapila*, and one strain of *P. citrimaxima* and *P. mangiferaceae*. In this study, *P. citrimaxima* (as isolated from *Citrus maxima*) and *P. mangiferaceae* (as isolated from *Mangifera indica*) were described as new species discovered from Thailand based on the data of multigene analysis. A phylogenetic tree of 160 *Phyllosticta* strains is also presented in this study to clarify the taxonomy of *Phyllosticta* species. Publications obtained from my study are as follows:

Wikee, S., Cai, L., Noireung, P., McKenzie, E. H. C., Su, Y. Y., Chukeatirote, E., Thi H. N., Bahkali, A. H., Moslem, M. A., & Abdelsalam, K. (2011). "Colletotrichum species from Jasmine (*Jasminum sambac*)" *Fungal Diversity*, 46(1), 171-182.

Wikee, S., Udayanga, D., Crous, P. W., Chukeatirote, E., McKenzie, E. H. C., Bahkali, A. H., Dai, D. Q. & Hyde, K. D. (2011a). *Phyllosticta*—an overview of current status of species recognition. *Fungal Diversity*, 51, 43–61.

Wikee, S., Wulandari, N. F., McKenzie, E. H. C. & Hyde, K. D. (2011b). *Phyllosticta ophiopogonis* sp. nov. from *Ophiopogon japonicas* (*Liliaceae*). *Saudi Journal of Biological Sciences*, 19(2), 13–16.

Liu, J. K., Phookamsak, R., Doilom, M., Wikee, S., Li, Y. M., Ariyawansha, H., Boonmee, S., Chomnunti, P., Dai, D. Q., Bhat, D. J., Romero, I. A., Zhuang, W. Y., Monkai, J., Jones, E. B. G., Chukeatirote, E., Ko-Ko, T. W., Zhao, Y. C., Wang, Y. & Hyde K. D. (2012). Towards a natural classification of Botryosphaeriales. *Fungal Diversity*, 57, 149-210.

Wikee, S., Lombard, L., Crous, P.W., Nakashima, C., Motohashi, K., Chukeatirote, E., Alias, S. A., McKenzie, E. H. C. & Hyde, K. D. (2013). *Phyllosticta capitalensis*, a widespread endophyte of plants. *Fungal Diversity*, 60, 91-105.

Wikee, S., Jaidee, P., Wongkam, S., Chukeatirote, E., McKenzie, E. H. C. & Hyde, K. D. (2013). Antimicrobial activity of crude extracts of *Phyllosticta* spp. (In press).

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APPENDICES

APPENDIX A

MEDIA COMPOSITION AND PREPARATION

SNA (Synthetic Nutrient-poor Agar)

KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5g
KCl	0.5g
glucose	0.2g
saccharose	0.2g

Autoclaved pieces of filter paper may be added as carbon source.

PNA (Pine Needle Agar)

Agar	15g

Media are generally made up with 1 litre distilled water. The media are sterilised by autoclaving at 121°C for 15 min. For PNA, pine needle was separately sterilised by autoclaving at 121°C for 15 min and then placed on the agar.

APPENDIX B

POLYMERASE CHAIN REACTION DIAGRAM (PER 1 REACTION)

General PCR protocol

95 °C	5.0	min
95 °C	0.30	min
52 °C	0.30	min*
72 °C	1.0	min
72 °C	7.0	min
10 °C	Hold	

Run for 40 cycles

* For actin and elongation factor 1- alfa annealing temperature is 55 °C

Touchdown PCR condition for GPDH

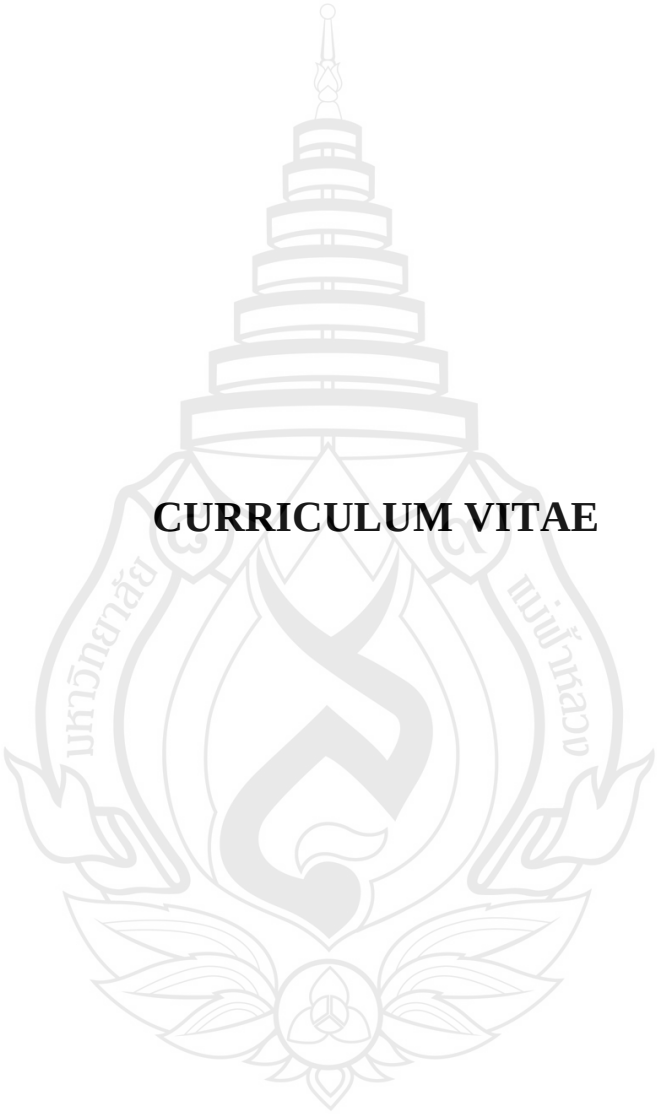
94 °C	1.0	min
65 °C	0.30	min
72 °C	0.30	min

Run for 13 cycles

94 °C	0.30	min
55 °C	0.30	min
72 °C	0.30	min

Run for 23 cycles

10 °C	Hold



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